The University of Reading

Thesis submitted for the degree of Doctor of Philosophy

Automation of Protein Annotation to Scan for Potential Drug Targets — a Case Study of MEP Pathway and Apicoplast Proteins

Sri Vishnu Vardhan Deevi

School of Biological Sciences

November 2007

Abstract

The basic aim was to automate the process of protein annotation in order to evaluate protein sequences of the Methyl Erythritol Phosphate (MEP) pathway and the apicoplast for purposes of Structure Based Drug Design (SBDD). Two novel tools were developed: APAT and TAPAS.

APAT is an extensible system to execute many serially independent annotation and prediction tools. XML formats were designed to capture the required input and output of a wide variety of annotation tools. Wrappers were written for tools running both locally and remotely and a display tool was written to generate HTML output. TAPAS is a specialized pipeline for ranking targets based on their suitability for SBDD (at basic level devoid of detailed structural studies). A ranking is provided based on the presence or absence of human hits, enzyme hits, structure hits, ligand hits, and transmembrane regions.

In an effort to improve transmembrane predictions, a combined neural network predictor was developed using the output from three of the best transmembrane predictors: TMHMM, MEMSAT and DAS-TMfilter. Performance of the individual programs and the combined predictor was evaluated amd the effect of masking signal peptide residues was assessed. At the residue level, the combined predictor performed only marginally better than TMHMM and signal peptide

masking reduced performance. At the whole protein level, TMHMM used alone with signal peptide masking provided the best performance.

In the analysis of the MEP pathway and apicoplast proteins, 58% of the 544 apicoplast proteins were given a high SBDD target score. However, out of the 544 sequences, only 36 (6.6%) were given the highest rank (6 out of 6) and further 36 (6.6%) were given the second highest rank (5 out of 6) because of the distribution of 58% of targets as a result of ranking. 12 out of the 13 (reviewed) top-ranked sequences are already being exploited as drug targets thus supporting the ranking scheme. The remaining protein is a hypothetical protein for which there is no known drug or inhibitor although there is a known ligand to which a number of drugs have over 80% 2D similarity. 8 sequences are ranked second and have known structures making them eminently suitable for SBDD. Another 28 sequences have no known structure, but otherwise rank highly and are therefore sensible choices to be solved by x-ray crystallography or NMR.

Declaration

I hereby declare that this is my own work and the use of all material from other sources has been properly and fully acknowledged to the best of my knowledge.

(Sri V. V. Deevi)

Abbreviations

The following abbreviations are used in this thesis:

Nucleic Acids

cDNA	complementary DNA
DNA	Deoxyribo Nucleic Acid
mRNA	messenger RNA
RNA	Ribo Nucleaic Acid
rRNA	ribosomal RNA
tRNA	transfer RNA

Amino Acids

A	Ala	Alanine	M	Met	Methionine
C	Cys	Cysteine	N	Asn	Asparagine
D	Asp	Aspartic acid	P	Pro	Proline
E	Glu	Glutamic acid	Q	Gln	Glutamine
F	Phe	Phenylalanine	R	Arg	Arginine
G	Gly	Glycine	S	Ser	Serine
H	His	Histidine	T	Thr	Threonine
I	Ile	Isoleucine	V	Val	Valine
K	Lys	Lysine	W	Trp	Trytophan
L	Leu	Leucine	Y	Tyr	Tyrosine

Miscellaneous

Å	Ångström
AC	Accession Code
AIDS	Acquired Immuno Deficiency Syndrome
ANN	Artificial neural networks
APAT	Automated Protein Annotation Tool
API	Application Program Interface
ATP	Adinosine TriPhosphate
BLAST	Basic Local Alignment Search Tool
CDP	Cytosine DiPhosphate
CGI	Common Gateway Interface
CTP	Cytosine TriPhosphate
DAG	Directed Acyclic Graph
DTD	Document Type Definition
EC	Enzyme Commission
E-value	Expectation value
GPCR	G-Protein Coupled Receptor
HIV	Human Immunodeficiency Virus
HMM	Hidden Markov Model
HTML	Hyper Text Markup Language
HTTP	Hyper Text Transfer Protocol
ID	Identifier
KEGG	Kyoto Encyclopedia of Genes and Genomes
MCC	Matthews' Correlation Coefficient
MEP	Methyl Erythritol Phosphate
NADPH	Reduced form of Nicotinamide Adinine Dinucleotide Phosphate
NMR	Nuclear magnetic resonance
PDB	Protein Databank
PIR	Protein Information Resource
Psi-BLAST	Position specific iterative BLAST
RDBMS	Relational DataBase Management System
RMSD	Root mean square deviation
Rprop	Resilient back-propagation
SBDD	Structure Based Drug Design
SNNS	Stuttgart Neural Network Simulator
SOAP	Simple Object Access Protocol
SQL	Structured Query Language
SVM	Support Vector Machine
TAPAS	Target Annotation Pipeline and Automated Selection
UniprotkB	Universal Protein Resource Knowledgebase
XML	eXtensible Markup Language

Acknowledgement

If there is one person whom I owe an enormous amount of gratitude for making this possible, it is my supervisor Dr. Andrew C. R. Martin, for showing tremendous levels of patience in motivating me and being a huge support through out. He has been and will continue to be a source of inspiration and I am blessed to have worked under the best supervisor one could possibly have.

I am very grateful and indebted to Felix trust for providing me with a scholarship and thus a wonderful oppurtunity to do my Ph.D.

I thank Dr. Mike Fry and Dr. Gail Hutchinson for being my Reading based supervisors and extending their help and advice when needed. My thanks are also due to my labmates and staff at the University of Reading and University College London, who have helped me in one way or the other.

I thank my parents and brother for their perennial love and faith. I also thank my friends across the globe for being my well-wishers.

Last but not least, my sincere thanks are due to Dhami for being my best friend and supporting me all the time.

Contents

List of Figures

List of Tables

Chapter 1

Introduction

There has been a tremendous rise in the number of protein sequences in public sequence databases as a result of an increase in genome sequencing projects — 82,853,685 entries were found in GenBank (Benson *et al.*, 2007) in the February 2008 release (Figure 1.1). While the number of entries in the PDB (Berman *et al.*, 2000) is also on the rise, it is relatively slow — the PDB had 49,295 structures in March 2008 (Figure 1.2). The widening gap between the number of proteins with known 3D structures and the number of proteins with known amino acid sequence is because of the experimental difficulties associated with obtaining protein crystals capable of diffracting at good resolution which is even more difficult in the case of transmembrane proteins whereas numerous genome sequencing projects are employing high throughput sequencing techniques to obtain sequence information (Cantor and Little, 1998). Despite recent advances in the field of X-ray crystallography and multidimensional nuclear magnetic resonance (NMR) spectroscopy, the gap is widening at an unprecedented pace. The completion of the Human Genome Project (HGP) in 2003 (Collins *et al.*, 2003) has set off a series of genome sequencing projects, but the revolutionary advances in the field of DNA

sequencing has marked the end of yesteryear's state-of-the-art sequencing technology (Sanger *et al.*, 1977) and the beginning of ultra-cheap DNA sequencing techniques (Metzker, 2005). The production of enormous amounts of sequence data also stresses the need for bioinformatics — automated annotation (genome annotation) and analysis of the hidden meaning in "A", "T", "G", and "C" to make any sense of the data.

Similarly, to derive biological meaning out of the 20 amino acid sequences, protein annotation is invaluable. Annotation can be defined as any piece of information associated with an amino acid sequence. Annotating protein sequences to assign them a function through homology, cross-linking them to various other databases, obtaining annotations from different prediction and annotation tools, and obtaining structural details is a tedious and error prone task to do manually. My project mainly deals with automating the process of protein annotation, designing a pipeline for automated drug target selection for Structure Based Drug Design (SBDD) and annotating the protein sequences of the apicoplast and the Methyl Erythritol Phosphate (MEP) pathway.

1.1 Isoprenoids

Isoprenoids (also called as terpenoids) are one of the oldest known biomolecules, recovered from sediments that are 2.5 billion years old (Lange *et al.*, 2000; Brocks *et al.*, 1999; Summons, 1999). They constitute the largest group of naturally occurring compounds with over 35,000 known compounds (Hunter, 2007; Dubey *et al.*, 2003; Dewick, 2002; Lange *et al.*, 2000; Sacchettini and Poulter, 1997). Isopentenyl pyrophosphate/Isopentenyl diphosphate (IPP) and its dimer, dimethylallyl pyrophosphate/dimethylallyl diphosphate (DMAPP) are basic C_5 isoprene units

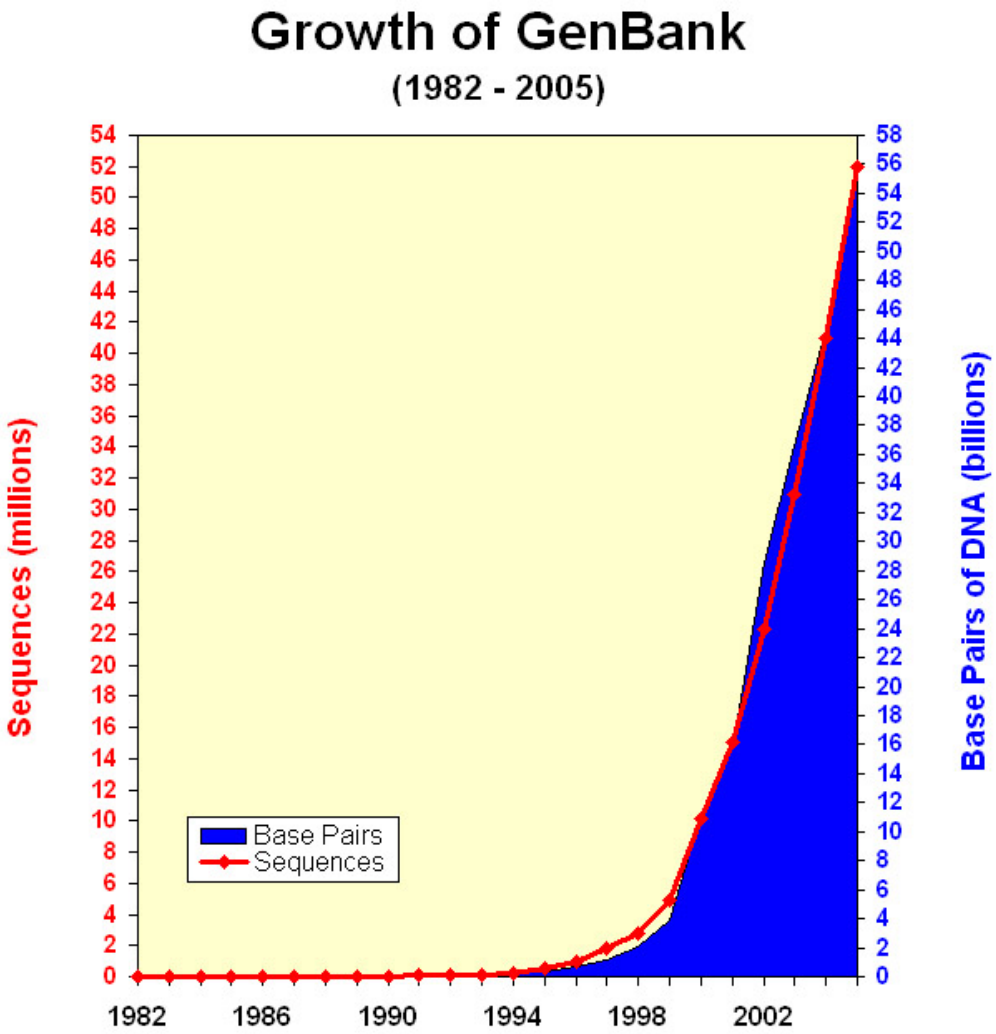


Figure 1.1: Growth of GenBank from 1982 to 2005 — reproduced from `http://www.ncbi.nlm.nih.gov/Genbank/genbankstats.html`.

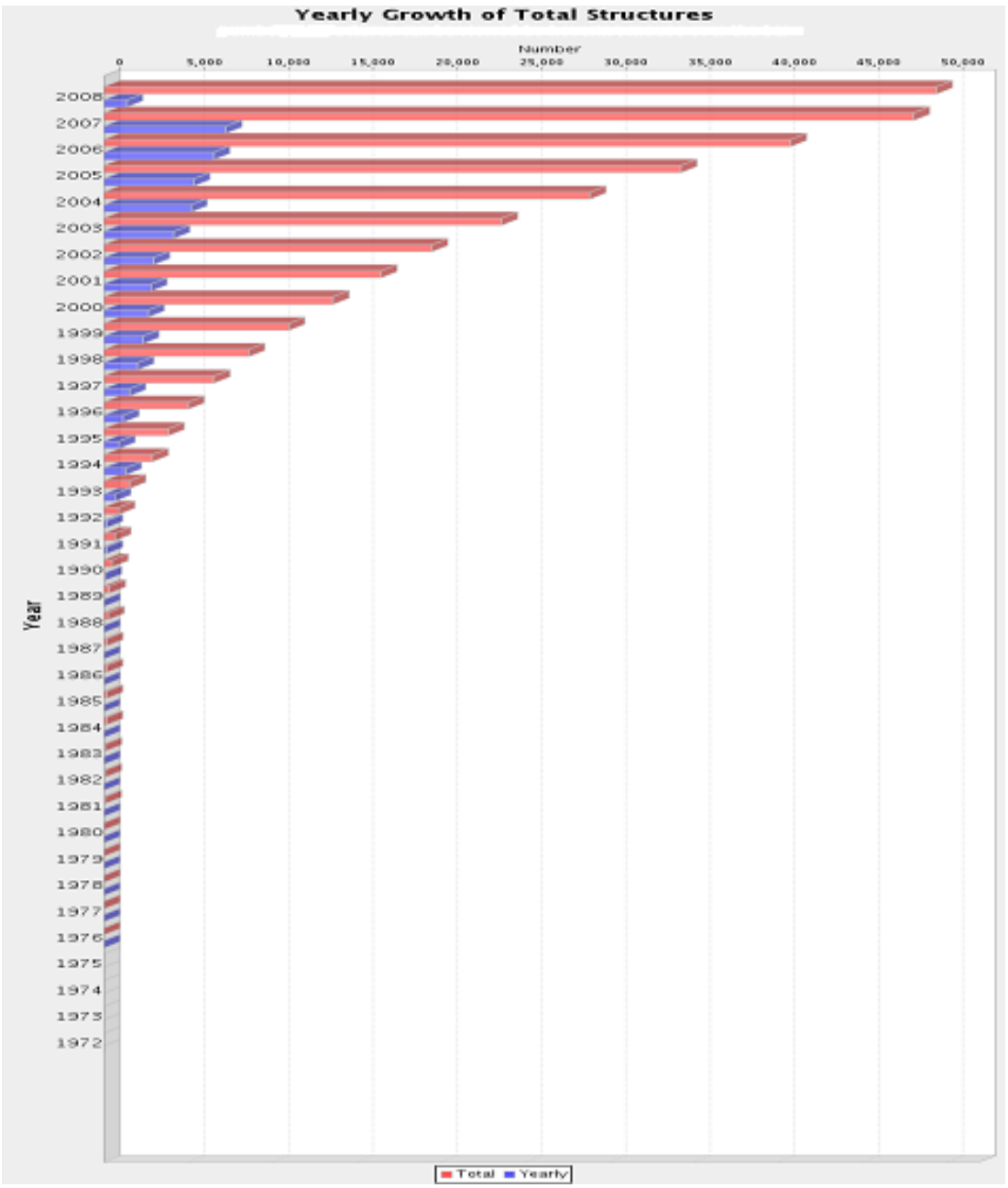


Figure 1.2: Growth of PDB from 1972 to 2008 — reproduced from `http://www.rcsb.org/pdb/statistics/contentGrowthChart.do?content=total&seqid=100`.

which act as precursors of natural products called isoprenoids.

Isoprenoids are the building blocks of various essential components of the cell and perform a wide variety of biochemical functions (Hunter, 2007; Dubey *et al.*, 2003; Mahmoud and Croteau, 2002; Wanke *et al.*, 2001; Lange *et al.*, 2000; Bach *et al.*, 1999; Sacchettini and Poulter, 1997). These include:

- respiration (electron transport) — ubiquinone,
- photosynthesis (pigments) — carotenoids, chlorophylls, and plastoquinones,
- structural components of biological membranes — prenyllipids (archaebacteria) and sterols (eubacteria and eukaryotes),
- hormones (growth and regulation) — in plants (gibberellins, brassinosteroids, abscisic acid, cytokinins, prenylated proteins) and in animals (steroid hormones and pheremones),
- defense system — in plants (monoterpenes, sesquiterpenes, diterpenes — essential oils which are also used as flavouring and fragrance agents in foods, beverages, cosmetics, perfumes, soaps) and in animals (apoptosis, protein cleavage and degradation),
- intracellular signal transduction and vesicular transport or subcellular transport — Ras proteins and Rab proteins (prenylated proteins),
- coenzymes — dolichols,
- regulation of transcription and post-translational processes, lipid biosynthesis, meiosis, and glycoprotein biosynthesis.

Isoprenoids are ubiquitous in all living organisms and were previously assumed to be synthesized only through the mevalonate (MVA) pathway (Buhaescu and Izzedine, 2007; Kobayashi *et al.*, 2007; Boucher and Doolittle, 2000; Bach *et al.*, 1999; Eisenreich *et al.*, 1998; Goldstein and Brown, 1990; Spurgeon and Porter, 1981; Beytía and Porter, 1976) until the recent discovery of an alternate methyl erythritol phosphate (MEP) pathway (Hunter, 2007; Xiang *et al.*, 2007; Seemann *et al.*, 2006; Eisenreich *et al.*, 2004; Rohdich *et al.*, 2004; Dubey *et al.*, 2003; Meyer *et al.*, 2003; Kemp *et al.*, 2002; Rodríguez-Concepción and Boronat, 2002; Lange *et al.*, 2000; Rohmer, 1999). Among these two different metabolic routes that lead to biosynthesis of IPP and DMAPP, the mevalonate pathway occurs in some eukaryotes (animals including all mammals), fungi, plant cytosol and mitochondria, archaebacteria, some eubacteria, and *Trypanasoma* and *Leshmania* (Hunter, 2007). The MEP pathway occurs in algae, cyanobacteria, most eubacteria, plant plastids (including chloroplast), apicomplexan parasites' apicoplast and *Mycoplasma penetrans* (unlike most other mycoplasmas) (Eberl *et al.*, 2004).

The Mevalonate pathway starts with condensation of acetyl-CoA molecules and uses seven enzymes during the biosynthesis of IPP and DMAPP whereas to synthesize the same compounds, the MEP pathway starts with condensation of pyruvate and D-glyceraldehyde 3-phosphate and uses nine enzymes (including two types of non-homologous IPP isomerases — IDI-I and IDI-II) to carry out eight reactions. (Figure 1.3).

1.1.1 MEP pathway

The following names of the pathway are all synonymous:

- MEP pathway — 2-C-methyl-D-erythritol 4-phosphate pathway

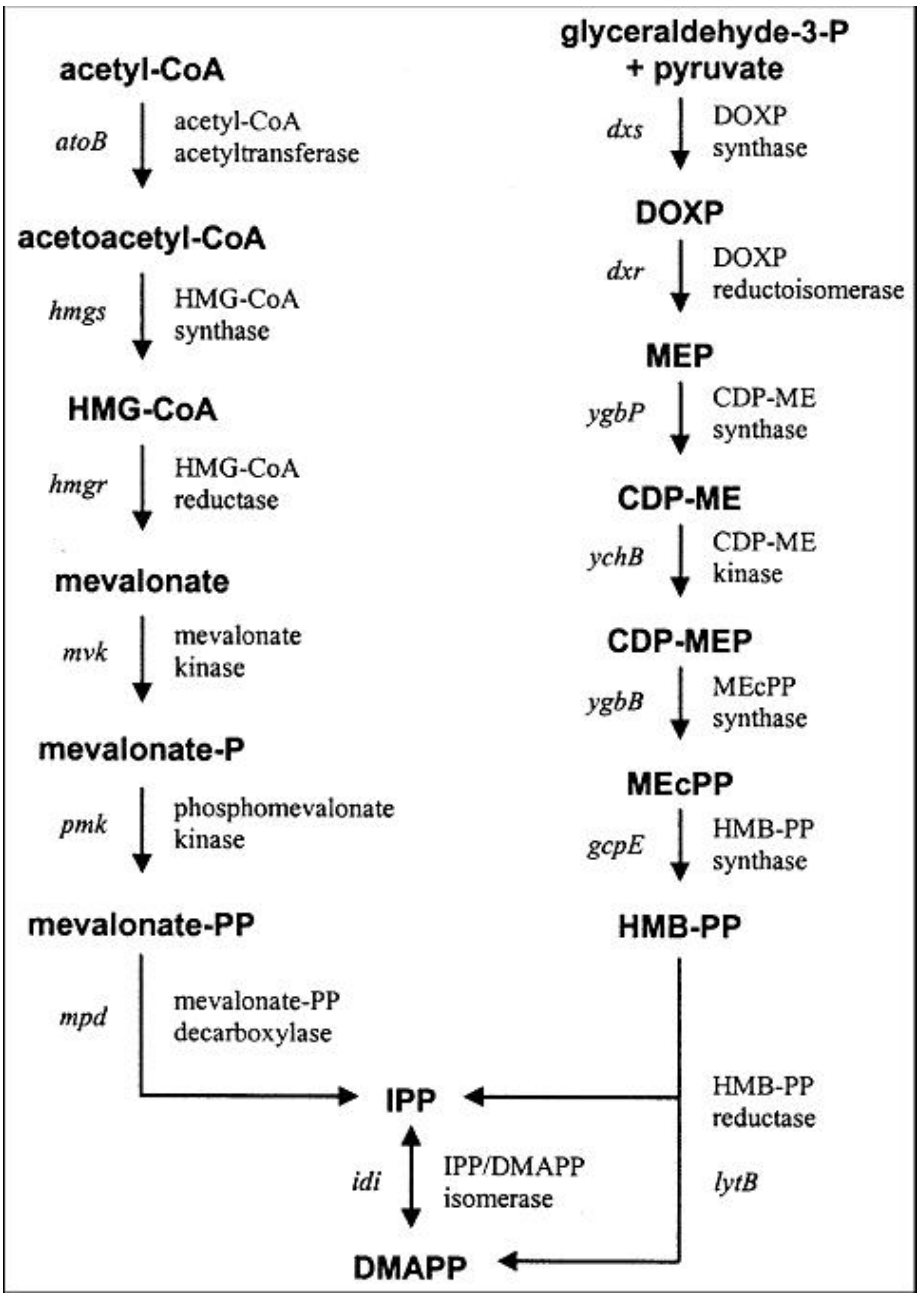


Figure 1.3: Isoprenoid biosynthesis in L. monocytogenes via the classical mevalonate pathway (left) and the alternative MEP pathway (right). CDP-ME, 4-diphosphocytidyl-2-C-methyl-D-erythritol; CDP-MEP, 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate; DMAPP, dimethylallyl pyrophosphate ; DOXP, 1-deoxy-D-xylulose 5-phosphate; MEcPP, 2-C-methyl-D-erythritol 2,4-cyclopyrophosphate; P, phosphate (figure and legend reproduced from (Begley *et al.*, 2004)).

- DXP/DOXP pathway — 1-deoxy-D-xylulose 5-phosphate pathway
- Non-mevalonate pathway

While the first two names originate from the intermediate compounds of the pathway, the last one is to distinguish it from the conventional mevalonate pathway.

The MEP biosynthetic pathway (shown in Figure 1.3 and in Figure 1.4 in more detail) is unique to, and important for, the survival of many pathogenic organisms that cause major diseases such as malaria, tuberculosis, leprosy, meningitis, bubonic plague, cholera, and typhoid. In addition, the pathway is also present in the unusual protozoan organelle, the apicoplast, which is present in many important pathogens such as *Plasmodium falciparum*, *Toxoplasma gondii*, and *Eimeria*. Since the isopernoids are synthesized through a totally different mevalonate pathway in humans and other higher animals, the MEP pathway enzymes and apicoplast proteins are of great pharmaceutical significance. Chances of drug cross-reaction will be minimal while targeting these proteins.

1. The MEP pathway commences with condensation of pyruvate and D-glyceraldehyde-3-phosphate (GAP) to form 1-deoxy-D-xylulose-5-phosphate (DXP) in the presence of 1-deoxy-D-xylulose-5-phosphate synthase (DXP Synthase). DXP is also a metabolite in biosynthesis of thiamine (vitamin B1) and pyridoxin (vitamin B6) (Xiang *et al.*, 2007; Sauret-Güeto *et al.*, 2006; Dubey *et al.*, 2003; Finkelstein and Rock, 2002; Richard *et al.*, 2002, 2001). Very recently a crystal structure of DXP synthase has been published by Xiang *et al.* (2007).

2. DXP is then converted to 2C-methyl-D-erythritol-4-Phosphate (MEP) in

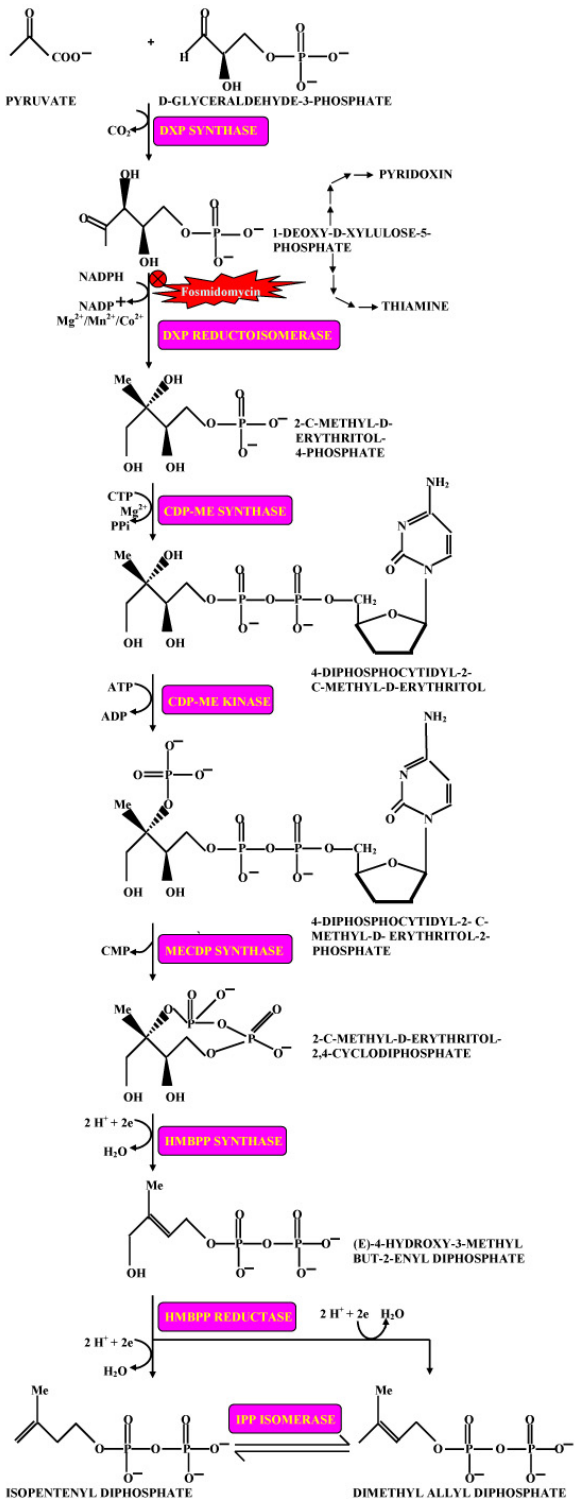


Figure 1.4: The MEP pathway in detail.

the presence of 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXPRI), NADPH and a divalent cation such as Mg^{2+}, Mn^{2+} or Co^{2+}. DXPRI is the most extensively studied enzyme of the MEP pathway because it is a target for the herbicide fosmidomycin (Cassera *et al.*, 2007; Na-Bangchang *et al.*, 2007; Lell *et al.*, 2003; Steinbacher *et al.*, 2003a; Wiesner *et al.*, 2003; Missinou *et al.*, 2002; Wiesner *et al.*, 2002; Shigi, 1989; Okuhara *et al.*, 1980). Crystal structures of DXPRI have been published by Ricagno *et al.* (2004), Henriksson *et al.* (2007), Steinbacher *et al.* (2003a), Yajima *et al.* (2007; 2002).

3. In the presence of CTP, 4-diphosphocytidyl-2C-methyl-D-erythritol cytidyltransferase (CDP-ME synthase) converts MEP to 4-diphosphocytidyl-2C-methyl-D-erythritol (CDP-ME). Crystal structures have been published by Gabrielsen *et al.* (2006), and Richard *et al.* (2001).

4. CDP-ME is then converted to 4-diphosphocytidyl-2C-methyl-D-erythritol-2-phosphate (CDP-MEP) in the presence of ATP and 4-diphosphocytidyl-2C-methyl-D-erythritol kinase (CDP-ME kinase). Crystal structures have been published by Miallau *et al.* (2003), and Wada *et al.* (2003).

5. CDP-MEP is then converted to 2C-methyl-D-erythritol-2,4-cyclodiphosphate (MECDP) in the presence of CTP and 2C-methyl-D-erythritol-2,4-cyclodiphosphate synthase (MECDP synthase). Crystal structures have been published by Crane *et al.* (2006), Sgraja *et al.* (2005), Ni *et al.* (2004), Gabrielsen *et al.* (2004), Kishida *et al.* (2003), Steinbacher *et al.* (2002), Richard *et al.* (2002), and Kemp *et al.* (2002).

6. MECDP undergoes a reduction reaction releasing a water molecule in

the presence of (E)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase (HMBPP synthase) (Seemann *et al.*, 2005; Altincicek *et al.*, 2002; Kollas *et al.*, 2002; Seemann *et al.*, 2002) to form (E)-4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP). No crystal structures have been published to date and the mechanism of action is not clearly understood.

7. HMBPP undergoes a reduction reaction releasing a water molecule in the presence of (E)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HMBPP reductase) (Lu *et al.*, 2007; Gräwert *et al.*, 2004; Wolff *et al.*, 2003) to form isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) in a ratio ranging from 3:1 to 6:1 (Hsieh and Goodman, 2006; Sauret-Güeto *et al.*, 2006; Wolff *et al.*, 2003; Adam *et al.*, 2002). No crystal structures have been published to date and the mechanism of action is not clearly understood.

8. In the final step, isomerization between IPP and DMAPP is carried out in the presence of isopentenyl diphosphate isomerase (IPP isomerase)/isopentenyl diphosphate isomerase:dimethylallyl diphosphate isomerase (IDI) (Anderson *et al.*, 1989). Until recently, only one type of IDI was known, but recently two non-homologous protein families were identified (Kaneda *et al.*, 2001).

   (a) Type I IDI (IDI-I) has been known for a long time (Agranoff *et al.*, 1960, 1959) and is the conventional IDI that is found in many organisms (Rohdich *et al.*, 2004) (eukaryotes and most eubacteria) which include *Arabidopsis thaliana* (Campbell *et al.*, 1998), *Saccharomyces cerevesiae* (Anderson *et al.*, 1989), *Escherichia coli* (Hahn *et al.*, 1999),

and humans (Hahn *et al.*, 1996). IDI-I requires only a divalent metal ion for its activity. IDI-I displays a compact α/β architecture. Crystal structures have been published by Durbecq *et al.* (2001), Wouters *et al.* (2005; 2004; 2003; 2003), Zhang *et al.* (2007), Zheng *et al.* (2007), de Ruyck *et al.* (2006), Oudjama *et al.* (2001), and Bonanno *et al.* (2001).

(b) Type II IDI (IDI-II) was recently discovered in *Streptomyces* sp. strain CL190 (Kaneda *et al.*, 2001) and is present in Archaea and some eubacteria. In addition to a divalent metal ion, it requires flavin mononucleotide (FMN) and NADPH for its activity. It is an octamer with a cage-like structure with each subunit displaying a triosephosphate isomerase (TIM) barrel fold ($\alpha_8\beta_8$). Crystal structures of *Bacillus subtilis* (Steinbacher *et al.*, 2003b) and *Thermus thermophilus* (de Ruyck *et al.*, 2005) have been published.

In the MEP pathway, both IPP and DMAPP are produced (in varied proportions) during the final step whereas in the mevalonate pathway, only IPP is produced which must be converted to DMAPP by an IPP isomerase/IDI. Although both the pathways use IDI for isomerization there is no simple correlation between the type of IDI (IDI-I or IDI-II) that occurs and the type of pathway an organism uses (Zheng *et al.*, 2007; Steinbacher *et al.*, 2003b). This can be best seen through a few example organisms (Steinbacher *et al.*, 2003b) as shown here:

Mevalonate pathway and IDI-I: Humans, *Saccharomyces cerevisiae*, and the cytosol of *Arabidopsis thaliana*,

Mevalonate pathway and IDI-II: Archaea, *Streptomyces* sp. CL190 or

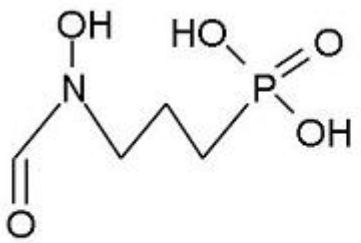
Gram-positive pathogens such as *Staphylococcus aureus*, *Streptococci*, *Enterococci*,

MEP pathway and IDI-I *Escherichia coli*,

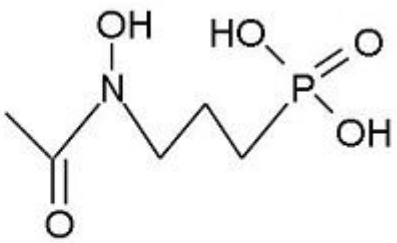
MEP pathway and IDI-II *Bacillus subtilis*, *Synechocystis* sp. PCC 6803 or *Deinococcus radiodurans*.

IDIs are critical for the survival of the organisms that use the mevalonate pathway for isoprenoid biosynthesis (essential — *S. cerevisiae* (mevalonate), non-essential — *E. coli* (MEP) (Steinbacher *et al.*, 2003b)). The variety in occurrence of a type of IDI and the metabolic route taken by the organism for isoprenoid biosynthesis coupled with the essentiality of IDI to the survival of an organism makes these enzymes an attractive drug target. For example, Humans and some Gram-positive multi-drug resistant bacterial strains of *S. aureus*, *Streptococci*, and *Enterococci* use the mevalonate pathway which makes IDI an essential enzyme, but the occurrence of IDI-I in humans in contrast to IDI-II in these pathogens is an interesting aspect for designing novel drugs against these pathogens (Steinbacher *et al.*, 2003b).

Fosmidomycin (FR-31564 — 3-(N-formyl-N-hydroxy) aminopropylphosphonic acid; Figure 1.5) is a phosphonic acid herbicide/antibiotic discovered in Fujisawa Research Laboratories in the fermentation broths of *Streptomyces lavendulae* (Shigi, 1989; Okuhara *et al.*, 1980). Fosmidomycin is the most extensively studied inhibitor of the MEP pathway and inhibits the second enzyme of the pathway, DXPRI. Crystal structures of DXPRI complexed with fosmidomycin (Henriksson *et al.*, 2007; Yajima *et al.*, 2007; Mac Sweeney *et al.*, 2005; Steinbacher *et al.*, 2003a) and its derivative FR-900098



Fosmidomycin



FR-900098

Figure 1.5: Chemical structures of Fosmidomycin (3-(N-formyl-N-hydroxy)aminopropylphosphonic acid) and FR-900098 (3-(N-acetyl-N-hydroxy)aminopropylphosphonic acid).

(3-(N-acetyl-N-hydroxy)aminopropylphosphonic acid; Figure 1.5) are available (Mac Sweeney *et al.*, 2005; Steinbacher *et al.*, 2003a). Fosmidomycin was proven to be effective against malaria (especially uncomplicated malaria (Missinou *et al.*, 2002)) but nevertheless it has shown an unacceptable disease reoccurence (Wiesner *et al.*, 2002) which lead to identifying a potential combination partner in clindamycin (Lell *et al.*, 2003). The combined therapy is very effective, and in the case of multidrug resistant *Plasmodium falciparum*, produced a 100% cure (Na-Bangchang *et al.*, 2007).

1.2 The Apicoplast

The apicoplast is a unique semi-autonomous organelle (plastid) thought to be reminiscent of the chloroplast and is found in apicomplexan group of protists (which mostly are obligate parasites). Although the origin (Waller *et al.*, 2003) of the apicoplast is still not very clear, it is best explained by endosymbiotic theory (Margulis and Bermudes, 1985; Cavalier-Smith, 1982; Margulis, 1981). It

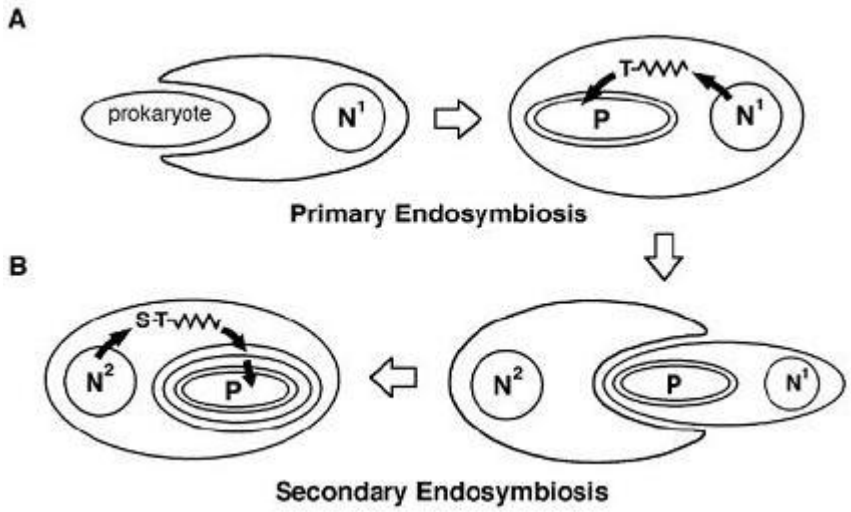


Figure 1.6: Plastid origins and protein targeting. (A) Primary endosymbiosis describes the uptake of a prokaryote by a eukaryote. Plastids derived by primary endosymbiosis are surrounded by two membranes and targeting of nucleus-encoded gene products to the endosymbiont is affected by an N-terminal transit peptide (T). (B) Secondary endosymbiotic plastid origin involves a heterotrophic eukaryote phagocytosing a photosynthetic eukaryote possessing a primary endosymbiont. The secondary endosymbiont's cytoplasm and nucleus (N1) are typically lost and the resulting plastid is surrounded by four membranes. Sometimes one of the two outer membranes is lost at this point, resulting in a total of three. Targeting of nucleus-encoded (N2) gene products to secondary plastids requires a signal peptide (S) to mediate protein passage across the outer membrane followed by a transit peptide (T) for import across the inner membranes. (figure and legend reproduced from (Waller and McFadden, 2005)).

is thought to have originated through secondary endosymbiosis (McFadden, 2001; Vellai *et al.*, 1998) (which would also explain the presence of four membranes (Köhler *et al.*, 1997) around the apicoplast) where the endosymbionts could be either green algae or red algae (contradictory evidence exists) (Palmer, 2003; Waller *et al.*, 2003; Funes *et al.*, 2002; McFadden, 1999; Köhler *et al.*, 1997).

During the process of endosymbiosis, which would have happened two times in the case of the apicoplast, a huge amount of genetic material was lost because of disintegration, leaving behind just 35kb of circular DNA (Wilson and Williamson, 1997; Wilson *et al.*, 1996, 1991). As a result of disintegration, the apicoplast has lost many functions including the ability to perform phostosynthesis, but it has acquired many new functions which clearly cannot be carried out by 35kb of DNA which has rRNA and tRNA genes and 28 open reading frames (Dahl *et al.*, 2006; Harb *et al.*, 2004; Wilson *et al.*, 1996). All of these take part in self-replication (unlike eukaryotic nuclear replication, it is prokaryotic DNA replication, transcription, translation) of the organelle (i.e., house keeping) (He *et al.*, 2001; Ralph *et al.*, 2001; Köhler *et al.*, 1997). Most of the apicoplast proteins are nuclear encoded and are later targetted to the apicoplast (Harb *et al.*, 2004; Ralph *et al.*, 2004a; van Dooren *et al.*, 2002; He *et al.*, 2001; Zuegge *et al.*, 2001; Waller *et al.*, 2000, 1998). Although the exact role of the apicoplast is not clear, it is essential for the survival of the organism and is the site for many biosynthetic pathways which include the MEP pathway for isoprenoid biosynthesis, type II fatty acid synthesis, Shikimate pathway for biosynthesis of aromatic amino acids and heme biosynthesis (Dahl *et al.*, 2006; Wilson, 2005; Ralph *et al.*, 2004b, 2001).

Pathogens having an apicoplast include:

- *Toxoplasma* species. These cause toxoplasmosis and congenital birth disorders in humans and livestock (Waller and McFadden, 2005; Wilson, 2005; Jenkins, 2001; Roizen *et al.*, 1995; Dubey and Welcome, 1988) and cause an opportunistic infection related to AIDS (He *et al.*, 2001; Luft *et al.*, 1993)

- *Plasmodium* species (Waller and McFadden, 2005; Wilson, 2005; He *et al.*, 2001; Wilson *et al.*, 1996). These cause malaria, annually infecting approximately 300 million people leading to the death of 1 million people out of which a large proportion are caused by the most virulent and multi drug-resistant *Plasmodium falciparum* (van Dooren *et al.*, 2002; W.H.O., 1999)

- *Eimeria* species. These cause coccidiosis in poultry and farm animals (Waller and McFadden, 2005; Wilson, 2005; Harb *et al.*, 2004; He *et al.*, 2001; Jenkins, 2001).

- *Babesia* (He *et al.*, 2001; Jenkins, 2001) and *Theilaria* (Waller and McFadden, 2005; Wilson, 2005; He *et al.*, 2001; Jenkins, 2001) are other apicoplast-bearing pathogens of livestock.

1.3 Structure Based Drug Design

Both 'receptor based drug design' and 'ligand based drug design' fall into 'structure based drug design' (SBDD) (Tintelnot-Blomley and Lewis, 2006; Acharya *et al.*, 2003; Anderson, 2003; Jones and Mongin-Bulewski, 2002; Saphire, 2002). Finding a new drug molecule that alters the activity of a protein, given its structure and/or its binding site is receptor based drug design, whereas finding a new

drug molecule that alters the activity of a protein, given an active ligand is ligand based drug design.

SBDD was earlier used for lead optimization, but now covers and supports virtually all steps in the drug discovery pipeline (Tintelnot-Blomley and Lewis, 2006). Building 3D computer models of proteins for SBDD is being commonly practiced (Karkola *et al.*, 2007; Volarath *et al.*, 2007; Singh *et al.*, 2006b). SBDD is being effectively used in combination with virtual screening (Li *et al.*, 2007), quantum mechanics (Raha *et al.*, 2007; Peters *et al.*, 2006), docking (Kroemer, 2007), and X-ray crystallographic studies (Kinoshita, 2007; Yan *et al.*, 2007) for understanding various catalytic mechanisms of enzymes (such as the MEP pathway enzymes (de Ruyck and Wouters, 2008)), finding new molecular drug targets (Singh *et al.*, 2006b; Swindells and Overington, 2002; Swindells and Fagan, 2001; Fagan *et al.*, 2001) and small molecule drugs many of which are in clinical trials or on the market (Fox *et al.*, 2007).

1.4 Druggability

The term "druggability" is often classified into two broad groups and is used in three distinct ways in the field of drug discovery:

1. While referring to molecular targets — druggable genome and druggable proteins. This can refer to:

   (a) the biological suitability of a target (Sugiyama, 2005; Swindells and Overington, 2002; Swindells and Fagan, 2001; Fagan and Swindells, 2000), or

   (b) the protein's physical suitability for binding a small molecule drug (Ha-

jduk *et al.*, 2005a,b; An *et al.*, 2004; Campbell *et al.*, 2003; Laskowski *et al.*, 1996)

2. While referring to druggable compounds — small molecule drugs (Cheng *et al.*, 2007; Sirois *et al.*, 2005; Sugiyama, 2005; Brown and Superti-Furga, 2003; Lipinski *et al.*, 2001).

Druggability in the former case can be defined as the ability of a portion of a genome (i.e., specific groups of proteins), or a protein, to be targetted by a drug, especially by a small molecule drug. In other words, the probability of regulating a target with a small molecule drug which is essential in determining the success of a hit along the drug discovery pipeline (Owens, 2007). While the biological macromolecules which could be modulated by small molecule drugs include proteins, polysaccharides, lipids and nucleic acids, macromolecules other than proteins are largely unaffected because they lack suitable potent compounds (with low toxicity and high specificity) that act against them (Hopkins and Groom, 2002). It has to be noted that a small, but nevertheless significant group of drug molecules succeeded in acting against DNA (Shaikh *et al.*, 2004; Gambari *et al.*, 2000; Kennard, 1993; Le Pecq *et al.*, 1975) (see Table 1.1). One such revolutionary anticancer DNA drug is 'cisplatin' — *cis*-diamminedichloridoplatinum(II) (Alderden *et al.*, 2006) which binds to DNA by a covalent cross-link (see Figure 1.7). Cisplatin's[1] pharmacological significance was discovered by Barnett Rosenberg (1965) when he noticed the inhibition of cell division in *Escherichia coli* by electrolysis products from a platinum electrode.

[1]Cisplatin was first synthesized by Michael Peyrone (1845) whose structure and configuration (also distinguishing between *cis* and *trans* forms) was correctly predicted by Alfred Werner while establishing his theory of coordination chemistry (1893). Werner received a Nobel Prize for Chemistry in 1913 (`http://nobelprize.org/nobel_prizes/chemistry/laureates/1913/index.html`).

S.No.	Drug	Action	Mode of Binding	PDB code
1	Hoechst 33258	Antitumor	Minor groove binding	264D
2	Netropsin	Antitumor, Antiviral	Minor groove binding	121D
3	Pentamidine	Active against *P. carinii*	Minor groove binding	1D64
4	Berenil	Antitrypanosomal	Minor groove binding	1D63
5	Guanyl bisfuramidine	Active against *P. carinii*	Minor groove binding	227D
6	Netropsin	Antitumor, Antiviral	Minor groove binding	121D
7	Distamycin	Antitumor, Antiviral	Minor groove binding	2DND
8	SN7167	Antitumor, Antiviral	Minor groove binding	328D
9	SN6999	Active against *P. falciparum*	Minor groove binding	144D
10	Nogalamycin	Antitumor	Intercalation	182D
11	Menogaril	Antitumor-Topoisomerase II poison	Intercalation	202D
12	Mithramycin	Anticancer antibiotic	Minor groove binding	146D
13	Plicamycin	Anticancer antibiotic	Minor groove binding	1BP8
14	Chromomycin A3	Anticancer antibiotic	Minor groove binding	1EKH
15	cisPlatin	Anticancer antibiotic	Covalent cross-linking	1AU5

Table 1.1: Drug, action and mode of binding for some DNA binding drugs (Table and caption reproduced from `http://www.scfbio-iitd.res.in/doc/preddicta.pdf`).

Recent advances in 3D structure determination of RNA along with studies which revealed surprising intricacy in RNA structure opened up the possibility of exploring RNA as a drug target for small molecule drugs (Klinck *et al.*, 2000; Ecker and Griffey, 1999) such as 'Hoechst 33258' which selectively inhibits group I intron self-splicing by affecting RNA folding (Disney *et al.*, 2004).

Among the ∼30,000 genes in the human genome only ∼3000 (10%) genes code for druggable proteins and only ∼600–1,500 (5%) genes are drug targets (i.e., both druggable and relevant to disease) (Hopkins and Groom, 2002) (See Figure 1.8).

In spite of monumental advances in molecular biology, X-ray crystallography and NMR techniques, completion of the human genome project, which should have unravelled many clues about diseases, and a sharp rise in research and development investments, clinical validation and marketing of drug targets is happening at a slower pace. Every year, on average, only four new drugs are being launched against novel targets (Hopkins and Groom, 2002) (See Figure 1.9).

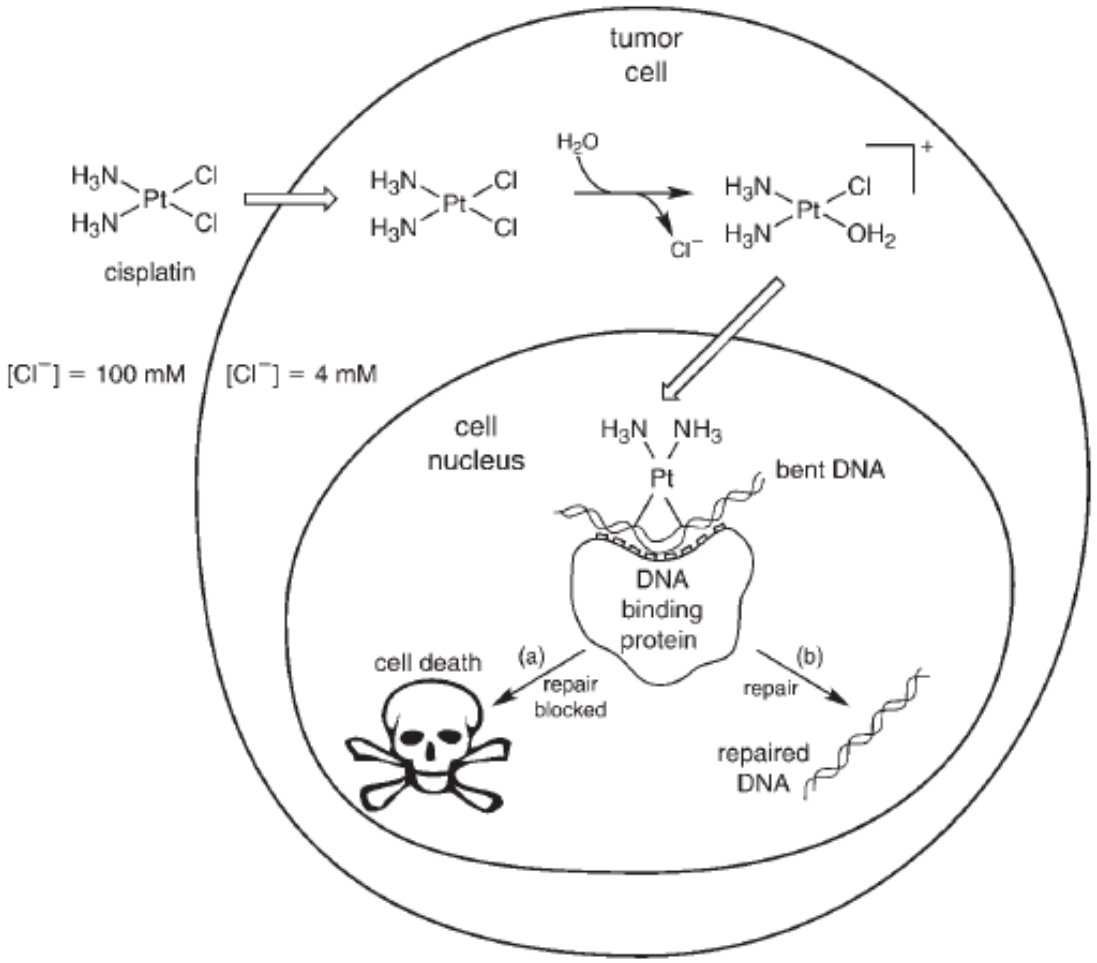


Figure 1.7: Schematic showing the cytotoxic pathway for cisplatin. After entering the cell, cisplatin is aquated, then binds to cellular DNA. If the DNA lesion is not repaired by the cell (path a), then cell death (apoptosis), can occur (Figure and caption reproduced from (Alderden *et al.*, 2006).

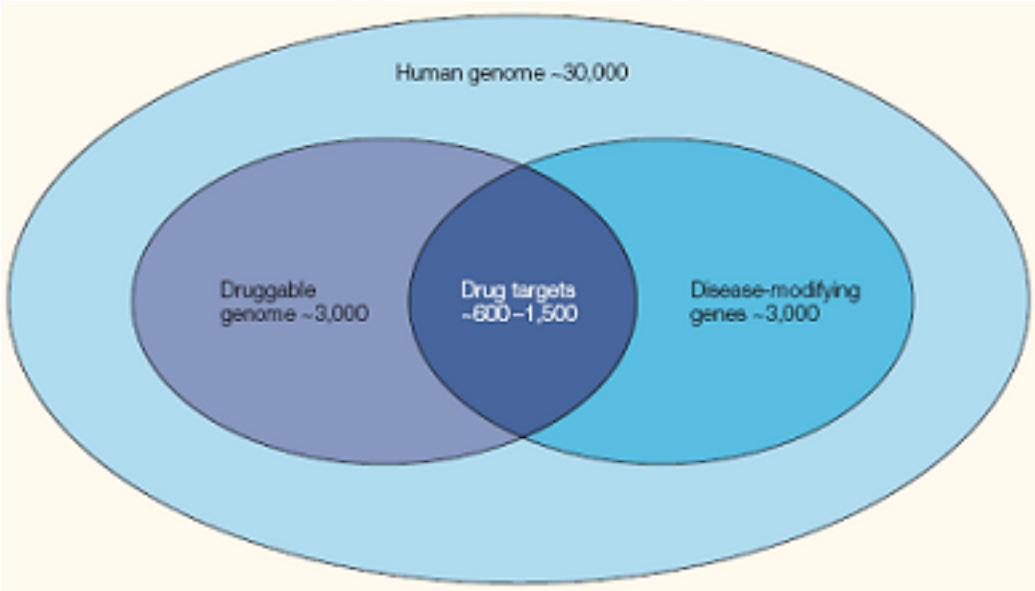


Figure 1.8: The effective number of exploitable drug targets can be determined by the intersection of the number of genes linked to disease and the 'druggable' subset of the human genome (Figure and caption reproduced from (Hopkins and Groom, 2002)).

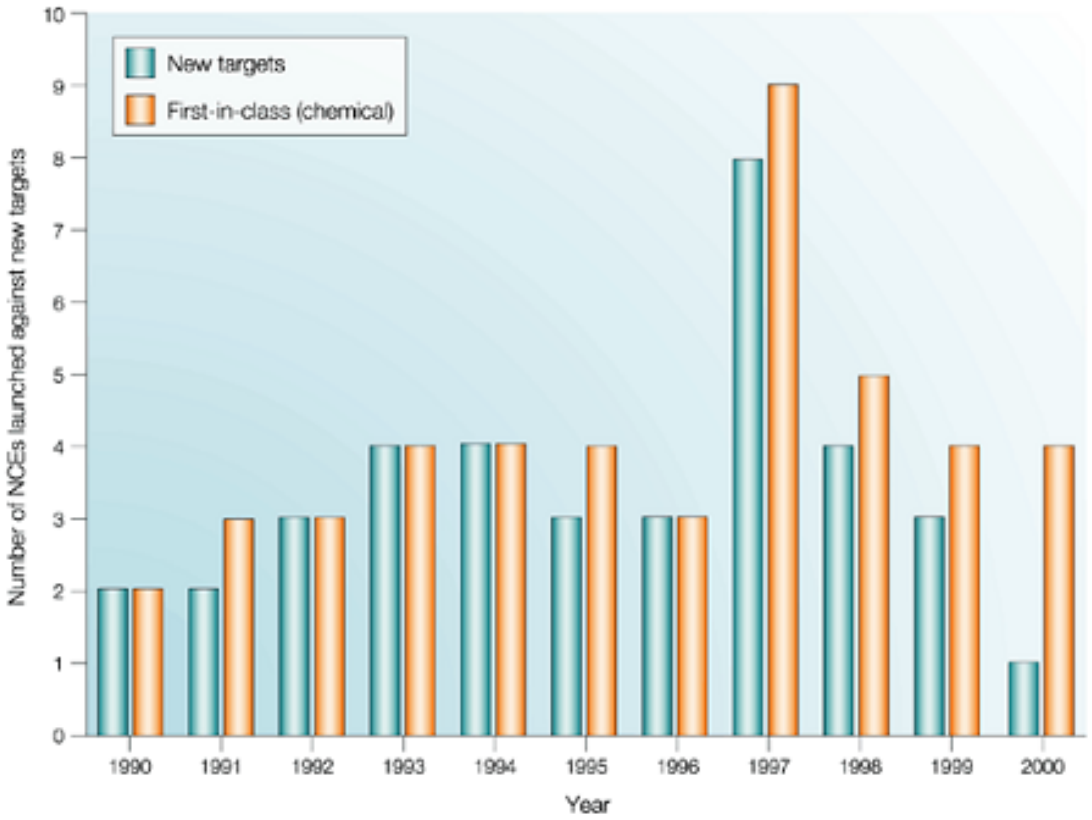


Figure 1.9: The graph shows the number of small-molecule, 'first-in-class' drugs and associated new drug targets that have been launched on the market in the past decade (data derived from collating annual "This Year's Drugs" reviews of Drug News & Perspectives, Prous Science). NCE, new chemical entity. (Figure and caption reproduced from Hopkins and Groom (2002))

Predicting protein druggability, ideally as early as possible in the drug discovery pipeline is an important advantage because it reduces expenditure and time loss. Various methods developed to achieve this include:

1. Classifying targets based on whether or not they belong to druggable gene families (Cheng *et al.*, 2007; Hopkins and Groom, 2002; Drews, 1996; Drews and Ryser, 1997a). Almost 50% of the targets fall into just six gene families — G-protein coupled receptors (GPCRs), serine/threonine and tyrosine protein kinases, zinc metallopeptidases, serine proteases, nuclear hormone receptors and phosphodiesterases (Hopkins and Groom, 2002). Nevertheless, other gene families have produced new targets and different proteins of a gene family differ in druggability (Fauman *et al.*, 2003).

2. Hajduk *et al.* (2005a) developed a method to predict the druggability of protein targets using indices derived from NMR-based screening data.

3. Predicting protein druggability based solely on 3D structure. Hajduk *et al.* (2005b) suggest potential utility of tools for characterizing protein targets (finding druggable protein pockets) and strategies for integrating data of protein druggability with bioinformatics approaches to select druggable targets.

4. Predicting the biological suitability (druggability) of a target by using computational tools and databases (computational proteomics), mathematical algorithms and chemical techniques to obtain as much relevant information as possible in the shortest possible time are becoming increasingly popular (Overington *et al.*, 2006; Maggio, 2002; Swindells and Overington, 2002; Chan and Weir, 2001; Fagan *et al.*, 2001; Maggio and Ramnarayan, 2001;

Drews and Ryser, 1997b).

The second definition of druggability is the ability of a compound to show drug-like properties which are the physicochemical (solubility, stability, etc.) and biological (absorption, distribution, metabolism, elimination and toxicity — ADME-Tox) characteristics that are consistent with good clinical performance (Sugiyama, 2005; Sirois *et al.*, 2005). Approximately 60% of drug candidates (Cheng *et al.*, 2007; Brown and Superti-Furga, 2003) are dropped during the passage from hit-to-lead because they fail to show drug-like properties and many pharmaceutical companies look out for these properties as early as possible. Lipinski *et al.* (2001) formulated a 'rule of five' (ROF) to look for physicochemical properties that increase the probability of a drug candidate's oral bioavailability. The ROF predicts that poor absorption or permeation is more likely for molecules having > 5 H-bond donors, 10 H-bond acceptors (sum of nitrogen and oxygen atoms), a molecular weight (MW) > 500 Da, and high lipophilicity (ClogP $>$ 5, or MlogP > 4.15. ClogP is a partition coefficient that indicates a molecule's hydrophobicity, and MlogP is an octanol-water partition coefficient). Pharmaceutical companies accept ROF as being a measure for good drug candidates. High throughput screening (HTS) of chemical libraries is another commonly used method for identifying hits (Sirois *et al.*, 2005) and associating HTS with NMR technology is even more powerful (Vogtherr and Fiebig, 2003). To minimize expenditure and time loss further, purely structure-based methods have been developed such as the 'structure based maximal affinity' model (Cheng *et al.*, 2007) which predicts small molecule druggability based solely on the crystal structure of the target binding site.

In the context of this project, druggability is used to refer to the biological suitability of targets with some incorporation of basic structural knowledge (i.e., known ligands suggest suitability for SBDD). This will be especially useful when dealing with a set of unannotated hypothetical sequences most of which do not have solved 3D structures. It does not address the suitability of the target protein in terms of pockets able to bind a drug.

Druggable targets or drug targets?

When referring to the human genome, or to other non-pathogenic eukaryotic organisms, proteins that are druggable are called 'druggable targets' whereas proteins must be related to a disease, in addition to being druggable, to be called 'drug targets' (Hopkins and Groom, 2002). This project deals with pathogenic bacteria and protists and thus all their druggable proteins may qualify as 'drug targets' because these organisms are disease-related from a human perspective. In addition to that they are regarded as essential for the survival of these pathogens. Thus, the term 'potential drug target' is used in the rest of this thesis.

For the reasons mentioned earlier, annotating the proteins of the apicoplast and the MEP pathway is of great significance. This led to the creation of the APAT tool and the TAPAS pipeline. In the next chapter, I will review various tools for mass sequence analysis.

Chapter 2

Introduction to Bioinformatics Tools - Mass Sequence Analysis

In this chapter, I present a review of various high throughput sequence analysis tools such as workflows/pipelines, highlighting their advantages and disadvantages.

2.1 Workflows

Sequence analysis which involves annotation of a whole genome, proteins of a particular pathway, or of an organelle, requires automatic execution of various prediction and annotation tools residing locally, or remotely, in a parallel or sequential manner followed by integration of results from these tools.

A workflow can be defined as a set of analyses to be performed on a single sequence, or set of sequences (Shah *et al.*, 2004). The characteristics of a workflow include:

1. the analyses can be tied such that output from one analysis can be used as

input to subsequent analyses,

2. analyses can accept outputs from more than one analysis as input, and

3. analyses that need not be run serially can be executed in parallel.

> "Workflow is concerned with the automation of procedures whereby files and data are passed between participants according to a defined set of rules to achieve an overall goal (Hollingsworth, 1995). A workflow management system defines, manages and executes workflows on computing resources. Imposing the workflow paradigm for application composition on Grids offers several advantages (Spooner *et al.*, 2004) such as:
>
> - Ability to build dynamic applications which orchestrate distributed resources.
> - Utilization of resources that are located in a particular domain to increase throughput or reduce execution costs.
> - Execution spanning multiple administrative domains to obtain specific processing capabilities.
> - Integration of multiple teams involved in managing of different parts of the experiment workflow — thus promoting inter-organizational collaborations." (Yu and Buyya, 2005b).

Shah *et al.* (2004) described three crucial aspects that are to be considered essential for building pipelines and these are summarized here as the salient features of a good pipeline:

1. a flexible architecture such that one system can analyse different datasets that may require different analysis tools,

2. allowance for the inclusion of new tools in a modular fashion so that, on the addition of new tools, the architecture does not require any alteration, and

3. provision of a framework to facilitate data integration of analysis results from different tools that were computed on the same input.

2.1.1 Taverna

One attempt to integrate diverse tools is Taverna (Oinn *et al.*, 2004) which is part of the ^{my}Grid project (`http://www.mygrid.org.uk`). This system provides a graphical tool for creating and running arbitrarily complex bioinformatics workflows consisting of interlinked processing units each of which transforms a set of input data into a set of output data. Workflows are created in a language called Scufl. Oinn *et al.* (2004) list six types of supported Taverna 'processors'.

1. Arbitrary WSDL types allow the use of tools provided as Web-services;

2. Soaplab types allow local tools to be wrapped within a Web-service (Senger *et al.*, 2003) and servers available via web pages may be wrapped using the Gowlab tool of SoapLab.

3. Talisman types allow access to Grid applications developed using the Talisman system for rapid application development (Oinn, 2003);

4. Nested workflow types allow child Scufl workflows to be invoked;

5. ***String constant types*** allow a constant value to be fed into an established workflow;

6. ***Local processor types*** allow new local functions to be used. These must be coded as classes which comply with a simple Java interface.

Oinn *et al.* (2004) state that invocation mechanisms other than Web-services require *"first, creating a plug-in for the Freefluo enactor to access the resource and, second, implementing a corresponding Scufl processor type"*. In this way, Taverna provides support for a number of mechanisms, including access to BioMart (Pruess *et al.*, 2005; Durinck *et al.*, 2005), an API consumer (which can cope with a variety of Java APIs) and scripting support via the beanshell. The beanshell *"is a small, free, embeddable Java source dynamic interpreter with object scripting language features, written in Java"*) (see `http://www.beanshell.org/intro.html`).

2.1.2 ToolBus

The ToolBus architecture (Eckart and Sobral, 2003), is another system which provides a generic, web-services based framework to deal with issues such as data and tool interoperability. ToolBus is a client-side interconnect, written in Java, which allows access to remote web-services as well as local programs and files. This provides data and analysis services, and allows examination of results using a wide variety of visualization tools. In addition, ToolBus enables users to form groupings of related information and to perform comparative analysis using these data groups in order to support the discovery of interesting inter-data relationships. PathPort (Pathogen Portal) is a collection of web-services

(including gene prediction and multiple sequence alignment) and visualization tools based around the ToolBus architecture (Eckart and Sobral, 2003).

2.1.3 Other tools

Other attempts to integrate heterogeneous resources include ISYS (Siepel *et al.*, 2001), Biopipe (Hoon *et al.*, 2003), Pegasys (Shah *et al.*, 2004), GPIPE (Garcia Castro *et al.*, 2005), GATO (Fujita *et al.*, 2005), PseudoPipe (Zhang *et al.*, 2006), and MPP (Davey *et al.*, 2007).

- ISYS (Siepel *et al.*, 2001) uses a decentralized, component-based approach with a design similar to CORBA[1] and SOAP[2]/WSDL[3]. It allows dynamic discovery of services via a broker. The data-model is heavily object-based and is implemented through a set of Java interfaces.

- Biopipe (Hoon *et al.*, 2003) is a flexible framework that aims to allow researchers to focus on designing an analysis pipeline. Analysis modules and configuration parameters are chosen and the protocol, data sources and modules are wrapped in XML. It integrates some analysis tools by using Bioperl API and MySQL.

- GPIPE (Garcia Castro *et al.*, 2005) is a graphical pipeline generator for PISE (The Pasteur Institute Software Environment (Letondal, 2001) which

[1]CORBA (Common Object Request Broker Architecture) is a standard architecture defined by the Object Management Group (OMG) that enables software components written in multiple computer languages and running on multiple computers to work together (as described at `http://en.wikipedia.org/wiki/CORBA`).

[2]SOAP is a simple XML based protocol to let applications exchange information over HTTP (for accessing a Web service) (as described at `http://www.w3schools.com/soap/soap_intro.asp`).

[3]WSDL is an XML-based language for describing Web services and how to access them (as described at `http://www.w3schools.com/wsdl/default.asp`).

generates a web interface for molecular biology programs in unix) that follows a task-flow[4] model and facilitates storage of metadata in XML based language.

- Pegasys (Shah *et al.*, 2004) is a modular and customizable workflow system for executing a variety of analysis tools and integration of results from them using a backend relational database management system. Pegasys is implemented in Java and uses a client/server model and a DAG[5] data structure for dynamically creating sequence analysis workflows via a graphical user interface.

- Wildfire (Tang *et al.*, 2005) is a graphical user interface for construction and execution of workflows implemented in Java. It uses GEL (Grid Execution Language (Lian *et al.*, 2005)) which can execute the workflow over a cluster, can run executables directly, or on a grid providing supercomputing power.

- ICENI (Imperial College e-Science Networked Infrastructure) (Furmento *et al.*, 2002) is service oriented/integrated Grid middleware[6] implemented in Java and Jini[7] for constructing, defining and executing workflows that are described in XML on a grid.

- ProGenGrid (Aloisio *et al.*, 2005) (Proteomics and Genomics Grid) is an-

[4] A task-flow lets one create an application that facilitates the execution and parameterization of a set of tasks.

[5] A Directed Acyclic Graph (DAG) is a directed graph with no cycles. For example, if there is a route from node A to node B then there is no way back. The Root is a node with no incoming edges whereas a leaf is a node with no outgoing edges.

[6] Middleware (as described at `http://www.s3.kth.se/~kallej/papers/runes_ejc07.pdf`) is a software abstraction layer that mediates the interactions of a component with its environment by providing a programming interface transparent to the operating systems and to the network protocols underneath.

[7] Jini is a network architecture with a programming model that facilitates distributed computing which was developed by Sun but now being developed as Apache River.

> other grid based workflow system for composing and executing tasks that simulate biological experiments.

It should be noted that 'Grid' (Foster and Kesselman, 1999) and 'application technologies' are increasingly being used to build many complex systems to handle and run large-scale scientific experiments on distributed and heterogenous resources (Yu and Buyya, 2005a,b). In these papers Yu and Buyya have presented a taxonomy of scientific workflow systems for Grid computing in which they highlight the design and engineering similarities and differences of state-of-the-art Grid workflow systems, along with the areas that need further research (Table 2.1).

The following text is an extract from Yu and Buyya's (2005a) taxonomy of scientific workflow systems for Grid computing:

> "The taxonomy characterizes and classifies approaches of scientific workflow systems in the context of Grid computing. It consists of four elements of a Grid workflow management system: (a) workflow design, (b) workflow scheduling, (c) fault tolerance and (d) data movement" (Yu and Buyya, 2005a).

Their taxonomy is illustrated in Figure 2.1 which is reproduced from their paper. The terminology used in their paper, Table 2.1 and Figure 2.1 is briefed in the following paragraphs.

Workflow design encompasses the 'structure' and 'model' (or 'specification') of the workflow and a 'composition system'. The 'structure' indicates the time dependency of the tasks and either can, or cannot, be represented by a DAG (Directed Acyclic Graph). DAG-based workflow structures allow sequential, parallel

Project	Workflow Design			Workflow Scheduling				Fault	Data
	Structure	Model	Composition System	Architecture	Decision Making	Planning Scheme	Strategies	Tolerance	Movement
DAGMan	DAG	Abstract	User-directed (Language-based)	Centralized	Local	Dynamic (Just-in-time)	Performance-driven	Task Level (migration, retrying) Workflow Level (Rescue workflow)	User-directed
Pegasus	DAG	Abstract	User-directed (Language-based) Automatic	Centralized	Local Global	Static (user-directed) Dynamic (Just-in-time)	Performance-driven	Based on DAGMan	Mediated
Triana	Non-DAG	Abstract	User-directed (Graph-based)	Decentralized	Local	Dynamic (Just-in-time)	Performance-driven	Based on GAT manger	Peer-to-Peer
ICENI	Non-DAG	Abstract	User-directed (Language-based, Graph-based)	Centralized	Global	Dynamic (Prediction-based)	Performance-driven Market-driven	Based on ICENI middleware	Mediated
Taverna	DAG	Abstract Concrete	User-directed (Language-based, Graph-based)	Centralized	Local	Dynamic (Just-in-time)	Performance-driven	Task Level (Retry, Alternate Resource)	Centralized
GrADS	DAG	Abstract	User-directed (Language-based)	Centralized	Local Global	Dynamic (Prediction-based)	Performance-driven	Task Level in rescheduling work in GrADS, but not in workflows.	Peer-to-Peer
GridFlow	DAG	Abstract	User-directed (Graph-based, Language-based)	Hierarchical	Local	Static (Simulation-based)	Performance-driven	Task Level (Alternate resource)	Peer-to-Peer
UNICORE	Non-DAG	Concrete	User-directed (Graph-based)	Centralized	User-defined*	Static (User-directed)	User-defined*	Based on UNICORE middleware	Mediated
Gridbus workflow	DAG	Abstract Concrete	User-directed (Language-based)	Hierarchical	Local	Static (User-directed) Dynamic (Just-in-time)	Market-driven	Task Level (Alternate resource)	Centralized Peer-to-Peer
Askalon	Non-DAG	Abstract	User-directed (Graph-based, Language-based)	Decentralized	Global	Dynamic (Just-in-time, Prediction-based)	Performance-driven Market-driven	Task Level (Retry, Alternate resource) Workflow level (Rescue workflow)	Centralized User-directed
Karajan	Non-DAG	Abstract	User-directed (Graph-based, Language-based)	Centralized		User-defined*		Task Level (Retry, Alternate resource, checkpoint / restart) Workflow Level (User-defined, exception handling)	User-directed
Kepler	Non-DAG	Abstract Concrete	User-directed (Graph-based)	Centralized		User-defined*		Task Level (Alternate resource) Workflow Level (User-defined exception handling, Workflow rescue)	Centralized Mediated Peer-to-Peer

Table 2.1: Taxonomy mapping to Grid workflow systems. *user-defined — the architecture of the system has been explicitly designed for user extension. DAGMan (Tannenbaum *et al.*, 2002), Pegasus (Deelman *et al.*, 2003), Triana (Taylor *et al.*, 2004), ICENI (Mcgough *et al.*, 2004), Taverna (Oinn *et al.*, 2004), GrADS (Berman *et al.*, 2001), GridFlow (Cao *et al.*, 2003), UNICORE (Almond and Snelling, 1998), Gridbus workflow (Yu and Buyya, 2004), ASKALON (Fahringer *et al.*, 2005), Karajan (Laszewski, 2005), Kepler (Ludscher *et al.*, 2005) (Table reproduced from (Yu and Buyya, 2005a))

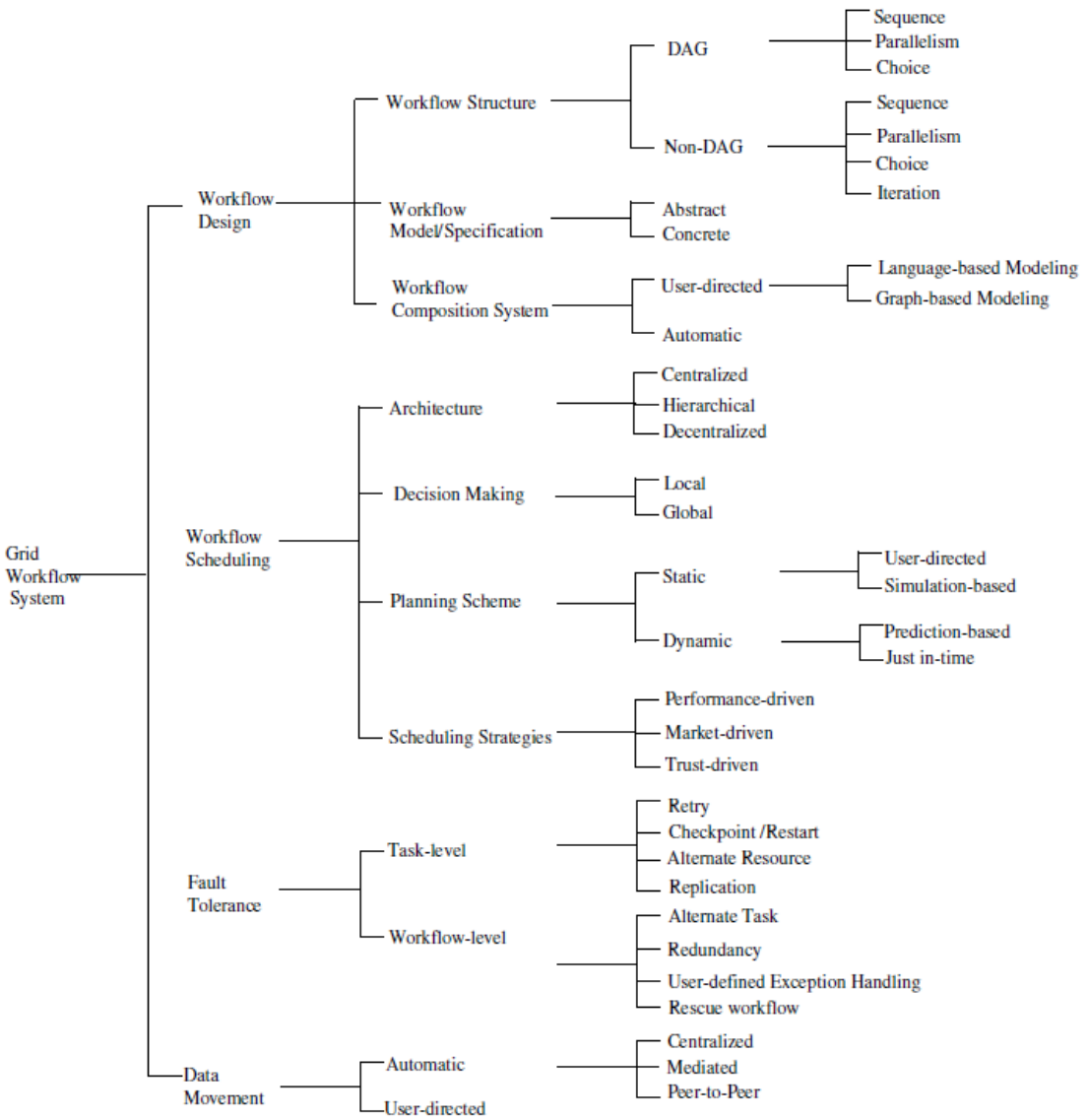


Figure 2.1: A taxonomy of scientific workflow systems for Grid computing (Figure reproduced from (Yu and Buyya, 2005a)).

or conditional execution of tasks. Non-DAG workflows also allow iterations - i.e. sections of the workflow can be repeated. The 'model' can either be 'abstract' or 'concrete'. An abstract workflow defines tasks without referring to specific resources, while a concrete model specifies the resources to be used for the tasks. The 'composition system' refers to the way in which users assemble the workflow and can either be 'user-directed' or 'automatic'. User-directed systems use a workflow language, or a graphical tool such as Kepler (Ludscher *et al.*, 2005). Automatic systems create a workflow without user intervention, given only a very high level view of the input and required output.

Workflow scheduling is the most complex element of workflow management and covers the 'architecture', 'decision making', 'planning scheme' and 'strategy' of the workflow:

- The 'architecture' is important for the scalability, autonomy, quality and performance of the system (Hamscher *et al.*, 2000) and may be one of three types: (i) 'centralized' in which one central scheduler makes scheduling decisions for all tasks in the workflow, (ii) 'hierarchical' in which there is one central manager and multiple lower-level sub-workflow schedulers, and (iii) 'decentralized' where there are multiple schedulers without any central controller.

- The 'decision making' process defines how workflows are mapped onto resources and is one of two types — (i) 'local' where decisions are made based on information of the current task, and (ii) 'global' where decisions are based on information about the entire workflow (Deelman *et al.*, 2004).

- The 'planning scheme' is the method by which abstract models are trans-

lated into concrete models and can be either 'static' in which concrete models are generated before execution, or 'dynamic' in which static and dynamic information about resources is used at run-time. Static schemes may be either (i) 'user-directed' in which users make resource mapping decisions according to their knowledge, preference and/or performance criteria, or (ii) 'simulation-based' in which the best schedule is achieved by simulating task execution on a given set of resources before the workflow starts execution. Dynamic schemes can be classified as either 'prediction-based' using dynamic information together with some prediction-based results, or 'just-in-time' where decisions are made at the time of task execution.

- The 'strategy' takes users constraints such as deadlines and budget into consideration when mapping tasks to resources. It can be (i) 'performance-driven' where resources are selected that achieve optimal execution performance, (ii) 'market-driven' in which market models are used to manage resource allocation (Geppert *et al.*, 1998) and (iii) 'trust-driven' in which properties such as security policy are given priority (Song *et al.*, 2005).

Fault tolerance considers techniques for handling failures in workflow execution and may be classified as either (i) 'task-level' in which the effects of execution failure of individual tasks are masked, or (ii) 'workflow-level' in which the workflow structure is manipulated in order to deal with errors (Hwang and Kesselman, 2003). Task-level techniques can be classified as 'retry' in which the same task is re-executed on the same resource, 'alternate resource' in which the task is re-executed on another resource, 'checkpoint/restart' in which failed tasks are transparently moved to other resources, and 'replication' where the same task runs simultaneously on different resources. Workflow-level techniques can be clas-

sified as 'alternate task' in which another implementation of a task is executed if the first one failed, 'redundancy' in which multiple alternative tasks are executed simultaneously, 'user-defined exception handling' where users specify how failures should be treated, and 'rescue workflow' where information about failed tasks is recorded during the first workflow execution.

Data movement deals with movement and availability of input and output files and is classified as either (i) 'user-directed' where users have to manage intermediate data transfer in the workflow specification, or (ii) 'automatic' where intermediate data are transferred automatically. The automatic approach is classified into 'centralized' where all intermediate data moves between resources via a central point, 'mediated' in which a distributed data management system manages the locations of the intermediate data, and 'peer-to-peer' where data are transferred directly between processing resources.
In addition to these general purpose workflows/pipelining tools, a number of more specialized workflows/pipelines have been developed. For example:

- The gene annotation tool (GATO) (Fujita *et al.*, 2005) is a pipeline for automatic annotation (preliminary DNA analysis) and access to annotated genes. GATO is implemented in PHP and Perl and annotations are obtained from web-accessible resources which are then stored in a local MySQL database. It permits individual sequence annotation using the GATO Web interface and large scale annotation using GATOALL.

- MPP (Davey *et al.*, 2007) is a phylogeny pipeline (Java application) that calculates the probability of existence of genes or markers within a genome by processing the data obtained from microarray experiments.

- MicroGen (Burgarella *et al.*, 2005) is a microarray specific web system for managing workflow information in the pipeline of spotted cDNA microarray experiments.

- PseudoPipe (Zhang *et al.*, 2006) is a homology-based pipeline implemented in Python for searching and identifying pseudogene[8] sequences in a mammalian genome using a pseudogene identification algorithm (Zhang *et al.*, 2003, 2004).

- BIPASS (Lacroix *et al.*, 2007) (Bioinformatics Pipeline Alternative Splicing Services) is a specialized pipeline for alternate splicing analysis.

- PROSPECT-PSPP (Guo *et al.*, 2004) is a specialized pipeline for protein 3D structure prediction implemented using SOAP (for sharing tools and resources), Perl (pipeline manager), MySQL (database for storing and accessing input, parameters and output at various stages) and PHP (for web interface). The pipeline is based on threading-based program, PROSPECT (Xu and Xu, 2000; Kim *et al.*, 2003) and it preprocesses sequences, predicts secondary structure, performs fold recognition and models 3D structure automatically.

2.2 Machine Learning Methods

"Machine learning is an area of artificial intelligence concerned with the study of computer algorithms that improve automatically through

[8]pseudogenes (Zhang *et al.*, 2006) are disabled copies (genomic fossils) of functional genes that have been retained in the genome by gene duplication or retrotransposition events and are important resources in understanding the evolutionary history of genes and genomes.

> experience. In practice, this involves creating programs that optimize a performance criterion through the analysis of data".

as described by Sewell at `http://www.machinelearning.net/machine-learning.pdf`.

Machine learning has a broad range of applications which include pattern recognition, search engines, natural language processing, medical diagnosis, bioinformatics, fraud detection, stock market analysis, speech and handwriting recognition, object recognition in digital vision, computer games and robotics.

There are numerous machine learning methods and some of the most commonly used methods include:

- Bayesian Methods
- Hidden Markov Models
- Decision Trees
- Support Vector Machines
- Artificial Neural Networks

2.2.1 Bayesian Methods

Bayesian Methods are based on Thomas Bayes' theorem (Bayes, 1763). Bayes' theorem relates the conditional and marginal probabilities of stochastic events A and B. Conditional probability is the probability of some event A, given the occurrence of some other event B, whereas Marginal probability is the probability of one event, regardless of the other event.

$$P(A|B) = \frac{P(B|A)P(A)}{P(B)} \tag{2.1}$$

where:

- P(A) is the marginal probability of A. It does not take into account any information about B.
- P(A|B) is the conditional probability of A, given B.
- P(B|A) is the conditional probability of B given A.
- P(B) is the marginal probability of B, and acts as a normalizing constant.

Judea Pearl coined the term Bayesian networks and presented the theory (Pearl, 1985, 1988). Bayesian networks are probabilistic directed acyclic graphs where each node represents a random variable. The Bayesian inference process starts with a prior knowledge of the distribution and the distribution is altered with each set of new presented data. Consequently, the reliability of the prior knowledge is crucial for performance and final outcome.

Figure 2.2 (Pearl, 2000) illustrates a simple, yet typical, Bayesian network. It describes the causal relationships among the season of the year (X1), whether it's raining (X2), whether the sprinkler is on (X3), whether the pavement is wet (X4), and whether the pavement is slippery (X5). Here, the absence of a direct link between X1 and X5, for example, captures our understanding that there is no direct influence of season on slipperiness — the influence is mediated by the wetness of the pavement. If freezing is a possibility, then a direct link could be added.

Pros:

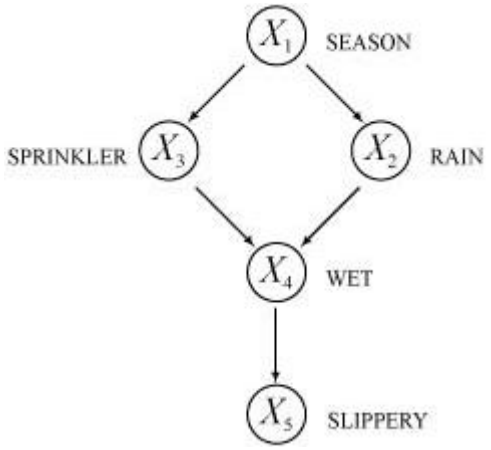


Figure 2.2: A Bayesian network representing casual influences among five variables (reproduced from `http://www.secondmoment.org/articles/bayesian.php`).

- It is straightforward to derive biological meaning.
- They can deal well with missing or partial data (`http://www.bayesit.com/docs/advantages.html`).

Cons:

- They require prior knowledge of the distribution of probabilities to make the initial assumption.
- They are associated with computational difficulties of exploring a previously unknown network. Calculation of the probability of any single branch of the network is not straightforward; all branches must be calculated (Niedermayer, 1998).

Prior knowledge of the distribution of probabilities is crucial for accuracy because any further predictions depend on these assumptions and it is generally difficult to make correct assumptions.

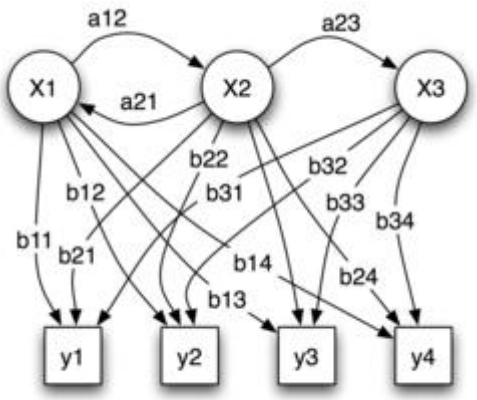


Figure 2.3: Probabilistic parameters of an example hidden Markov model where x - states, y - possible observations, a - state transition probabilities, and b - output probabilities (reproduced from `http://en.wikipedia.org/wiki/Hidden_Markov_model`).

2.2.2 Hidden Markov Models

Hidden Markov Models (HMMs) are a probabilistic model mainly used for recognition problems and has a Markov chain with hidden states (Rabiner, 1990). They are good at recognizing patterns of indefinite length (Gough, 2002). Markov models have a state which is directly visible to the observer, and the transition probabilities of a state are the only parameters. Hidden Markov models have a state that is not directly visible, but variables influenced by the state are visible. Because each state has a probability distribution over the possible output tokens, some information about the sequence of states is derived from the sequence of tokens generated by an HMM (Figure 2.3).

Pros:

- They are very good at pattern recognition.

- They are well suited for recognition of distant relationships when other methods fail to detect a very weak signal.
- They are modular - Smaller HMMs can be combined into larger HMMs.
- They are transparent - a good model increases understanding and one can read the model to make sense of it (`www.cs.ualberta.ca/~colinc/cmput606/606FinalPres.ppt`).

Cons:

- They are not ideal for cases of high similarity (i.e., where there is a strong signal).
- They are rather slow to train (because they enumerate all possible paths in a model) compared to other methods (`www.cs.ualberta.ca/~colinc/cmput606/606FinalPres.ppt`, `www.cnel.ufl.edu/files/1102356403.ppt`).
- Over-fitting can be a problem.

2.2.3 Decision Trees

Decision Trees accept an object or a situation associated with a set of properties as an input and produce yes/no as an output (Russell *et al.*, 1996; Russell and Norvig, 2003; Hall *et al.*, 1999). The inference process starts at the root node and tests an example against various attributes until it reaches a leaf node where it is finally segregated into a specific class. They are commonly used for data mining and classification. For example, Figure 2.4 illustrates a decision making process

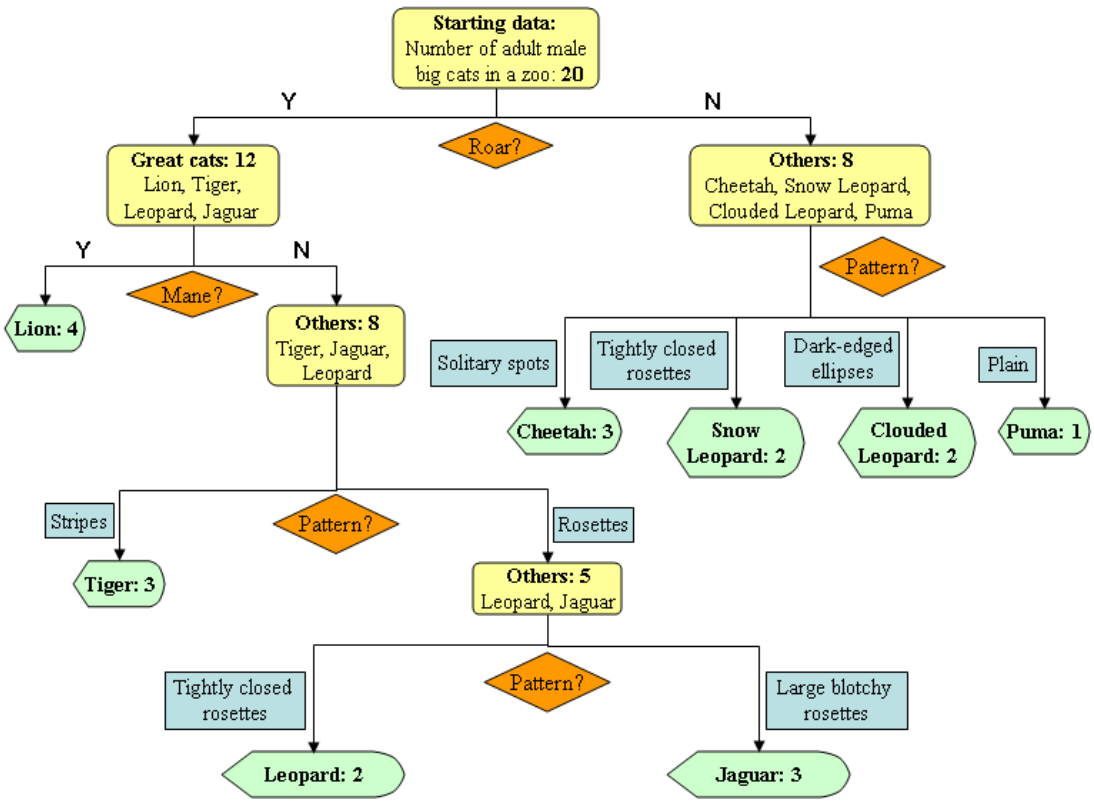


Figure 2.4: A decision tree of adult male big cats in a zoo displaying decision making based on various attributes.

which uses various attributes during the classification of adult male big cats in a zoo.

Pros:

- They are simple and easy to use, understand and interpret.
- They require minimal data preparation.
- They are computationally cheap.

Cons:

- Over-fitting can be a problem (`http://www.autonlab.org/tutorials/`

`dtree18.pdf`).

- They can perform poorly where data are difficult to segregate.

2.2.4 Support Vector Machines

Support Vector Machines(SVMs) are based on Vapnik's Statistical Learning Theory (Vapnik, 1995). Data are projected into a higher dimensional space where they are linearly separable. The points closest to the dividing line are the support vectors. The technique tries to maximise the distance between the points and the dividing hyperplane (see Figure 2.5).

Pros:

- They have good generalization accuracy (Spinosa and de Carvalho, 2005; Hearst *et al.*, 1998).
- Because of their good generalization, they are less susceptible to over-fitting than other methods and they achieve better results when dealing with new examples (Spinosa and de Carvalho, 2005).
- Their robustness in high dimensions makes them particularly interesting for applications where the datasets consist of a small number of examples and a high number of attributes (Spinosa and de Carvalho, 2005).
- They have a fast convergence rate (fast to learn) (Ding and Dubchak, 2001; Hearst *et al.*, 1998).
- They have a low false positive rate (Ding and Dubchak, 2001).

Cons:

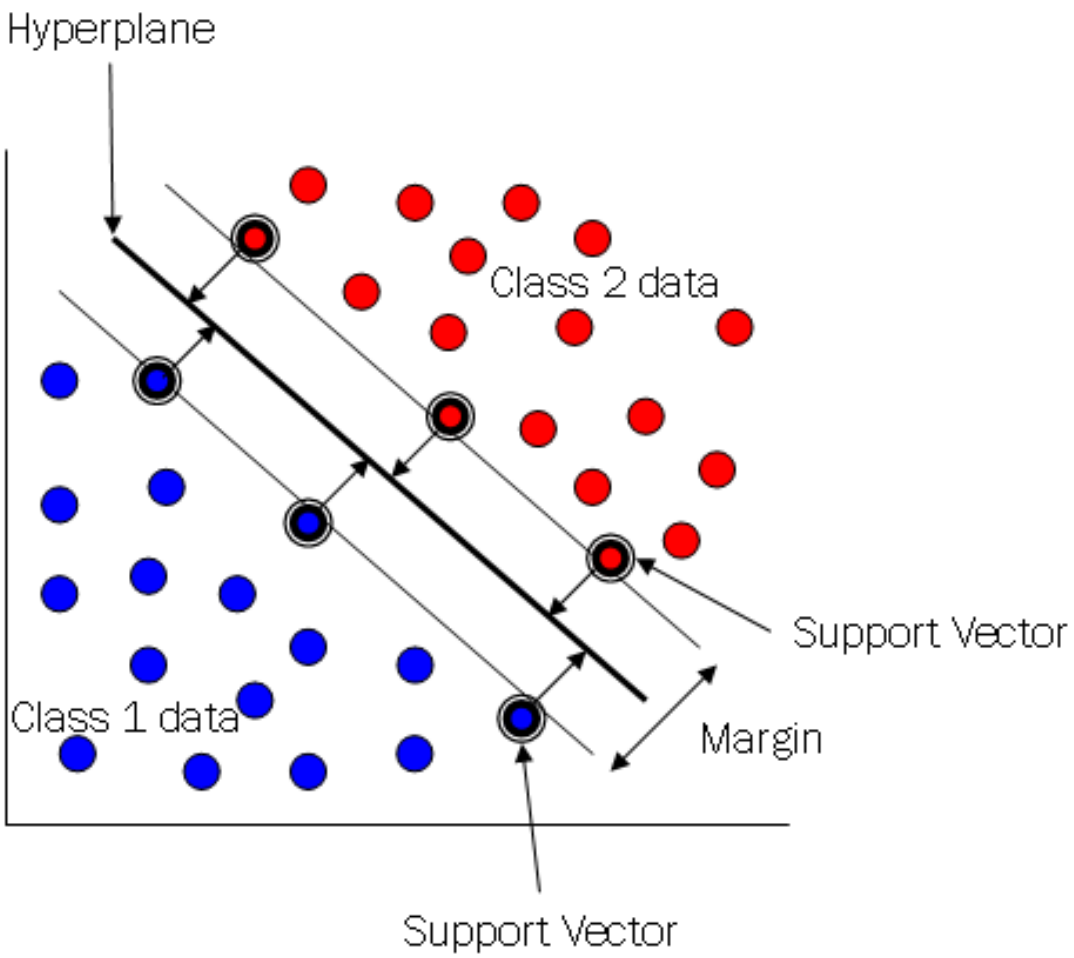


Figure 2.5: Support Vector Machines project the data into a space where it is linearly separable and then maximises the distance between the support vectors and the dividing hyperplane.

- Even though they have a low false positive rate, they also have a low true positive rate (Ding and Dubchak, 2001).
- They are binary classifiers, which makes them unsuitable for problems dealing with more than two outputs. However, some such problems can be split into multiple binary classifiers.
- They require lots of memory.

2.2.5 Artificial Neural Networks

An artificial neural network (ANN), often simply called a neural network (NN) is a computational method for information processing that is inspired by the way biological nervous systems process information (`http://www.doc.ic.ac.uk/~nd/surprise_96/journal/vol4/cs11/report.html`).

In general machine learning methods are capable of handling noisy data. Neural networks generally learn faulty examples later than others or reject them totally (Brunak, 1993). Neural networks were first used for prediction of secondary structure successfully, and better than other statistical methods, by (Qian and Sejnowski, 1988)

Pros:

- Their accuracy increases when multiple parameter datasets are used because of significant reduction of noise as compared with other methods (Ding and Dubchak, 2001).
- One can generally expect a network to train quite well when applied to problems with dynamic or non-linear relationships (`http://pages.cs.wisc.edu/~bolo/shipyard/neural/local.html`).

- They provide an analytical alternative to conventional techniques which are often limited by strict assumptions of normality, linearity, variable independence, etc. (`http://pages.cs.wisc.edu/~bolo/shipyard/neural/local.html`).

Cons:

- They can be slow to learn because of their slow convergence rate (Ding and Dubchak, 2001).
- They have high false positive rate when fewer-parameter datasets are used resulting in low accuracy.
- Generalization and over-fitting can be a problem.

Architecture

A neural network consists of a group of interconnected artificial neurons ('neurodes', or 'nodes') and learns a set of weights from examples (a training set) to predict an output for a given input (a test set) as shown in Figure 2.6.

Each node has n inputs. The inputs may be represented therefore as $x_1, x_2, x_3, \ldots, x_n$ and the corresponding weights for the inputs as $w_1, w_2, w_3, \ldots, w_n$. The summation of the weights multiplied by the inputs can be written as:

$$a = \sum_{i=1}^{n} w_i x_i \tag{2.2}$$

where a is the activation value (see Figure 2.7). Typically, the output will be 1, if the activation value $>$ threshold, whereas it will be 0, if the activation value $<$ threshold (`http://www.ai-junkie.com/ann/evolved/nnt3.html`).

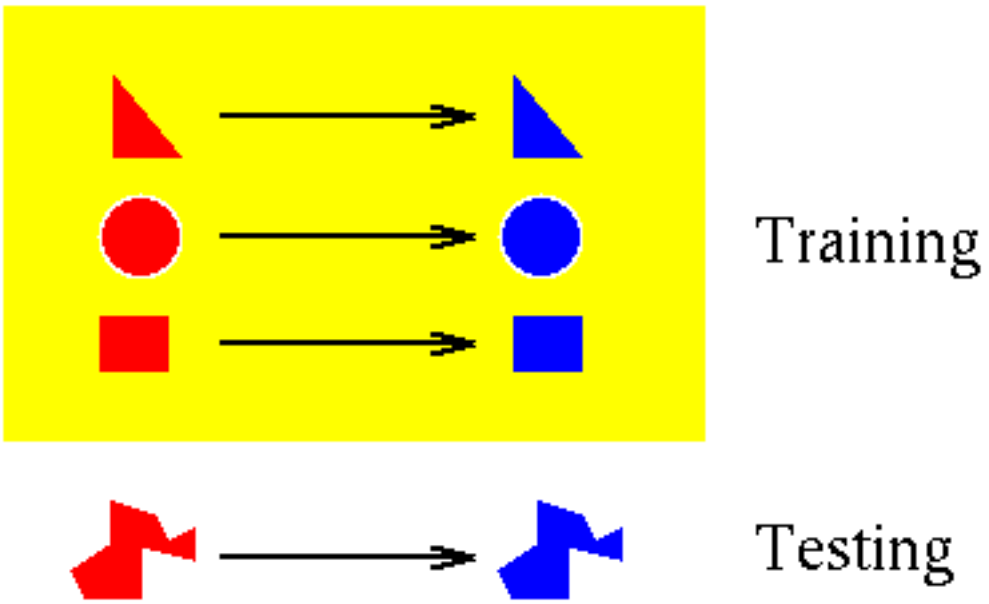


Figure 2.6: A simple pattern recognition example of what a neural network can do (Figure adapted from `http://www.gc.ssr.upm.es/inves/neural/ann1/concepts/app.htm`).

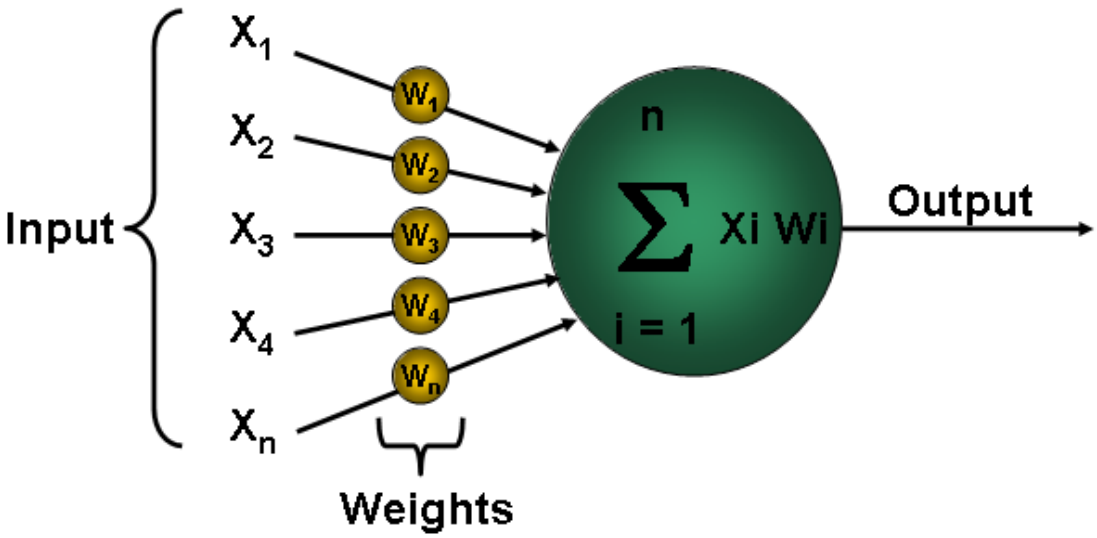


Figure 2.7: The process of handling data by a node — simple input, data processing and output generation.

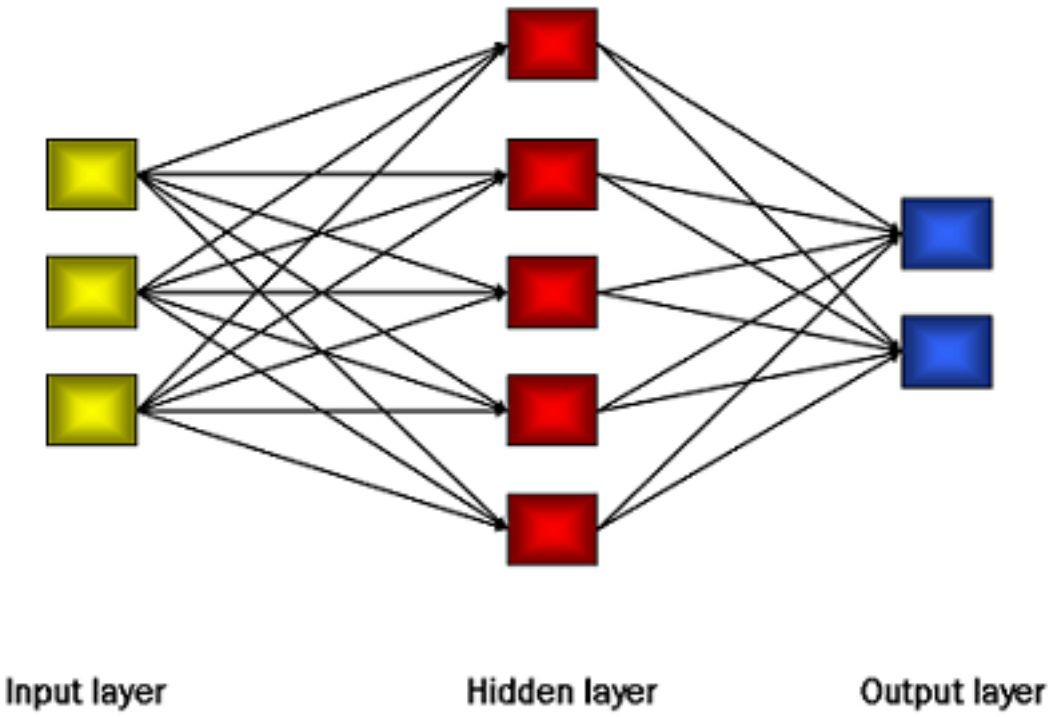


Figure 2.8: The architecture of a simple feed-forward neural network. The coloured boxes represent nodes where yellow corresponds to the input layer, red to the hidden layer and blue to the output layer. The black arrows represent the connections and data flow between various nodes.

A typical neural network consists of a number of nodes segregated into three types of layers: the input layer, the hidden layer, and the output layer (see Figure 2.8).

A part of this project is carried out using a simple feed-forward neural network (Rumelhart and McClelland, 1986) using supervised training with the Rprop algorithm (Riedmiller and Braun, 1993).

Feed-forward networks

Feed-forward networks are the most popular and most widely used neural networks. They have the following characteristics

(`http://cse.stanford.edu/class/sophomore-college/projects-00/neural-networks/Architecture/feedforward.html`):

- Nodes are arranged in layers, with the first layer taking in inputs and the last layer producing outputs. The middle layers have no connection with the external world, and hence are called hidden layers.
- Each node in one layer is connected to every node on the next layer. Hence information is constantly fed forward from one layer to the next.
- There is no connection among nodes in the same layer.

Resilient back-propagation

The training method used in this research was resilient back-propagation (Rprop) (Riedmiller and Braun, 1993, 1992). In general, the resilient back-propagation algorithm is faster than traditional back-propagation (Liu *et al.*, 2002). It uses individual dynamically tuned learning rates during the training of the neural network. Shiffmann *et al.* (1993) reported that Rprop outperforms all other learning algorithms in both speed and quality. In a study by Anastasiadis *et al.* (2003), it is also found to be one of the best learning methods in terms of accuracy and robustness with respect to its parameters. The basic principle of Rprop is to eliminate the harmful influence of the size of the partial derivative on the weight step (Anastasiadis *et al.*, 2003; Zell *et al.*, 1995). The weight-specific $\Delta_{ij}^{(t)}$ value that the size of the weight change is determined by:

$$\Delta_{ij}^{(t)} = \begin{cases} -\Delta_{ij}^{(t)} & \text{if} \frac{\theta E^{(t)}}{\theta w_{ij}} > 0 \\ \Delta_{ij}^{(t)} & \text{if} \frac{\theta E^{(t)}}{\theta w_{ij}} < 0 \end{cases} \tag{2.3}$$

where $\frac{\theta E^{(t)}}{\theta w_{ij}}$ denotes the summed gradient information over all patterns in the pattern file (Zell *et al.*, 1995).

The second step of the learning method is to determine the new update-values $\Delta_{ij}^{(t)}$ (Zell *et al.*, 1995). This is calculated by:

$$\Delta_{ij}^{(t)} = \begin{cases} \eta^+ \times \Delta_{ij}^{(t-1)} & \text{if} \frac{\theta E^{(t-1)}}{\theta w_{ij}} \times \frac{\theta E^{(t)}}{\theta w_{ij}} > 0 \\ \eta^- \times \Delta_{ij}^{(t-1)} & \text{if} \frac{\theta E^{(t-1)}}{\theta w_{ij}} \times \frac{\theta E^{(t)}}{\theta w_{ij}} < 0 \\ \Delta_{ij}^{(t-1)} & \text{otherwise} \end{cases} \tag{2.4}$$

where η^- and $\eta+$ denote decrease and increase factors respectively whose standard values are 0.5 and 1.2 (Riedmiller and Braun, 1992).

Applications

In addition to computational biology and bioinformatics, ANNs are being used in a variety of fields such as speech recognition, pattern recognition, image analysis, gaming, fraud control, and spam filters. A few applications of neural networks in bioinformatics include:

- secondary structure prediction of proteins (Guimarães *et al.*, 2003; McGuffin *et al.*, 2000; Cuff and Barton, 1999; Cuff *et al.*, 1998; Chandonia and Karplus, 1995; Qian and Sejnowski, 1988)

- transmembrane protein prediction (Rost, 1996; Lohmann *et al.*, 1994; Jacoboni *et al.*, 2001; Gromiha *et al.*, 2004)

- prediction of post-translational modifications (Bendtsen *et al.*, 2004; Nielsen *et al.*, 1997; Blom *et al.*, 1999; Julenius *et al.*, 2004)

- prediction of the drug resistance of HIV protease mutants (Drăghici and Potter, 2003)

In this chapter, I have reviewed available tools for high throughput protein annotation as well as machine learning methods and highlighted their strengths and weaknesses. In general, existing methods pose problems of significant time investment, difficulties associated with learning, modifying and implementing pipelining tools, or some methods are designed to perform only a specific task. This led to the development of an "automated protein annotation tool" (APAT) and "target annotation pipeline and automated selection" (TAPAS) which are described in the following chapters. In addition to these, an effort was made to improve prediction of transmembrane proteins by a combined neural network predictor.

Chapter 3

An Extensible Automated Protein Annotation Tool — APAT

Some of the material in this chapter has been published elsewhere (Deevi, S.V.V and Martin, A.C.R. (2006) An extensible automated protein annotation tool: standardizing input and output using validated XML, *Bioinformatics*, **22**:291–296).

In this chapter, I describe the development of the APAT (Automated Protein Annotation Tool) system, which automates protein annotation by running various prediction and annotation tools situated either locally or remotely. APAT standardizes input and output using validated XML and also provides uniform display of the results. While biologists and bioinformaticians can use all the wrappers or choose among the wrappers provided by APAT, bioiformaticians can easily extend the number of tools that can be accessed by APAT by writing additional

wrappers to other tools.

3.1 Introduction

In the analysis of sequence data, whether from genomics, transcriptomics, proteomics, or a more specific interest in a small set of proteins from a single pathway or those targeted to an organelle, there is a frequent need to apply a wide range of prediction and annotation tools to one or more sequences. Using numerous web-based or local tools, and collating and comparing their outputs is a laborious and error-prone task.

Given a protein sequence, one generally starts by looking in SwissProt (Bairoch and Apweiler, 2000) to see whether the sequence has already been annotated by expert hand-curation (Boeckmann *et al.*, 2003). Failing that, a close homologue may be available from which annotations can be transferred. However, if the sequence (or a close homologue) is not present in SwissProt, or the specific type of required annotation is not included, then one may need to run a selection of prediction tools, either across the web, or locally.

A number of web-based tools exist which provide integrated annotation/prediction systems. However, all of these systems suffer restrictions of one form or another. Very few are extensible: many provide pre-calculated annotations (frequently at the genome or complete proteome level), or provide only a fixed set of tools that can be run on a protein sequence. Where a sequence can be submitted for predictions to be made, it is relatively unusual that more than one sequence can be submitted at a time, especially where more than one type of annotation is provided. For example, there are a few tools such as the 'DAS-TMfilter' (Cserzo *et al.*, 2004) and 'NetPhos 2.0' (Blom *et al.*,

1999) which accept more than one sequence at a time, but they provide only one type of annotation (i.e., prediction of transmembrane regions (DAS-TMfilter) or phosphorylation sites (NetPhos)). There are only a few tools which accept one sequence at a time and produce more than one type of annotation such as the 'PredictProtein Server' (Rost *et al.*, 2004; Rost, 1996).

My project involves automating the process of protein annotation which includes execution of a variety of tools for protein annotation and prediction tools on protein sequences of the MEP pathway and apicoplast which include many hypothetical proteins and less annotated proteins. This requires using an assortment of prediction or annotation tools such as post-translational modification predictors, transmembrane predictors, secondary structure predictors, motif predictors, or sub-cellular location predictors. I also aim to have all these results combined to be displayed in a uniform and visually well-presented manner. Obtaining these various kinds of annotations for each of the protein sequences manually is a tedious task. To accomplish this task one does not need a complex pipeline/workflow where the output from one tool becomes the input for another, but rather one needs a system capable of executing multiple tools on a single sequence. APAT was primarily designed to play an important role in providing annotations and can be used by other research groups as an annotation "fan". While biologists can use all the wrappers or choose among the wrappers included in APAT, bioinformaticians can additionally write simple wrappers (which mainly take care of the XML output so that it fits into APAT specific DTD) to any tool they wish to run or to webservices provided by Taverna (Oinn *et al.*, 2004) or the EBI (Labarga *et al.*, 2007; Rice, 2007). Wrappers can be written in any programming language and simply need to read and write XML that complies with

the APAT DTD.

Precalculated annotations

There are many examples of pre-calculated annotations. For example, ENSEMBL (Hubbard *et al.*, 2002), the eukaryotic genome database project, provides annotations of genome data including limited annotation of the translated proteins. DAS (`http://www.biodas.org/`) is a distributed annotation system that allows pre-calculated annotation of genomes (including the encoded proteins) to be decentralized among multiple third-party annotators and integrated by client-side software (Dowell *et al.*, 2001). ENSEMBL also provides annotations served via DAS. In principle, this allows anyone to add their own annotations, but this requires pre-calculation to be performed in-house — there is no support for on-the-fly annotations. PEDANT (Protein Extraction, Description and ANalysis Tool) (Frishman *et al.*, 2001) also provides pre-calculated annotations for a wide range of complete and incomplete genomes with integration of both functional and structural information. Another server, Integr8 (`http://www.ebi.ac.uk/integr8/`) assigns annotations from various sources including InterPro (Apweiler *et al.*, 2001) and Gene Ontology (GO) (Ashburner *et al.*, 2000) terms to gene products in completed genomes and proteomes.

Similarly there are tools that work at the protein level. For example PDBSUM (Laskowski, 2001) is a pre-calculated set of annotations of structures from the Protein Data Bank; GRASS (Nayal *et al.*, 1999) provides graphical representation and analysis of structures; SAS (Milburn *et al.*, 1998) and STING-M (Neshich *et al.*, 2003) are web-based tools for integrating structural information with sequence analysis and alignment.

Web-based annotation servers

The 'PredictProtein Server' (Rost *et al.*, 2004; Rost, 1996) can take a protein sequence and perform predictions using a set of tools, but this toolset cannot be extended and only one sequence can be processed at a time. In addition there are large numbers of individual servers which allow a sequence to be submitted over the web and predictions of properties such as secondary structure, post-translational modification sites, solvent accessibility and transmembrane regions. Representative lists are available at `http://www.expasy.org/tools/` and `http://www.up.univ-mrs.fr/~wabim/english/logligne.html`. A few servers, such as DAS-TM (Cserzo *et al.*, 2004) and NetPhos 2.0 (Blom *et al.*, 1999) allow a batch of sequences to be submitted.

SEView (Junier and Bucher, 1998) is a Java applet that provides an attractive graphical representation of annotation on a protein or nucleotide sequence, but does not, itself, perform annotations.

Output from tools

Another aspect of the currently available tools is that the results are all presented in different forms. It would be much easier for the biologist wanting to scan the results if different tools provided results in a consistent format. Similarly for the bioinformatician wishing to write code to integrate and analyze the results from a number of different prediction tools, it would be advantageous if the results were available in a consistent form.

A number of proposals have been made for XML formats in which to store sequence data and related annotation information. One example is the DAS XML specification (`http://www.biodas.org/`). Others include the GAME XML spec-

ification implemented by flybase (`http://flybase.bio.indiana.edu/annot/`) and OmniGene (`http://omnigene.sourceforge.net/`). Another XML specification for annotation of sequences has been used for PathPort/ToolBus (Eckart and Sobral, 2003). While the DASGFF and DASSTYLE elements of the DAS XML specification come close to the requirements of this project, they still do not provide a simple, concise and consistent annotation format that can be used for the output of a large range of protein sequence annotation tools where results may need to be represented as numbers, text and graphs.

3.1.1 Workflows and pipelines

Various workflow based systems were described in Section 2.1. It is probable that Taverna or ICENI could be used for most of my requirements. However these are extremely powerful tool with aims which are much more wide-ranging and complex than supporting the simple desire to scan one or more sequences against a set of prediction servers. For most of my purposes, there is no requirement for a true workflow: no data output by one tool becomes the input for another tool. While Taverna could clearly be used in this way, extending the Taverna system to access local and web-based (non Web-service) tools, either using SoapLab, or local processor types are complex procedures and require a considerable investment of time to develop the expertise required. Further, Taverna makes no attempt to enforce a common presentation of predictions for a sequence allowing the scientist to obtain a summary of all the predictions in a common format.

Figure 3.1 and Figure 3.2 show a generalized view of two important and different themes. Figure 3.1 describes the theme where the input for a tool is reliant on the output from the preceding tool — a workflow/pipeline. Figure 3.2

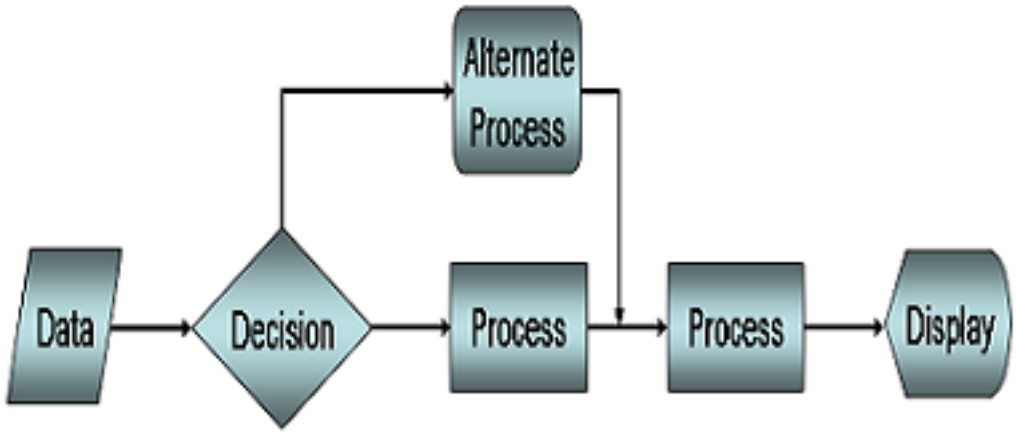


Figure 3.1: Simple depiction of a workflow/pipeline.

shows the theme employed by a software that runs multiple processes (potentially in parallel) where the input of one tool is independent of the output from other tools — an annotation "fan".

As described above, my requirement was not to transform the output of one tool into input for another tool (see Figure 3.1), but simply to feed the same type of data (a protein sequence) into a number of separate analysis tools (see Figure 3.2).

As there is a frequent need to apply a large range of local and/or remote prediction and annotation tools to one or more sequences, a tool able to dispatch sequences to assorted services by defining a consistent XML format for data and annotations was created and made available for download by the user community.

3.2 Software requirements

The basic requirements for APAT were:

1. allowing one or more sequences to be analyzed in a single run,

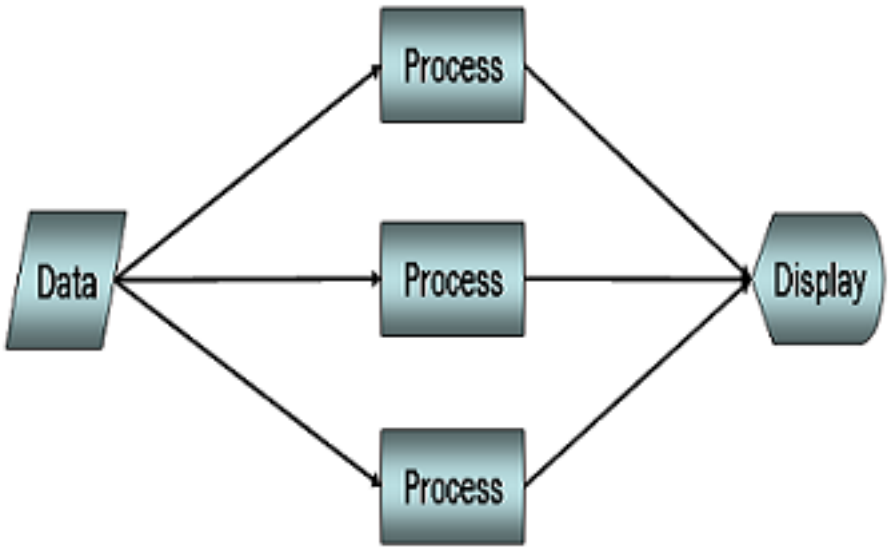


Figure 3.2: Simple depiction of a tool with minimal flow of data from one process to another — annotation fan (e.g. APAT).

2. using multiple prediction/annotation tools residing locally or over the web through normal CGI scripts or Web-services,

3. obtaining the results in a common format for further analysis,

4. providing consistent visual presentation of results and

5. making the implementation of wrappers to additional tools as straightforward and language-independent as possible.

A decision was taken to wrap the input and output of each tool in XML. The system then submits one or more sequences to a set of prediction tools whose output is converted to a standard XML format (APATML) which can be further analyzed, or displayed as HTML via a display program.

3.3 Approach and methods

3.3.1 Analysis of input and output of various annotation and prediction tools

A number of web-based and local tools were analyzed to discover the types of information required as input and returned as output. These tools were investigated in order to determine the data types and not to evaluate their performance (see Table 3.1).

The tools included NetPhos (Blom *et al.*, 1999), NetOGlyc (Julenius *et al.*, 2004), DAS-TM Filter (Cserzo *et al.*, 2004), TargetP (Emanuelsson *et al.*, 2000), PsiPred (McGuffin *et al.*, 2000), InterProScan (Zdobnov and Apweiler, 2001), LOCtree (Nair and Rost, 2004), Predotar (Small *et al.*, 2004), BLAST (Altschul *et al.*, 1990, 1997), FingerPrintScan (Scordis *et al.*, 1999), TMHMM (Krogh *et al.*, 2001), PATS (Zuegge *et al.*, 2001), ScanProsite (Gattiker *et al.*, 2002; Hulo *et al.*, 2004) and SMART (Schultz *et al.*, 1998).

3.3.2 Input data

Many of the programs have different options, but supply defaults for the vast majority of these. For purposes of bulk scanning of sequence data, using these default values is considered as acceptable and is commonly practiced (Rost *et al.*, 2004). It is a trivial matter to modify the wrappers to use different values. From the tools that were examined, in addition to the sequence, the only data that must be supplied are: an identifier for the sequence, an email address and an indication of whether or not a sequence is of plant origin.

A very simple DTD which is used to encode the input sequence and any

	Residue level		Domain level		Sequence level	
Tool	number	text	number	text	number	text
NetPhos 2.0	√					
NetOGlyc	√					
DAS-TM	√		√	√	√	
TargetP					√	√
LOCtree					√	√
PsiPred	√	√				
Predotar					√	√
BLAST					√	√
FingerPrintScan			√	√		
TMHMM	√			√	√	
PATS					√	√
ScanProsite			√	√		
SMART			√	√		
InterProScan			√	√		

Table 3.1: Annotation types returned by a number of example tools.

parameters required by the annotation/prediction programs was designed. A Perl script 'mix.pl' which converts a Fasta file into this format and accepts any additional parameters on the command line or interactively was also created.

Some programs may have more extensive input requirements. By definition, XML is extensible so the DTD for input data can easily be extended to allow for additional input requirements of specific wrappers. These additional tags will simply be ignored by wrapper scripts which don't need them.

An example XML file is shown in Figure 3.3 (also on the web at `http://www.bioinf.org.uk/apat/`) while the DTD is shown in Figure 3.4.

3.3.3 Output data

After careful analysis of various annotation tools, as described above, it was found that the annotations provided by these programs could all be described by 6 data types:

```
<input>
   <sequenceid>1 NULL 1-deoxy-D-xylulose 5-phosphate
        reductoisomerase
   </sequenceid>
   <sequence>
       MKKYIYIYFFFITITINDLVINNTSKCVSIERRKNNAYINYGIGYN
       GPDNKITKSRRCKRIKLCKKDLIDIGAIKKPINVAIFGSTGSIGTN
       ALNIIRECNKIENVFNVKALYVNKSVNELYEQAREFLPEYLCIHDK
       SVYEELKELVKNIKDYKPIILCGDEGMKEICSSNSIDKIVIGIDSF
       QGLYSTMYAIMNNKIVALANKESIVSAGFFLKKLLNIHKNAKIIPV
       DSEHSAIFQCLDNNKVLKTKCLQDNFSKINNINKIFLCSSGGPFQN
       LTMDELKNVTSENALKHPKWKMGKKITIDSATMMNKGLEVIETHFL
       FDVDYNDIEVIVHKECIIHSCVEFIDKSVISQMYYPDMQIPILYSL
       TWPDRIKTNLKPLDLAQVSTLTFHKPSLEHFPCIKLAYQAGIKGNF
       YPTVLNASNEIANNLFLNNKIKYFDISSIISQVLESFNSQKVSENS
       EDLMKQILQIHSWAKDKATDIYNKHNSS
   </sequence>
   <emailaddress>s.v.v.deevi@rdg.ac.uk</emailaddress>
   <parameter server='targetp' param='origin' value='non-plant' />
   <parameter server='psort' param='origin' value='animal' />
   <parameter server='subloc' param='origin' value='eukaryotic' />
</input>
```

Figure 3.3: An example of the XML format used for input to the APAT system.

```
<!ELEMENT emailaddress ( #PCDATA ) >

<!ELEMENT input ( sequenceid, sequence, emailaddress, parameter+ ) >

<!ELEMENT parameter EMPTY >
<!ATTLIST parameter param CDATA #REQUIRED >
<!ATTLIST parameter server CDATA #REQUIRED >
<!ATTLIST parameter value CDATA #REQUIRED >

<!ELEMENT sequence ( #PCDATA ) >

<!ELEMENT sequenceid ( #PCDATA ) >
```

Figure 3.4: APATINML DTD.

```
<result program='NetPhos' version='2.0'>
   <function>Protein Phosphorylation sites Prediction</function>
   <run>
      ...Lists any parameters supplied to the program...
      <date>Fri Nov 19 15:30:38 GMT 2004</date>
   </run>
   <predictions>
      <perres-number name = 'P-score' clrmin = '0.0' clrmax = '1.0'
                    graph='1' graphtype='bars'>
         <value-perres residue='1'>0.215567</value-perres>
         ...
      </perres-number>
      <threshold>
         ...<description> describes threshold...</description>
         ...<thr-res> tags list the positive prediction residues</thr-res>
      </threshold>
   </predictions>
</result>
```

Figure 3.5: Summary of the key aspects of an APATML output file for per-residue annotations.

1. Residue level-Textual data

2. Residue level-Numeric data

3. Domain region level-Textual data

4. Domain region level-Numeric data

5. Whole sequence level-Textual data

6. Whole sequence level-Numeric data

Table 3.1 shows examples of the annotations returned by different tools. In the case of residue-level annotations, a value is often provided for every residue in a sequence. Typical examples are secondary structure prediction, transmembrane prediction, glycosylation and phosphorylation site prediction. In some cases, graphical display of such annotations in the form of both line-charts and bar-charts can be useful and it is necessary that this requirement can be flagged.

Domain-level annotations can be viewed as an extension of residue-level annotations in which discrete continuous stretches of residues are given the same label. However, the semantic meaning is somewhat different. While a residue-level annotation applies to that residue in isolation, a domain-level annotation is not meaningful in the context of a single residue. For example, domain-level annotations are generally used for the results of pattern or profile searches such as FingerPRINTScan (Scordis *et al.*, 1999), ProSite (Gattiker *et al.*, 2002; Hulo *et al.*, 2004), InterProScan (Zdobnov and Apweiler, 2001), and SMART (Schultz *et al.*, 1998). It would not be meaningful to say that a single residue matched one of the patterns which these tools recognise. From a presentational viewpoint, one generally wishes such annotations to be provided in a tabular form rather

```
<result program='InterProScan' version='1.0'>
   <function>Protein domain Prediction</function>
   <run>
      ...Lists any parameters supplied to the program...
      <date>Fri Nov 19 15:50:04 GMT 2004</date>
   </run>
   <predictions>
      <perdom class='PRINTS' name = 'SH2_DOMAIN' highlight='1'
              rangemin='23' rangemax='150'>
         <value-perdom label='e-value'>1.7e-17</value-perdom>
      </perdom>
      <perdom class='PROSITE' name = 'ASN_GLYCOSYLATION' highlight='0'
              rangemin='48' rangemax='51'>
         <value-perdom label='match'>NLTV</value-perdom>
      </perdom>
      <perdom-description class='PROSITE' name = 'ASN_GLYCOSYLATION'>
         Potential N-linked glycosylation site identified by ProSite pattern.
      </perdom-description>
   </predictions>
</result>
```

Figure 3.6: Summary of the key aspects of an APATML output file for per-domain annotations.

```
<result program='TargetP' version='1.01'>
   <function>Protein subcellular location Prediction</function>
   <run>
      ...Lists any parameters supplied to the program...
      <date>Fri Nov 19 15:47:04 GMT 2004</date>
   </run>
   <predictions>
      <perseq name = 'mTP-pred'>
         <description>Mitochondrial targeting peptide (mTP) prediction
                     score</description>
         <value-perseq highlight='0'>0.031</value-perseq>
      <perseq>
      <perseq name = 'Loc-pred'>
         <description>SUBCELLULAR LOCATION PREDICTION</description>
         <value-perseq highlight='1'>SECRETORY PATHWAY, i.e. THE SEQUENCE
                                     CONTAINS A SIGNAL PEPTIDE,SP.
         </value-perseq>
      </perseq>
      ...
   </predictions>
</result>
```

Figure 3.7: Summary of the key aspects of an APATML output file for per-sequence annotations.

than indicated on the sequence itself. Taking FingerPRINTScan as an example, for each fingerprint matched, the server at `http://www.ebi.ac.uk/printsscan/` returns 7 numbers (the number of motifs matched; the number of motifs in the fingerprint; SumID; AveID; ProfScore; P-value; E-value) and 2 strings (the fingerprint name; an indication of which motifs within the fingerprint match). In addition, one additional string is returned for each motif matched indicating the matched residues. From this a residue range can be calculated.

Sequence-level annotations provide a value which is applicable to the whole sequence. Examples are protein localization predictions such as TargetP (Emanuelsson *et al.*, 2000), Predotar (Small *et al.*, 2004), LOCtree (Nair and

Rost, 2004), PATS (Zuegge *et al.*, 2001) and the results of BLAST (Altschul *et al.*, 1990, 1997) searches. Semantically this is similar to a domain-level annotation, but the presentation requirements are rather different.

Some servers provide annotations at more than one level. For example, TMHMM (Krogh *et al.*, 2001) provides per-residue values indicating the probability that an individual residue is in a transmembrane region. In addition, it summarizes ranges of residues predicted to form transmembrane helices indicating their orientation together with an overall significance value. Finally it generates a number of pieces of summary data such as the number of amino acids predicted to be in transmembrane helices and the number within the first 60 amino acids. It therefore has annotations at all three levels: per-residue, per-domain and per-sequence.

Since XML makes no distinction between numeric and character data (everything is stored as plain text), a decision was taken to simplify this scheme further by treating the numeric and text annotations for domains and for sequences as single types. However, the distinction for per-residue annotations was retained since one may wish to generate graphs of numeric data while there will be no such requirement for character data.

Therefore APAT has just four data types which can be used to encapsulate the annotations from all the tools likely to be encountered:

1. per-residue numbers
2. per-residue strings
3. per-domain values
4. per-sequence values

As described above, the use of the DAS XML format (`http://www.biodas.org/documents/spec.html`) for the annotation requirements was considered, but decided against for a number of reasons.

- First, simplicity was a priority to allow additional service wrappers to be written easily. Being designed primarily for DNA-level annotations, DAS is unnecessarily complex for my purposes having many redundant fields. Also DASGFF and DASSTYLE elements need to be combined to achieve the simple task of indicating visual annotations.

- Second, there is no direct way within the DASSTYLE elements to specify a requirement for a graph to be displayed. One would either have to extend the DAS XML specification or co-opt existing glyph styles to have non-standard meanings.

- Third, providing the semantics are easily transferable, conversion between XML formats is straightforward using XSLT, so a specific format can be chosen to ease the burden of implementing a particular system.

Consequently, a new XML format, APATML was designed as shown in the DTD in Figure 3.8, with simplified examples of per-residue, per-domain and per-sequence annotations in Figure 3.5, Figure 3.6 and Figure 3.7 respectively. Details of the meaning of each XML tag are provided in Table 3.2.

The actual output of many web-based servers provides visual highlighting, graphs and extensive text. Only the essential information from this is captured — alternative presentation issues can be addressed in a display program. However, in addition to the pure annotation data, the storage of limited meta-data about what the annotations mean is allowed. For example, at the server level, the name and

Tags	Description	subtags	Attributes
<results>	The root tag that wraps various kinds of annotations produced by different servers	<input> <result>	Nil
<input>	Contains details of input sequence	<seqid> <seq> <emailaddress> <parameter>	Nil
<seqid>	Contains protein sequence identification details	Nil	Nil
<seq>	Contains amino acids of the input protein (one residue per tag)	Nil	Nil
<emailaddress>	Contains email address entered by users	Nil	Nil
<parameter>	This is an empty tag containing the information about parameters in its attributes	Nil	server: name of the server, param: type or name of the parameter, value: value stored
<result>	Includes the output from one particular server	<function> <info> <run> <predictions>	program: name of the annotation server, version: (optional) version of the annotation server
<function>	Describes the function of an annotation server	Nil	Nil
<info>	Describes the tool being used and is hyperlinked to the actual server if it is a web-based tool or just says it is a local server if it is one	Nil	Nil
<run>	Describes run parameters and date	<params> <date>	Nil
<params>	Contains tags that represent the run parameters	<param>	Nil
<param>	Contains name and value associated with the parameter as attributes (One or more tags)	Nil	name: name of the parameter, value: value assigned for the parameter
<date>	Contains details about day, date, time of run	Nil	Nil
<predictions>	Contains predictions from the server	<link> <perres-number> <perres-character> <threshold> <perdom> <perseq> (all optional)	Nil
<link>	Contains link for prediction from the actual webserver being used in it's unparsed form	Nil	href: contains the URL for output page from the webserver
<perres-number>	Describes numeric annotation at the per-residue level	<value-perres>	name: name of the type of numeric data stored in the array (like 'p-score'), clrmin: minimum value for colouring range, clrmax: maximum value for colouring range, graph: (optional) whether a graph is required or not (1 for yes and 0 for no), graphtype: (optional, required if graph = 1) type of graph to be used 'bars' or 'lines'
<value-perres>	These tags specify a value to be assigned to each residue	Nil	residue: sequential number of the residue in the protein sequence
<perres-character>	Describes text annotation at the per-residue level	<value-perres>	name: name of the type of numeric data stored in an array (like 'p-score')
<threshold>	Used to indicate residues that pass some prediction threshold (those that are considered 'positive' predictions and should therefore be highlighted)	<description> <thr-res>	Nil
<description>	Description of what the threshold represents	Nil	Nil
<thr-res>	Stores residue numbers (one per tag) of those residues that pass the threshold value	Nil	Nil
<perdom>	Describes annotations (either numeric or character) that apply to a continuous range of residues	<value-perdom> <perdom-description>	class: (optional) contains name of the server; used for servers that produce multiple types of annotation, name: (optional) annotation name applied to the domain, highlight: whether to highlight the value or not (1 for yes and 0 for no), rangemin: minimum sequence number of the range of the annotated region, rangemax: maximum sequence number of the range of the annotated region
<value-perdom>	Includes either numeric or character data of annotation; typically used for indicating confidence or match region	Nil	label: description of what the value refers to (e.g. Confidence, e-value, match pattern)
<perdom-description>	Provides an extended general description of the kind of domain predicted	Nil	class: (optional) contains name of the server and is used for servers that produce multiple types of annotation, name: annotation name applied to the domain
<perseq>	Sequence level annotation that contains either number or character output from the server	<value-perseq> <description>, name: name of the prediction/type of output	
<value-perseq>	Contains the actual value (output) of prediction	Nil	highlight: whether to highlight the value or not (1 for yes and 0 for no)
<description>	More detailed description of the prediction/output	Nil	Nil

Table 3.2: A detailed list of XML tags included in APATML.

version number of the program, the run-time parameters and a textual description of the program's function is stored. At the per-sequence annotation level, one can store extensive text associated with annotations and at the per-domain level a description may be stored associated with a prediction. This accounts for servers such as InterProScan which potentially identify more than one region of a protein using a number of underlying databases/algorithms. Simple storage of the annotated residue range is allowed, together with a database name (e.g. PRINTS) and annotation (e.g. SH2_DOMAIN) which is stored separately from an explanation of what a 'PRINTS SH2_DOMAIN' actually is.

Although APAT provides an easy means of comparison between the output from various tools (which itself is a summarized output from the actual tool) through uniform output format, it makes no attempt to compare the results from related prediction tools and does not generate any form of consensus prediction. However, this could be achieved by a post analysis script. For example, results from similar tools were combined using a post analysis script as described in Chapter 5. APAT is a simple sequence annotation "fan" with standardized XML input and output where the choice of tools is left for the user. Usage of the display program to obtain HTML output is optional and could be replaced by any type of post analysis script.

For example, APAT was used in TAPAS (shown in Chapter 4) and also in the work of improving transmembrane prediction (shown in Chapter 5). The output from APAT is handled differently in both cases. While the display program of APAT was used to produce HTML output in TAPAS which is later used to obtain the number of transmembrane proteins predicted (along with the link provided for viewing output from other tools) the display program of APAT was excluded in

the transmembrane work where the XML output from APAT was used by a post analysis script to produce input files for neural networks having the prediction values from various transmembrane predictors. The choice of tools used in both cases is also different.

Handling complex schemes

In the case of numeric per-residue annotations, the DTD also allows one to indicate whether a graph (either a line chart or a bar chart) should be provided to display the data. In addition, a mechanism by which individual residues can be highlighted as 'positive' predictions is provided. Initially the hope was simply to provide some threshold value such that any per-residue numeric scores higher than the threshold could be flagged by the display program. However, some of the servers have much more complex threshold schemes. For example NetOGlyc makes a positive prediction if one score (the 'G-score') is > 0.5 or, for threonines, if the G-score is < 0.5, but the 'I-score' is > 0.5 and there are no other sites predicted within 10 residues. Therefore it was decided that the DTD should include a list of the residues considered as positive predictions together with a description of how such residues are identified. This moves the logic of indicating a positive prediction back to the service wrapper rather than the display program.

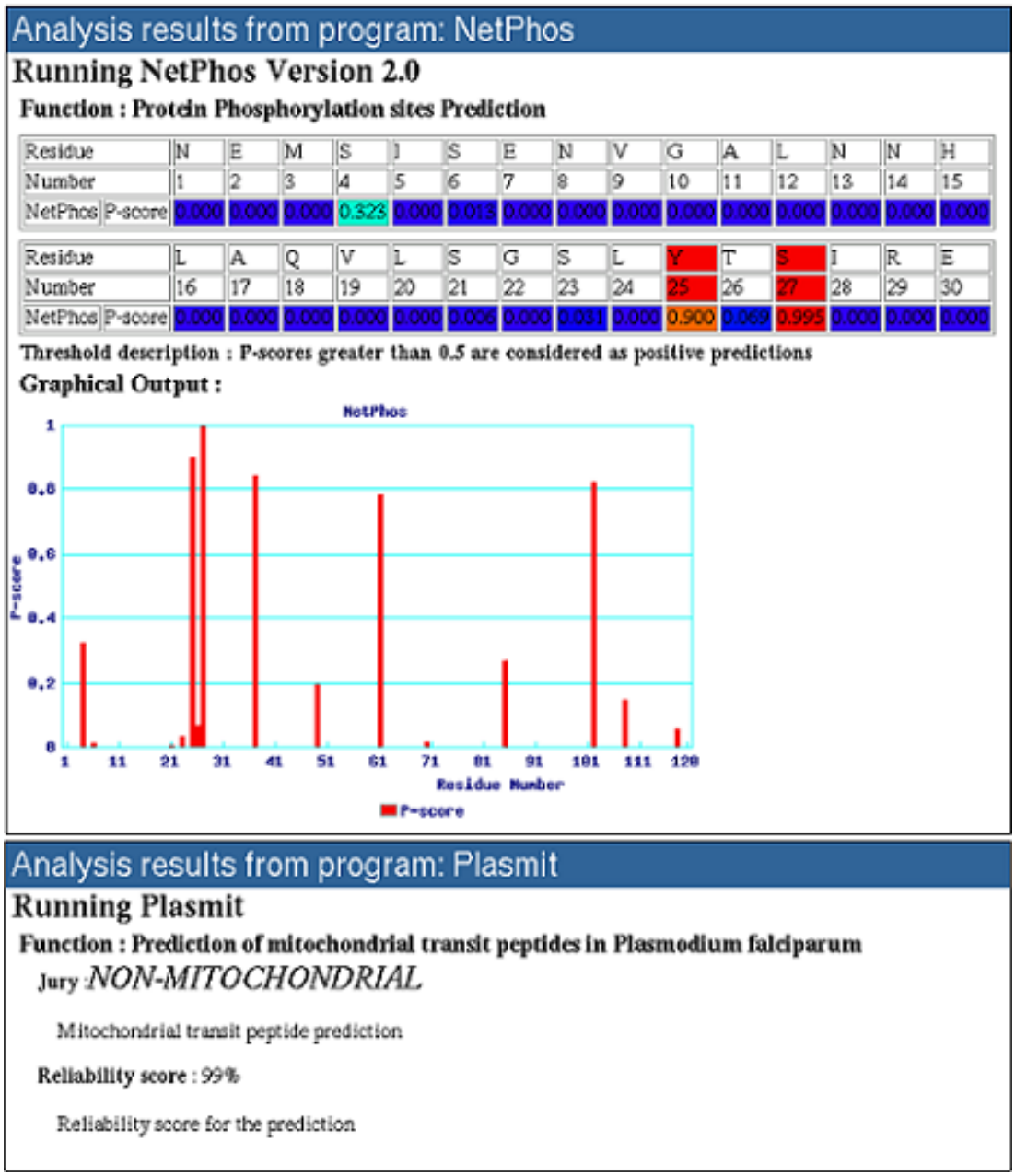
3.3.4 System architecture

The overall architecture of the APAT system is shown in Figure 3.10. The system is implemented in Perl using the XML::DOM module for parsing XML files. The software consists of 3 major components:

1. a 'master' script which reads an XML input file containing the sequence

```
<!ELEMENT date ( #PCDATA ) >
<!ELEMENT description ( #PCDATA ) >
<!ELEMENT emailaddress ( #PCDATA ) >
<!ELEMENT function ( #PCDATA ) >

<!ELEMENT info ( #PCDATA ) >
<!ATTLIST info href CDATA #IMPLIED >

<!ELEMENT input ( seqid, seq+, emailaddress, parameter+ ) >

<!ELEMENT link ( #PCDATA ) >
<!ATTLIST link href CDATA #REQUIRED >

<!ELEMENT param EMPTY >
<!ATTLIST param name CDATA #REQUIRED >
<!ATTLIST param value CDATA #REQUIRED >

<!ELEMENT parameter EMPTY >
<!ATTLIST parameter param CDATA #REQUIRED >
<!ATTLIST parameter server CDATA #REQUIRED >
<!ATTLIST parameter value CDATA #REQUIRED >

<!ELEMENT params ( param+ ) >

<!ELEMENT perdom ( value-perdom+ ) >
<!ATTLIST perdom class CDATA #IMPLIED >
<!ATTLIST perdom highlight CDATA #IMPLIED >
<!ATTLIST perdom name CDATA #IMPLIED >
<!ATTLIST perdom rangemax CDATA #REQUIRED >
<!ATTLIST perdom rangemin CDATA #REQUIRED >

<!ELEMENT perdom-description ( #PCDATA ) >
<!ATTLIST perdom-description class CDATA #IMPLIED >
<!ATTLIST perdom-description name CDATA #REQUIRED >

<!ELEMENT perres-character ( value-perres+ ) >
<!ATTLIST perres-character name CDATA #REQUIRED >
<!ELEMENT perres-number ( value-perres+ ) >
<!ATTLIST perres-number clrmax CDATA #REQUIRED >
<!ATTLIST perres-number clrmin CDATA #REQUIRED >
<!ATTLIST perres-number graph CDATA #IMPLIED >
<!ATTLIST perres-number graphtype CDATA #IMPLIED >
<!ATTLIST perres-number name CDATA #REQUIRED >

<!ELEMENT perseq ( description, value-perseq ) >
<!ATTLIST perseq name CDATA #REQUIRED >

<!ELEMENT predictions (link | perdom | perdom-description | perres-character | perres-number | perseq | threshold)* >

<!ELEMENT result ( function, info, run, predictions ) >
<!ATTLIST result program CDATA #REQUIRED >
<!ATTLIST result version CDATA #IMPLIED >

<!ELEMENT results ( input, result+ ) >
<!ELEMENT run ( params, date ) >
<!ELEMENT seq ( #PCDATA ) >
<!ELEMENT seqid ( #PCDATA ) >
<!ELEMENT thr-res ( #PCDATA ) >
<!ELEMENT threshold ( description, thr-res* ) >

<!ELEMENT value-perdom ( #PCDATA ) >
<!ATTLIST value-perdom label CDATA #REQUIRED >

<!ELEMENT value-perres ( #PCDATA ) >
<!ATTLIST value-perres residue CDATA #REQUIRED >

<!ELEMENT value-perseq ( #PCDATA) >
<!ATTLIST value-perseq highlight CDATA #REQUIRED >
```

Figure 3.8: APATML DTD.



Analysis results from program: NetPhos

Running NetPhos Version 2.0

Function : Protein Phosphorylation sites Prediction

Residue	N	E	M	S	I	S	E	N	V	G	A	L	N	N	H
Number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
NetPhos P-score	0.000	0.000	0.000	0.323	0.000	0.013	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Residue	L	A	Q	V	L	S	G	S	L	Y	T	S	I	R	E
Number	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
NetPhos P-score	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.031	0.000	0.900	0.069	0.995	0.000	0.000	0.000

Threshold description : P-scores greater than 0.5 are considered as positive predictions

Graphical Output :

Analysis results from program: Plasmit

Running Plasmit

Function : Prediction of mitochondrial transit peptides in Plasmodium falciparum

Jury : *NON-MITOCHONDRIAL*

Mitochondrial transit peptide prediction

Reliability score : 99%

Reliability score for the prediction

Figure 3.9: Sample HTML output from the APAT system showing per-residue and per-sequence annotations. The per-residue annotation has been edited to two lines for brevity. A full example may be seen on the APAT web site.

and dispatches it to each service wrapper,

2. wrappers for each annotation/prediction service,

3. a display program which converts the APATML output from the annotation/prediction services to HTML for display.

This display program can be replaced by any number of post-analysis scripts. In addition, to allow the system to be used to process a batch of sequences, a short script was implemented which will run through all the input XML files in a directory and process each in turn using the 'master' script. While the system is not really designed for online use, a simple web interface was implemented primarily for demonstration purposes, although this could prove useful for intranet installations.

The Display Program

The most complex part of the system is the display program which provides a uniform display for all the annotation services. The APATML file is read using XML::DOM. Per-sequence annotations, which are applied to the whole sequence, are simply displayed as text, while per-domain annotations are displayed as a simple table of results with associated descriptions following the table. Per-residue annotations are presented in the form of a table in which numeric values are coloured on a scale from blue through green to red. In addition, residues marked in the APATML as 'positive predictions' are highlighted and, where indicated by the APATML, the GD::Graph Perl module is used to provide graphical display of per-residue annotations. A sample of HTML output from the display program is shown in Figure 3.9).

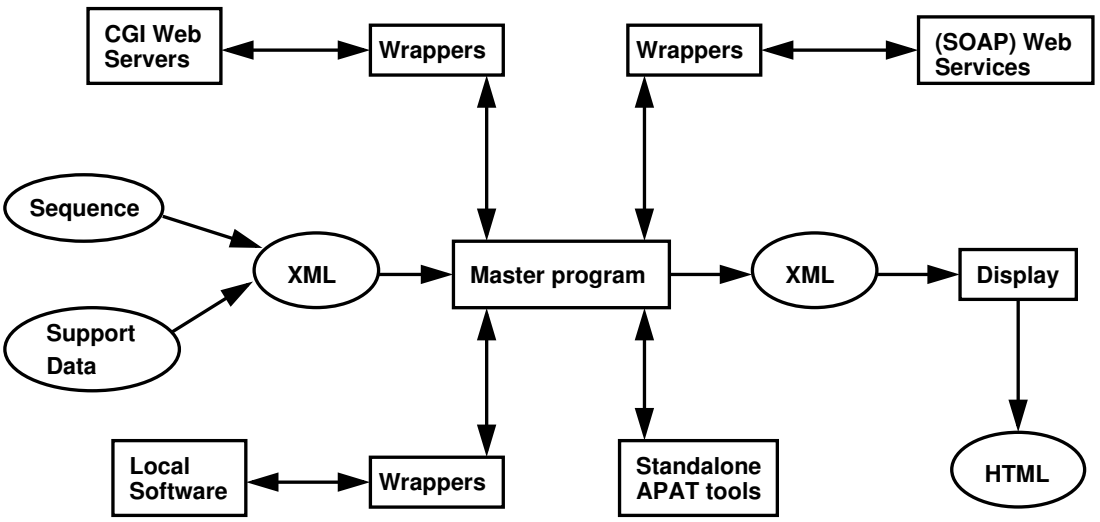


Figure 3.10: Overall architecture of the APAT system.

Service Wrappers

Each of the service wrappers is implemented as a stand-alone 'plug-in'. The master program simply identifies all the plug-ins available and runs each in turn. This design allows individual service wrappers to be implemented and debugged as stand-alone code and simply placed in a standard directory for integration into the system. Plug-ins can be implemented such that they provide a self-contained annotation service, but in practice they are generally wrappers to some other tool. Such tools may reside locally or remotely, either as Web-services or CGI-based servers on the web. Remote services may be accessed via SOAP or by 'screen-scraping' of web pages respectively. Since the only requirement of the plug-ins is that they read and write XML, they can be implemented in any programming language and integrated seamlessly into the APAT system. A number of plug-in service wrappers are implemented in Perl for which the SOAP::Lite and LWP packages make access to Web-services and CGI-based servers straightforward.

Tool	Web address
NetPhos	`http://www.cbs.dtu.dk/services/NetPhos/`
PsiPred	`http://bioinf.cs.ucl.ac.uk/psipred/` (running locally)
TargetP	`http://www.cbs.dtu.dk/services/TargetP/`
TMHMM	`http://www.cbs.dtu.dk/services/TMHMM/`
ChloroP	`http://www.cbs.dtu.dk/services/ChloroP/`
NetOGlyc	`http://www.cbs.dtu.dk/services/NetOGlyc/`
DAS-TMfilter	`http://www.enzim.hu/DAS/DAS.html`
PrositeScan	`ftp://us.expasy.org/databases/prosite/tools/ps_scan/` (running locally)
Plasmit	`http://gecco.org.chemie.uni-frankfurt.de/plasmit/`
PSORT	`http://psort.nibb.ac.jp/form.html`
SubLoc	`http://www.bioinfo.tsinghua.edu.cn/SubLoc/`

Table 3.3: Wrappers are made available for the tools listed here along with their web addresses.

It is also possible to use wrappers on top of web services made available by Taverna (Oinn *et al.*, 2004), the EBI (Labarga *et al.*, 2007), or other sources, to obtain results from various tools. Writing simple wrappers to access these services avoids the need to write a wrapper from scratch. All that is required is a simple wrapper to take care of XML formatting of the output so that it fits into the XML DTD of APAT.

Implementation of additional service wrappers is relatively straightforward and validation against the APATML DTD ensures that the results can be converted to HTML using the display program. The DTD was prepared with the aid of the XML-to-DTD conversion utility from Hit Software available at `http://www.hitsw.com/xml_utilites/` before careful manual checking and modification. A guide to writing wrappers with explanation and examples is provided on the CD-ROM and the APAT website. A list of wrappers written to date is provided in Table 3.3.

3.3.5 Web interface

A simple web interface was created which allows a single sequence to be submitted to a small number of prokaryotic and eukaryotic prediction tools and was provided as a demonstration (accessible at `http://www.bioinf.org.uk/apat/`). An example of HTML output from the tool was also provided on the web for a quick overview. Users can run the tool, although it does not include all the plugin wrappers provided in the downloadable version. A facility for submitting additional wrappers written by users was also provided. An XML DTD for input and output along with other documentation about writing wrappers and the source code of APAT was also provided for download.

3.4 Summary and Discussion

APAT is designed to perform a very simple but repetitive task in a straightforward manner: it allows one or more sequences to be presented to a number of different annotation/prediction servers, collating the results and presenting them in a consistent format for automated or visual analysis. This approach contrasts with Taverna (Oinn *et al.*, 2004) and ToolBus (Eckart and Sobral, 2003). These are hugely capable workflow-based systems which, while clearly capable of similar things, come with an overhead of complexity requiring some considerable investment in time to learn how they can be extended. In addition, Taverna, in its current form, is not designed to highlight the key information needed by a Biologist in a simple and consistent format.

The output produced by a wide range of protein sequence and annotation tools was analyzed and it was determined that all annotations can be expressed in one

of four ways (character or numeric per-residue annotations, or annotations at the per-domain or per-sequence level). On the basis of this analysis, an XML DTD was designed to abstract and encode the annotations provided by any prediction server. On the basis of this DTD, a display tool and wrappers to a number of annotation/prediction services running both locally and remotely were designed and implemented.

APAT provides an easy way of comparing the output from various tools through uniform output format, but does not suggest which particular tool among a group of similar tools provides the best answer. It also does not provide a final summary of output for each sequence. APAT is an annotation fan where the choice of tools and interpretation of output is left with users.

Wrappers are provided for some of the currently available state-of-the-art tools. Usage of the display program to obtain HTML output is optional and could be replaced by any type of post analysis scripts.

APAT was used in TAPAS (Chapter 4) and also in the work of improving transmembrane prediction (Chapter 5).

The system is designed to be downloaded and run locally allowing the user to run many annotation/prediction services on one or more sequences without manual intervention. Users can easily choose which plug-in annotation services they wish to use and implementation of additional service wrappers is straightforward using the existing wrappers as examples. While this may not be possible for the average Biologist, it should take an experienced Bioinformatician no more than a couple of hours to implement an additional wrapper. Compliance of the resulting XML can be checked against the APATML DTD using a validating parser to ensure compatibility.

While the system allows multiple single sequences to be sent to prediction servers, it makes no attempt to handle servers which require multiple sequences (for example, multiple sequence alignments and phylogeny). Similarly there is no ability to deal with servers which return much more complex data such as three-dimensional models built by comparative modelling. The display tool presents the results of each annotation/prediction server separately; a further possible enhancement would be to display multiple residue-level annotations on a single view of the sequence as is done in DAS (`http://www.biodas.org/`).

Source code for the master and display programs and for a number of plug-in service wrappers has been made available for download from the web site together with the DTD and documentation. The download also provides the scripts for converting a sequence to the input XML format and for running all the input XML files in a specified directory through the APAT system. An installation script (written by Dr. Andrew Martin) is provided which installs the software and, optionally, the web interface. Documentation includes detailed descriptions of the APATML format and a guide to implementing service wrappers. As a demonstration, a web-based tool that allows a single sequence to be submitted to a small number of prokaryotic and eukaryotic prediction tools is also provided. The system may be accessed at `http://www.bioinf.org.uk/apat/`.

Frishman (2007) in his review of protein annotation on a genomic scale has written about tools such as APAT having the advantage of being highly configurable and flexible. The following is an extract from Frishman's review of protein annotation:

> "In recent years, the idea of protocol-based genome data processing has been popularized, which draws parallels between the organization

> of routine bioinformatics analyses and experimental lab work. Just as wet experiments are carefully planned and then executed following a defined sequence of steps, tools such as BioPipe and APAT allow for the creation of customized workflows from standard modules, which typically include XML parsers for a variety of input and output formats, wrappers for running external applications, interfaces to SQL databases and batch processing systems, and facilities for transporting the results to the end user via standard exchange protocols, such as Web services. In contrast to conventional integrated genome analysis systems, protocol-based analysis pipelines have the advantage of being highly configurable and flexible, but their users are required to have a good understanding of software technologies as well as substantial system administration and bioinformatics skills. Recent releases of major genome analysis systems, such as PEDANT, have also been equipped with workflow-based process management (Frishman et al., in preparation)" (Frishman, 2007).

At the time of writing (June 2008), APAT had been downloaded by >600 users.

Chapter 4

Target Annotation Pipeline and Automated Selection — TAPAS

In the previous chapter, I described a tool (APAT) that despatches an input sequence to many annotation and prediction tools to obtain assorted annotations which are displayed in a uniform manner for visual analysis. Results from various tools are independent of one another because they are not serially dependent. In this chapter, I describe a specialized pipeline tool that despatches different inputs to different tools that are serially dependent on results obtained from the previous tool. The output from one tool would be the input for another tool except while executing APAT which is also integrated as a part of this tool. It is appropriate to mention that Taverna is an extremely powerful tool which could have been an alternative to TAPAS. TAPAS is a simple pipeline developed to perform a specialized task of ranking proteins for their suitability for SBDD, whereas Taverna is a general purpose Grid workflow management system which is capable of performing tasks similar to TAPAS and also a wide range of other tasks.

In spite of the existance of general purpose workflow systems such as Taverna, which are capable of building customized workflows, there are a number of specialized pipelines being developed. For example, GATO (Fujita *et al.*, 2005), MPP (Davey *et al.*, 2007), MicroGen (Burgarella *et al.*, 2005), PseudoPipe (Zhang *et al.*, 2006), BIPASS (Lacroix *et al.*, 2007), PROSPECT-PSPP (Guo *et al.*, 2004) are all specialized pipelines, developed very recently. A few possible reasons for this could be because: a) a custom solution is a low-risk strategy in terms of time needed to understand, implement and extend these general purpose workflow systems, b) there is no real need for all the complex features (which include Grid computing, distributed annotation, etc.) provided by such tools, c) an in-house pipeline could be the ideal one for their needs — simple in terms of developement and usage, and precise in terms of fulfilling their needs. For similar reasons a decision was made to create a specialized in-house system.

4.1 Introduction

If one would like to analyze the protein sequences of an organelle, a pathway, or a genome and screen them for potential drug targets, then one needs to perform a set of repetitive tasks (sequentially, or in parallel) such as running BLAST to find homologues, screening for human hits, checking whether or not a protein is an enzyme, looking for KEGG pathway maps, checking whether or not it is a transmembrane protein, or looking for availability of 3D structures (of the protein or of homologues) preferably with a bound ligand. Clearly this is a tedious job to do manually. A computer analysis eliminates the risk of being incomplete as computers are ideally suited for performing repetitive tasks and are better at pattern matching for large sets of data.

Having preliminary information about whether or not a protein could be a good drug target is very useful for the pharmaceutical sector, especially for Structure Based Drug Design (SBDD). My aim was to look for potential drug targets among protein sequences belonging to a particular organelle, the apicoplast and a pathway, the MEP pathway.

While it is true that around 50% of drugs bind to membrane bound receptors, the characteristics of good microbial druggable targets for which one intends to design a drug by SBDD include:

(a) no human homologue: not having any human homologue is an ideal condition because the chances of any cross-reaction owing to structural similarity is minimal.

(b) known ligands: having known ligands from other data sources is useful because they can be used as lead molecules on which one can modify the chemical groups in such a way that they bind to the intended drug target, often with increased specificity.

(c) availability of structural data: availability of structural data for the drug target or for a close homologue from which a model can be built (ideally with known ligands in bound and unbound states to understand conformational changes) is of great importance and a prerequisite in understanding the interactions with the active site residues for SBDD. In the absence of pre-existing data, ideally the structure would have to be solved by X-ray crystallography or NMR. Computer built models of protein structure are quite accurate when the sequence identity is >50% (Baker and Sali, 2001) (Root Mean Square Deviation (RMSD) for main-chain atoms of about 1 Å

which is comparable to medium-resolution NMR structure or low-resolution X-ray structure). Comparative models of moderate-accuracy can be built when the sequence similarity is between 30–50%. Since structure is better conserved than sequence during evolution, useful models can be produced even at lower sequence identity. Normally, if the sequence similarity between the model and the template is lower than 30%, it is difficult to obtain a model of good quality. However, Class A GPCRs are an exception, because each helix contains one or two highly conserved residues which permit an unambiguous alignment (Oliveira *et al.*, 2004). Thus, good models can be obtained for Class A GPCRs even when the sequence similarity is as low as 20% (Oliveira *et al.*, 2004).

(d) known Enzyme commission (EC) number: Members of a protein family having high structural and sequence similarity can perform different functions while proteins with dissimilar structures can perform identical biochemical roles (Todd *et al.*, 1999). Strictly speaking an EC number corresponds to enzymatic reaction, but is used as a numerical classification scheme for enzymes where enzymes are classified hierarchically (4 levels). Having a known EC number indicates that the protein is an enzyme which provides the opportunity to design inhibitors, especially competitive inhibitors that bind to the active site. However, these do not make the best drugs because their effect diminishes as substrate concentration builds up. Enzymes represent >47% of launched drug targets (Hopkins and Groom, 2002; Swindells and Overington, 2002). Here, the EC number helps to identify ligands for other enzymes having similar EC numbers as these enzymes are likely to have similar substrates and thus similar inhibitors,

(e) membrane bound: having information about whether a protein is membrane bound or not is useful. Despite the fact that ∼50% of today's prescription drugs target membrane proteins (Elofsson and Heijne, 2007; Terstappen and Reggiani, 2001; Flower, 1999; Gudermann *et al.*, 1995), these are not ideally suited for traditional SBDD because it is difficult to obtain a high resolution 3D structure through X-ray crystallography or multi-dimensional NMR spectroscopy.

Numerous factors must be considered while selecting a suitable target for SBDD which can broadly be grouped into three major categories:

1. Biological suitability
   (a) In the case of antimicrobials, one should avoid targets which have host homologues especially functionally equivalent orthologues.
   (b) In the case of host targets, one should avoid targets with close paralogues.
   (c) Knowledge of function (e.g., enzyme class) helps to suggest leads.
   (d) Knowledge of ligands and ligands that bind to (even distant) homologues helps to provide leads.
2. General structural suitability
   (a) Is a structure known?
   (b) Is the structure of a homologue known? (from which a model might be built).

(c) If no structural data are known, is the protein an integral membrane protein (in which case it is unlikely structural data can be obtained), or could a structure be solved?

3. Detailed structural suitability

(a) Does the protein have a cleft/binding pocket that could be exploited?

(b) Is the protein involved in interactions which could be disrupted?

(c) Is a structure known with a ligand bound that could be modified to form a drug?

The TAPAS pipeline essentially addresses the first two categories. It uses various runs of BLAST/PSI-BLAST to look for homologues, it looks for structural data and identifies transmembrane proteins that may not be suitable for structural analysis. It ranks supplied sequences on the basis of these factors such that the better looking sequences can then be taken forward for detailed structural analysis.

To obtain further insight into the druggability of a protein, one must examine various detailed structural features, such as the presence of hydrophobic deep clefts which provide increased surface area thus increasing the probability of a protein's binding ability for a small drug molecule (Laskowski *et al.*, 1996; Lewis, 1991). A tool that can automate the process of selection and annotation of drug targets before embarking on detailed structural analysis and SBDD, would enable one to process a set of protein sequences belonging to a particular pathway, organelle, or genome, to obtain annotated proteins along with specific details useful for determining whether or not a protein is a potential drug target at a basic level.

The general requirements for such a tool are: allowing one or more sequences to be analyzed in a single run, using multiple prediction/annotation tools residing locally, or over the web through normal CGI scripts or Web-services; cross-linking data from various databases and handling inconsistency; obtaining an insight into whether or not a protein is a possible drug target; and providing a compact output summary (ideally in HTML) with key information about the parameters that determine the selection of a possible drug target along with links to more detailed output.

Various pipeline and workflow based systems were discussed in Chapter 2. The analysis of other pipelines and workflows mentioned in the context of APAT is also applicable to TAPAS.

Target Annotation Pipeline and Automated Selection (TAPAS) was therefore designed mainly to enable a set of sequences to be presented to a number of different search, annotation and prediction tools which are run sequentially. Output from one tool was parsed and written into an intermediate file. This was then passed as input to another tool. Annotations and predictions were handled by APAT, which was integrated into the TAPAS pipeline as a standalone tool. Annotations of a protein sequence obtained from a set of tools were matched and tabulated in an HTML file. This provides a quick overview of results which were also hyperlinked for obtaining additional information from the world wide web or a local file. This HTML table includes key information about the presence of human hits (with E-values), structures (also ligands and heteroatoms), whether or not a hit is an enzyme, and whether or not a protein is a transmembrane protein (Swindells and Overington, 2002; Swindells and Fagan, 2001; Fagan and Swindells, 2000). These are the key features that were considered in this study

for obtaining some preliminary information about druggability of a protein for SBDD. This is a stage preceding detailed structural studies of proteins for assessing their suitability for druggability. Note that this project does not involve any predictions of function, or regions of proteins such as the active site, but just looks at some key biological characteristics of protein targets and ranks them for SBDD at a basic level devoid of detailed structural studies. One could then concentrate on those highly ranked proteins to further determine whether or not it is a 'druggable' target.

4.2 Specific software requirements

The main requirements for the TAPAS software were:

1. allowing one or more protein sequences (particularly proteins that belong to a particular pathway or an organelle) to be analyzed in a single run,

2. running multiple search, prediction and annotation tools (BLAST, PSI-BLAST, and tools integrated into the APAT system — NetPhos, NetOGlyc, TargetP, ChloroP, TMHMM, DAS-TMfilter, PSORT, SubLoc, PlasMit, PsiPred, PrositeScan) residing locally or over the web,

3. parsing output from one program and piping it as input for another to obtain: GenBank style IDs (from BLAST output) $\Longrightarrow$ SwissProt ACs $\Longrightarrow$ Species name $\Longrightarrow$ EC numbers $\Longrightarrow$ KEGG pathway maps,

4. cross-linking and tabulating data obtained from the steps described above,

5. providing a compact HTML output page with key information about the characteristics that determine the selection of a possible drug target for

SBDD (at a basic level devoid of detailed structural studies) along with hyperlinks for more detailed output.

These software requirements were devised by us to meet our needs of obtaining preliminary information about specific characteristics of a protein (Chen and Chen, 2008; Hopkins *et al.*, 2006; Swindells and Overington, 2002; Fagan *et al.*, 2001; Weir *et al.*, 2001). Thus TAPAS is a specialized pipeline where the tools to be used and the data flow was determined by us in a way we envisioned would be more useful for reaching our goals. TAPAS uses state-of-the-art tools for a specific annotation in a systematic way in order to obtain a diverse set of annotations. It is advantageous to wrap as many tools as possible within APAT (which is integrated into TAPAS) even if they have no effect on ranking the proteins as suitable drug targets.

The output design was such that the compact HTML page has details about the key features being considered in this study which are a) human homologues b) EC number c) known structure d) known ligand e) transmembrane. These details are later used for ranking proteins for their suitability for SBDD.

4.3 Approach and methods

4.3.1 Workflow and overall architecture

The workflow of TAPAS is shown in Figure 4.1. This provides a brief overview of tools used in the pipeline and the direction of flow of data in the workflow.

The overall architecture of TAPAS is shown in Figure 4.2. The system is implemented in Perl and uses the XML::DOM module for parsing XML files output from APAT (See Chapter 3 and Deevi and Martin (2006)). The pipeline

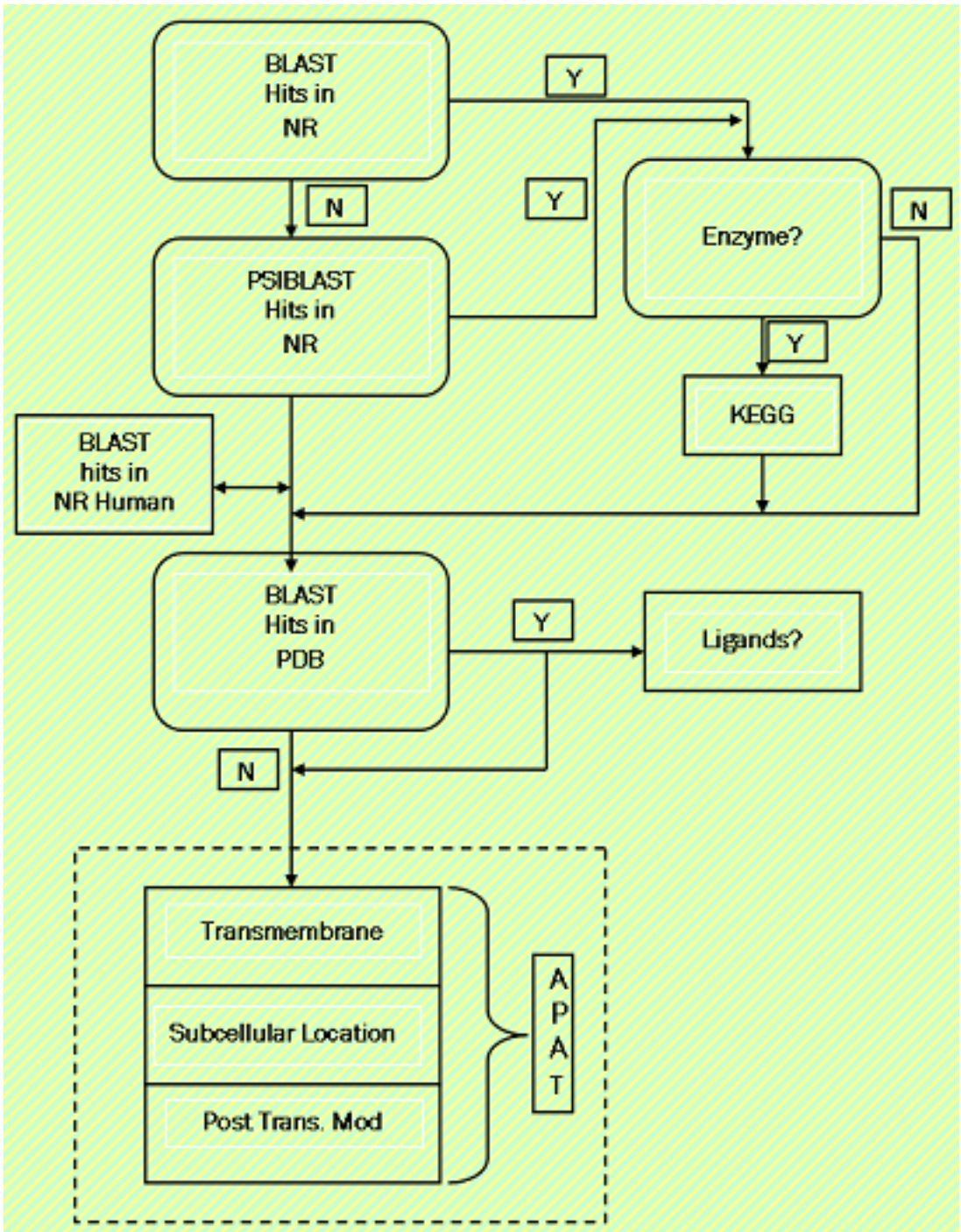


Figure 4.1: Workflow of TAPAS.

contains 4 major steps:

1. applying annotations in a stepwise manner by a master script,

2. integrating APAT into the system as a standalone tool for further annotations,

3. cross-linking the results from heterogeneous tools to make an HTML table provided with hyperlinks for additional information,

4. making a compact drug target selection table (with hyperlinks for relevant websites or local files to obtain additional information) containing useful information for obtaining insight into the selection of a protein as a possible drug target (Swindells and Overington, 2002; Swindells and Fagan, 2001; Fagan *et al.*, 2001).

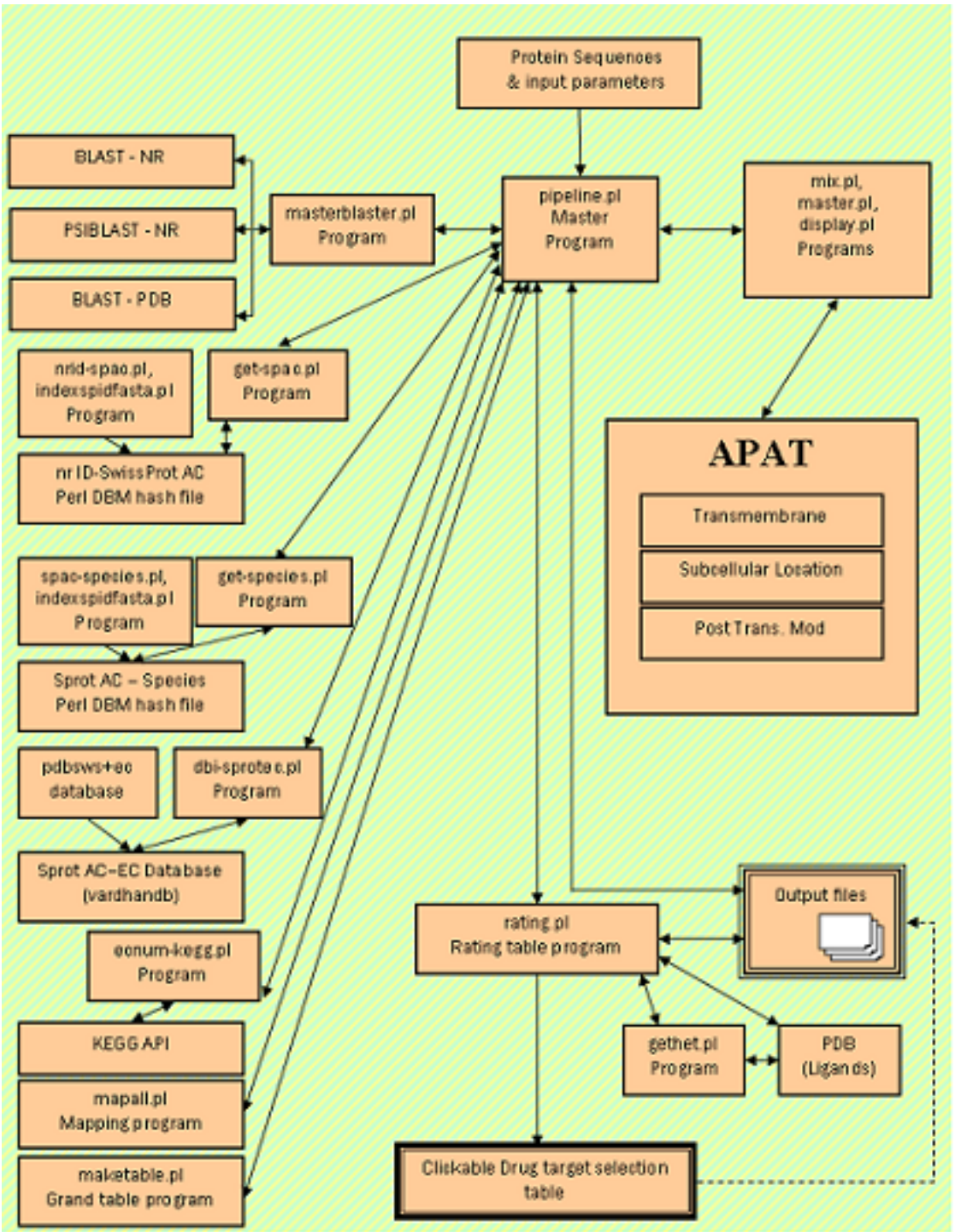


Figure 4.2: Overall architecture of TAPAS.

4.3.2 Applying annotations in a stepwise manner

Different types of annotations could be applied to a number of sequences in a stepwise manner. The master program, accepts

1. a definition file containing information about origin of sequences as required for running APAT,
2. a file containing protein sequences in Fasta format,
3. the output directory name for the results of running the pipeline.

A set of programs is then called one after another by this master program. The output from one program is parsed and used as input for the next except when running the APAT annotation fan which takes Fasta sequences as input. The pipeline runs BLAST (Altschul *et al.*, 1990), links GenBank (Benson *et al.*, 2007) style identifiers (IDs) to SwissProt (Bairoch and Apweiler, 2000) accession codes (ACs), SwissProt ACs to species name, SwissProt ACs to Enzyme Commission (EC) numbers (Bairoch, 2000), EC numbers to KEGG pathway maps (Kanehisa *et al.*, 2002), runs APAT, interlinks data from heterogeneous tools and tabulates all these results in an HTML table, and makes a drug target selection table by screening species names for human hits, looking for known structures and ligands, and checking whether or not a protein is transmembrane.

Running BLAST

The master program calls another program to perform a set of BLAST runs:

- BLAST against the nr (non-redundant) protein database[1],

[1]The nr protein database maintained by the NCBI as a target for their BLAST search services is a composite of SwissProt, SwissProt updates, PIR, and PDB. Entries with absolutely identical sequences have been merged.

```
ABE11004.1  :  Q1PKM6
ABE10930.1  :  Q1PKS6
GAA02238.1  :  Q1WYT7
AAR99081.1  :  Q6R3F4
AAP56260.3  :  Q7XYT1
ABH08964.1  :  Q0MW94
CAB43344.1  :  Q9XFS9 Q9M6U2
AAL37560.1  :  Q8W250 Q9FTN0
AAW28998.1  :  Q5MJZ5
```

Figure 4.3: Sample extract from the intermediate output file having GenBank style IDs cross-linked to their corresponding SwissProt ACs.

- PSI-BLAST (Altschul *et al.*, 1997) against the nr protein database, and
- BLAST against the PDB (Berman *et al.*, 2000).

The output from the BLAST runs is parsed and written into a file in a specified directory which is later used for extracting GenBank style IDs and E-values (Expectation-value)[2].

Linking GenBank style IDs to SwissProt ACs

The master program calls another program, which parses GenBank style IDs from the BLAST output file and, where possible, links these IDs to their corresponding SwissProt ACs. SwissProt ACs are obtained from a pre-created Perl DBM Hash file which indexes GenBank style IDs and SwissProt ACs. A sample view of the intermediate output file obtained at this stage is shown in Figure 4.3.

[2]The E-value describes the number of hits with a given score or better one can expect to see by chance when searching a given database of a particular size.

```
Q1PKM6  :   uncultured Prochlorococcus marinus clone ASNC612
Q1PKS6  :   uncultured Prochlorococcus marinus clone ASNC3046
Q1WYT7  :   Pelotomaculum thermopropionicum SI
Q6R3F4  :   Plectranthus barbatus
Q7XYT1  :   Cistus creticus
Q0MW94  :   Nicotiana tabacum (Common tobacco)
Q9XFS9  :   Arabidopsis thaliana (Mouse-ear cress)
Q9M6U2  :   Arabidopsis thaliana (Mouse-ear cress)
Q8W250  :   Oryza sativa (Rice)
```

Figure 4.4: Sample extract from the intermediate output file having SwissProt ACs mapped to their species name.

Linking SwissProt ACs to species name

The master program calls another program, which links SwissProt ACs to their corresponding species names. Species names are obtained from a pre-created Perl DBM Hash file having SwissProt ACs indexed against species names. A sample view of the intermediate output file obtained at this stage is shown in Figure 4.4.

Linking SwissProt ACs to EC numbers

The master program calls another program, which links SwissProt ACs to their corresponding EC numbers. EC numbers are obtained from a pre-created PostgreSQL (`http://www.postgresql.org/`) database which has a copy of the 'acac' table and 'sprotec' table from databases 'PDBSWS' (Martin, 2005) (which provides residue level mapping between PDB entries and UniProtKB/SwissProt entries) and 'PDBSprotEC' (Martin, 2004) (which provides mapping between PDB chains and EC numbers via SwissProt ACs) respectively. The PostgreSQL relational database also contains two more pre-created tables 'sprot_ec' and 'sprot_species' and all four tables are accessed through the Perl::DBI module.

```
Q9XFS9 : 1.1.1.267
Q9M6U2 : 1.1.1.267
Q8W250 : 1.1.1.267
Q9FTN0 : 1.1.1.267
Q57T35 : 1.1.1.267
P45568 : 1.1.1.267
P77209 : 1.1.1.267
Q8KMY5 : 1.1.1.267
P45568 : 1.1.1.267
```

Figure 4.5: Sample extract from the intermediate output file having SwissProt ACs matched with their EC numbers.

Cross-references in various databases not being updated in other databases is a complicating factor. For example, in SwissProt, outdated accession codes are retained as 'secondary accession codes' of a SwissProt entry (which now has a new primary accession code), but the Enzyme database, which is not updated as frequently as SwissProt, may maintain the outdated accession codes making the mapping more complex as both 1°and 2°accessions must be checked. In the PDB-SWS database, the 'acac' table links secondary SwissProt ACs to their primary ACs while the 'sprotec' table links the primary AC to its EC number which will then enable one to obtain an EC number from a new primary accession code of a SwissProt entry via its secondary accession code. A sample view of the intermediate output file obtained at this stage is shown in Figure 4.5. Format changes of databases has also been a concern because they break any scripts relying on the old format. For example, SwissProt has twice changed the allowed combinations of characters in ACs and has changed the format of its Fasta sequence dump headers during this project.

```
1.1.1.267 : path:map00100
1.1.1.23  : path:map00340
6.3.2.3   : path:map00251
6.3.2.3   : path:map00480
1.1.1.3   : path:map00260
1.1.1.3   : path:map00300
```

Figure 4.6: Sample extract from the intermediate output file having EC numbers matched with their KEGG pathway maps.

Searching for pathway maps in KEGG from EC numbers

The master program calls another program, which links EC numbers to their corresponding KEGG pathway maps, using SOAP webservices via the KEGG API. A sample view of the intermediate output file obtained at this stage is shown in Figure 4.6.

4.3.3 Integrating APAT into the pipeline

APAT (Chapter 3 and Deevi and Martin (2006)) was integrated into the pipeline as a stand-alone tool to provide more annotations by including the required wrappers in the APAT system.

Having knowledge about subcellular location, post-translational modifications, whether or not a protein is transmembrane, 3-dimensional structure, and ligands is useful for assessing accessibility of drug targets (for example, if the drug target is inside a membrane bound organelle then the drug needs to cross the extra bilipid barrier of the membrane to reach its target).

The following tools were included in APAT as used by the pipeline:

1. transmembrane predictors — TMHMM (Krogh *et al.*, 2001), DAS-TMfilter (Cserzo *et al.*, 2004), and MEMSAT 2 (Jones *et al.*, 1994)

2. subcellular location predictors — SubLoc (Chen *et al.*, 2006), TargetP (Emanuelsson *et al.*, 2000), PSORT (Nakai and Horton, 1999), ChloroP (Emanuelsson *et al.*, 1999) and PlasMit (Bender *et al.*, 2003)

3. post translational modification predictors — NetPhos (Blom *et al.*, 1999) and NetOGlyc (Julenius *et al.*, 2004)

4. secondary structure predictors — PsiPred (McGuffin *et al.*, 2000)

5. motif predictors — PrositeScan (Gattiker *et al.*, 2002)

The TAPAS master program calls three programs consecutively to complete the execution of APAT:

- First, protein sequences in Fasta format and information about the origin of the sequence are combined to form an input file for APAT in a defined XML format (APATINML — DTD shown in Figure 3.4),

- Second, this XML input file is then processed by APAT to produce heterogeneous annotations in a defined XML format (APATML — DTD shown in Figure 3.8), and

- Third, the XML output from APAT is converted to HTML by the 'display.pl' program for visual analysis.

4.3.4 Cross-linking and tabulating data

Data obtained from various annotation/prediction tools and databases are cross-linked and assembled. These data are written into an HTML table to provide a compact and quick overview of the results from the various tools. Details about

Type of BLAST	Significant BLAST hit	E-value	SwissProt AC	Species name	EC number	KEGG map
INPUT: O84468|ISPD_CHLTR 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase - Chlamydia trachomatis [APAT output: APAT.html]						
Blast	NP_219975.1	1e-113	undefined	undefined	undefined	undefined
Blast	NP_297120.1	8e-96	undefined	undefined	undefined	undefined
Blast	B71511	1e-88	O84468	Chlamydia trachomatis	2.7.7.60	path:map00100
Blast	YP_219590.1	7e-63	undefined	undefined	undefined	undefined
Blast	YP_515762.1	2e-60	undefined	undefined	undefined	undefined

Figure 4.7: Sample extract from the intermediate output file from the cross-linked data table.

the type of BLAST run (BLAST or PSI-BLAST), significant BLAST hits along with their corresponding E-values, SwissProt ACs, species names, EC number, and KEGG pathway maps are included in the table. Most of these data are hyperlinked to obtain further details from relevant websites or local files. A sample view of the intermediate output file obtained at this stage is shown in Figure 4.7.

4.3.5 Making a drug target selection table

The conclusions for the output of all the sequences submitted to the pipeline during a particular execution are provided in the form of a drug target selection table. Details about the input protein, a list of human hits (if present) along with the E-value, EC numbers of enzyme hits, PDB code of the best structural hit based on E-value, a list of ligands and hetero atoms, and whether or not it is a membrane protein are included in the table. These details are some of the

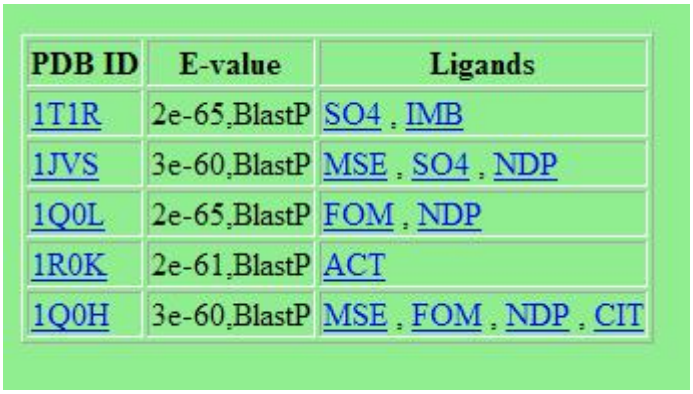
Input Protein	Human Hits(e-val)	Is Enzyme(all hits)	Structure(Best hit)	Ligands(Hetatm)	Is Membrane
inputseq1, allhitsmap, APAT output	NO	2.2.1.7	2O1X	MG , TDP , All_ligs	NO
inputseq2, allhitsmap, APAT output	NO	1.1.1.267	1R0K	ACT , All_ligs	NO
inputseq3, allhitsmap, APAT output	EAL24288.1, 7e-05 BlastP, A4D126;	2.7.7.60	1H3M	CL , N2P , All_ligs	NO
inputseq4, allhitsmap, APAT output	NO	2.7.1.148	NO	NO, All_ligs	NO

Figure 4.8: Sample extract from the final output file from the drug target selection table.

most crucial pieces of information needed for obtaining insight into the selection of a protein as a target for SBDD. A sample view of the final output file is shown in Figure 4.8. All ligand hits along with their PDB codes and e-values are shown on the ligands page obtained by clicking the 'All ligs' link from the final output page (Figure 4.9).

4.4 Summary and Discussion

TAPAS is designed mainly to enable a set of protein sequences to be presented to a number of different database searches, annotation and prediction tools are run sequentially to screen for potential drug targets. Output from one tool was parsed and written into an intermediate file which was then passed as input for



PDB ID	E-value	Ligands
1T1R	2e-65,BlastP	SO4 , IMB
1JVS	3e-60,BlastP	MSE , SO4 , NDP
1Q0L	2e-65,BlastP	FOM , NDP
1R0K	2e-61,BlastP	ACT
1Q0H	3e-60,BlastP	MSE , FOM , NDP , CIT

Figure 4.9: Sample extract from the ligand page obtained by clicking 'All ligs' link from the final output page of drug target selection table.

another tool. Sequence level annotations and predictions were obtained from APAT which was incorporated into the pipeline as a standalone tool. Details of a protein sequence from various tools were matched and written into an HTML file for a quick overview of results and were also hyperlinked for obtaining additional information from the world wide web or a local file. Key information for selecting a protein as a possible drug target was presented in an HTML table and includes the presence of human hits (along with E-value), availability of structure (also presence of ligands and heteroatoms), and whether or not a protein is transmembrane.

This approach of creating a specialized pipeline contrasts with tools like Taverna, ToolBus, BioPipe, GPIPE, ICENI and Pegasys. TAPAS is more similar to PseudoPipe (Zhang *et al.*, 2006), BIPASS (Lacroix *et al.*, 2007), PROSPECT-PSPP (Guo *et al.*, 2004), and MicroGen (Burgarella *et al.*, 2005), which are more specialized pipelines rather than general purpose pipelining systems.

In this Chapter, I have described the TAPAS pipeline. The results of applying TAPAS to the MEP pathway and to the apicoplast proteins are discussed in

Chapter 6 together with the development of a ranking scheme for scoring potential targets processed by the TAPAS pipeline.

Chapter 5

Improving Prediction of Transmembrane Proteins

One of the most important annotations applied to a protein sequence by the TAPAS system described in Chapter 4 is whether or not a protein is transmembrane and where the transmembrane segments are likely to occur.

In this chapter, I describe a combined neural network predictor developed by me to improve prediction ability. The analysis includes stressing the need for masking signal peptides to improve the prediction because they result in most of the false positives.

5.1 Introduction

In the post-genome world, there is an urgent need to annotate protein sequences of unknown structure and function. One particularly important class of proteins are those with transmembrane regions.

5.1.1 Membrane Proteins

All biological cells have a bounding plasma membrane which consists of lipids and proteins, and acts as a barrier between the exterior (extracellular fluid or matrix) and the interior of the cell. This phospolipid bilayer, which consists predominantly of amphiphilic phospholipids (such as phosphotidyl ethanolamine), is arranged such that the hydrophobic fatty acid tails face each other, whereas their hydrophilic phosphate polar heads face the exterior and interior of the cell. In addition, eukaryotic cells have membrane-enclosed organelles such as the nucleus, mitochondria, chloroplasts, and plastids.

Proteins that interact with membranes of a cell or an organelle are called 'membrane proteins'. Based on the type of their interaction with the membrane, membrane proteins are classified into two broad categories (shown in Figure 5.1).

(a) Integral membrane proteins (also called intrinsic membrane proteins) are tightly bound to the membrane. Based on the type of binding, they are further divided into:

1. *Transmembrane proteins* are integral membrane proteins that traverse the membrane one or more times extending into the aqueous medium on both sides of the membrane. The most common types span the membrane 7 times ("7 TMs", for example the G-protein coupled receptors, GPCRs) or once (e.g., Signal Anchor proteins). Based on the secondary structure of the transmembrane region, they are divided into:

   (a) α-helical membrane proteins — The membrane spanning regions consist of α-helices. They are present in all biological membranes

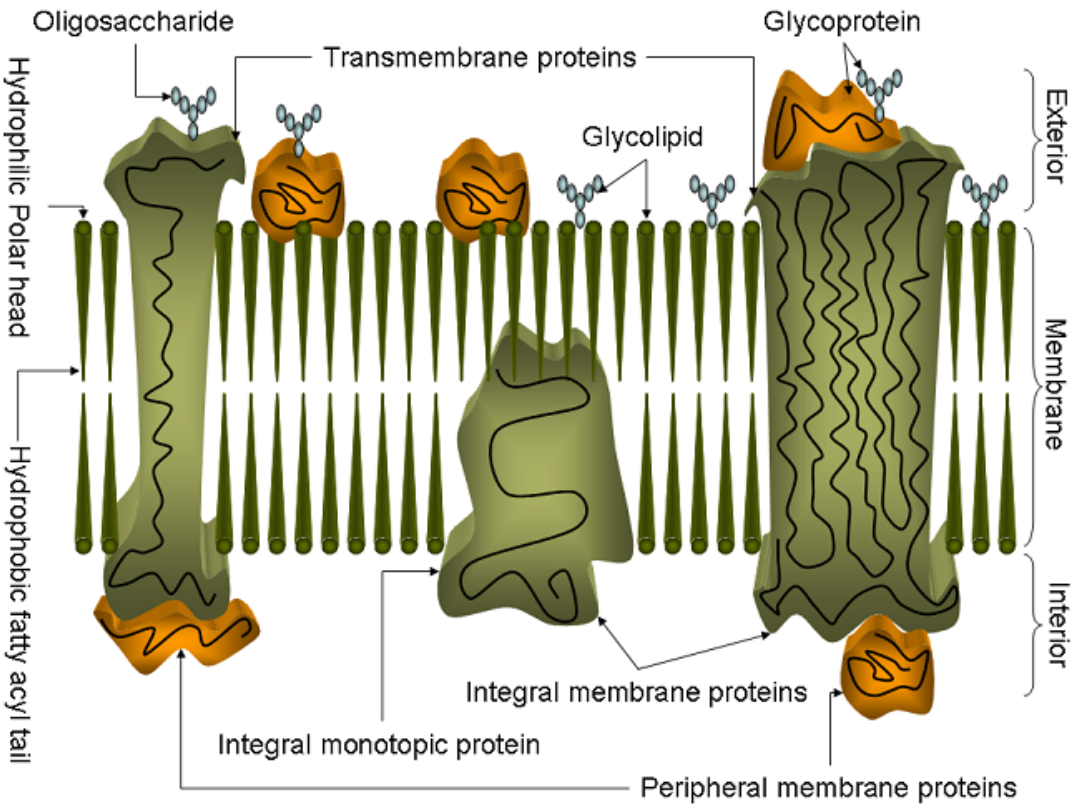


Figure 5.1: Membrane protein architecture.

and constitute the major group of transmembrane proteins.

(b) β-barrel membrane proteins — The membrane spanning regions consist of antiparallel β-sheets that form a barrel-like structure. Unlike the widespread α-helical membrane proteins, they form only a minor group of transmembrane proteins (See Figure 5.2).

2. *Integral monotopic proteins* are integral membrane proteins that do not traverse the phospholipid bilayer. Instead, they partially penetrate the membrane from one side. There are only four monotopic proteins whose crystal structures are available and all of them are pharmaceutically significant drug targets (Fowler and Coveney, 2006):

   - prostaglandin H2 synthase (Picot *et al.*, 1994),
   - squalene-hopene cyclase (Wendt *et al.*, 1997),
   - monoamine oxidase (Binda *et al.*, 2002), and
   - fatty acid amide hydrolase (Bracey *et al.*, 2002).

(b) Peripheral membrane proteins (also called extrinsic membrane proteins) are loosely bound to the membrane and do not interact with the hydrophobic core of the membrane (Lomize *et al.*, 2007; Cho and Stahelin, 2005; Goñi, 2002). Instead they bind to the polar heads of the phospholipid bilayer displaying amphitropic properties, or to other integral membrane proteins (Johnson and Cornell, 1999). Many hormones, toxins and inhibitors temporarily associate with the lipid bilayer before binding to their actual targets. A few examples of peripheral membrane proteins are:

- enzymes — Carboxypeptidase E (Rindler, 1998), Sialidase NEU3 (Sial-

idases or Nueraminidases) (Zanchetti *et al.*, 2007), Adenylate Cyclase in Yeast (Mitts *et al.*, 1990), Protein-Tyrosine Kinases (Quintrell *et al.*, 1987).

- polypeptide hormones (Ryan *et al.*, 2002; Massagué and Pandiella, 1993) — Transforming growth factor (TGF-α), epidermal growth factor (EGF), tumor necrosis factor, TNF-α,
- antimicrobial peptides — Lactoferricin B (Samuelsen *et al.*, 2005; Vorland *et al.*, 1999), Lantibiotics (Breukink, 2006; Chatterjee *et al.*, 2005; van Kraaij *et al.*, 1999), Defensins (Oppenheim *et al.*, 2003),
- Biotoxins (Chugh and Wallace, 2001; Rochet and Martin-Eauclaire, 2000; Schmitt *et al.*, 1999)

Transmembrane Proteins

Transmembrane proteins play vital roles in living cells by participating in cell signalling, cell-cell interactions, self recognition mechanisms, energy transduction, solute and ion transport across membranes by forming ion channels and pores, and acquired drug resistance mechanisms. They constitute around 20–30% of all proteins in fully sequenced genomes (Elofsson and Heijne, 2007; Bagos *et al.*, 2004a; Arai *et al.*, 2003; Krogh *et al.*, 2001; Jones, 1998; Wallin and von Heijne, 1998). They are of great interest to pharmaceutical research; membrane-bound receptors (GPCRs — G-protein coupled receptors) and channels constitute approximately 50% of successful drug targets (Elofsson and Heijne, 2007; Terstappen and Reggiani, 2001; Flower, 1999; Gudermann *et al.*, 1995). However, transmembrane proteins constitute only $\sim$0.5% (Elofsson and Heijne, 2007; White, 2004; Berman *et al.*, 2000) of the known structures in the Protein Databank

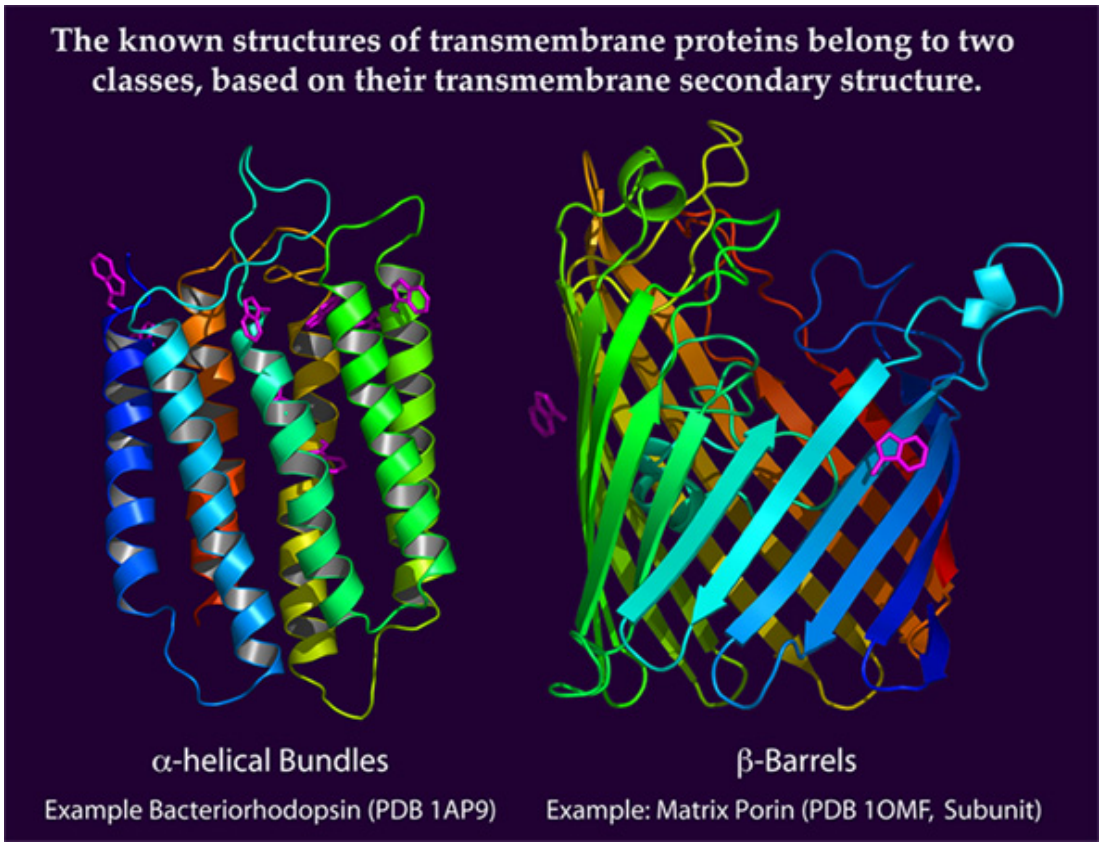


Figure 5.2: Structural differences in the two types of transmembrane proteins — alpha helical bundles and beta barrels. (Figure adapted from `http://www.biologie.uni-konstanz.de/folding/Membrane%20ProtStructure.html`).

(PDB) (Berman *et al.*, 2000), because in general they are difficult to crystallize and do not yield high quality diffracting crystals for study by X-ray crystallography and are unsuitable for analysis by multidimensional nuclear magnetic resonance (NMR) spectroscopy because of their size and solubility issues (Arora *et al.*, 2001). They are not generally suited for SBDD owing to the limited quantity of available 3D structures.

As described above, transmembrane proteins fall into two categories based on the secondary structure of the transmembrane regions (Figure 5.2).

(i) Alpha-helical transmembrane proteins have stretches of 15–35 predominantly hydrophobic amino acid residues (Bagos *et al.*, 2005; Käll *et al.*, 2004; Taylor *et al.*, 2003; von Heijne, 1999). They are the most common type of transmembrane proteins and are present in both prokaryotic and eukaryotic cell membranes. They are sometimes called helix-bundle proteins because when there is more than one TM α-helix they pack into a bundle. Features such as the continuous stretches of largely hydrophobic amino acids and simple rules such as the positive inside rule[1] (von Heijne, 1992) have allowed easier detection of α-helical transmembrane proteins (Bagos *et al.*, 2005, 2004a). Their abundance and importance in nature has resulted in a large number of tools being developed for their detection and for topology prediction. These include KKD (Klein *et al.*, 1985), TopPred II (Claros and von Heijne, 1994), SOSUI (Hirokawa *et al.*, 1998), ALOM2 (Nakai and Kanehisa, 1992), MPEx (White and Wimley, 1999), TMpred (Hofmann and Stoffel, 1993), SPLIT4 (Juretić *et al.*, 2002, 1993), PRED-TMR2 (Pasquier *et al.*, 1999; Pasquier and Hamodrakas, 1999), TM Finder (Deber *et al.*, 2001), TMAP (Persson and Argos, 1997), PHD (Rost, 1996), TMHMM 2.0 (Krogh *et al.*, 2001), HMMTOP 2.0 (Tusnády and Simon, 2001, 1998)), DAS-TMfilter (Cserzo *et al.*, 2004; Cserzö *et al.*, 1997), and MEMSAT 2 (Jones, 1998; Jones *et al.*, 1994).

(ii) Beta-barrel transmembrane proteins have shorter segments of 6–22 (typically 12) moderately hydrophobic amino acid residues (Garrow *et al.*, 2005). As stated earlier, they occur only in the outer membrane of Gram-negative

[1] A tendency for positive charged residues to be inside the cell and negative charged residues to be outside.

bacteria, outer membrane of acid-fast Gram-positive bacteria, and outer membranes of mitochondria and chloroplasts (plant plastids) (Bagos *et al.*, 2005; Garrow *et al.*, 2005; Bagos *et al.*, 2004b,a; Zhai and Saier, 2002). Their presence in eukaryotic organelles like mitochondria and chloroplasts is explained by the endosymbiotic theory (Bagos *et al.*, 2005; Cavalier-Smith, 2000; Moreira *et al.*, 2000; Gray *et al.*, 1999; Vellai *et al.*, 1998). As their name suggests, they are antiparallel β-sheets that form a barrel like structure by rolling themselves up in a way that the first and the last sheets are next to each other with both N and C termini towards the periplasmic side. Though they are not as easily detected as α-helices, there have been some tools developed for their prediction such as TMB-Hunt (Garrow *et al.*, 2005), BBF (Zhai and Saier, 2002), PRED-TMBB (Bagos *et al.*, 2004b,a), PROFtmb (Bigelow and Rost, 2006), TMBEETA-NET (Gromiha *et al.*, 2004), evaluation and consensus predictions (Bagos *et al.*, 2005) and others (Martelli *et al.*, 2002; Jacoboni *et al.*, 2001).

5.1.2 Transmembrane Prediction

Transmembrane prediction can be divided into three classes:

i) residue level — whether or not an individual residue falls into a transmembrane region,

ii) topological — whether an approximate segment of the sequence forms a transmembrane region and which end of each segment is in the inside of the cell,

iii) protein level — is a protein a transmembrane protein?

The first and third levels are the most useful with the information in the second level being available from a complete residue-level prediction once the orientation of the first transmembrane regions has been established.

Various transmembrane prediction methods

Transmembrane prediction techniques have employed a plethora of different methodologies. For example,

- simple hydropathy based methods based on hydrophobicity scales such as the Kyte and Doolittle (KD) hydrophobicity scale (1982), Whole-residue hydrophobicity scale (WW scale) — MPEx (White and Wimley, 1999), and the Augmented WW scale (aWW scale) (Jayasinghe *et al.*, 2001a)

- hydropathy in combination with: a) more refined propensity indices and a discriminant function to set boundaries for transmembrane regions — KKD (Klein *et al.*, 1985), b) positive inside rule — TopPred II (Claros and von Heijne, 1994), c) amphiphilicity — SOSUI (Hirokawa *et al.*, 1998), d) a rule based system — ALOM2 (Nakai and Kanehisa, 1992), e) weight matrices and statistical analysis of TMBase (Hofmann and Stoffel, 1993) — TMpred (Hofmann and Stoffel, 1993), f) cytoplasmic location of basic charged clusters — SPLIT4 (Juretić *et al.*, 2002, 1993), g) detection of probable starts and ends of transmembrane regions — PRED-TMR (Pasquier *et al.*, 1999) and a pre-processing by neural networks — PRED-TMR2 (Pasquier and Hamodrakas, 1999), and h) Nonpolar Phase Helicity Scales — TM Finder (Deber *et al.*, 2001),

- multiple sequence alignment (e.g. TMAP (Persson and Argos, 1997))

- machine learning methods such as neural networks (e.g. PHD (Rost, 1996)) or Hidden Markov Models (e.g. TMHMM 2.0 (Krogh *et al.*, 2001), and HMMTOP 2.0 (Tusnády and Simon, 2001, 1998)),

- combinatorial strategies and differences in distribution of amino acids (e.g. DAS-TMfilter (Cserzo *et al.*, 2004; Cserzö *et al.*, 1997), and MEMSAT 2 (Jones, 1998; Jones *et al.*, 1994) which additionally uses dynamic programming).

Evaluation of methods and combined predictions

There have been efforts to compare a number of existing transmembrane predictors, evaluate their performance and assess and improve predictions using consensus or majority-voting methods. A recent evaluation was performed by Möller *et al.* (2001) declaring TMHMM as the best performing transmembrane prediction method. Another assessment of ten methods was performed by Ikeda *et al.* (2002) who noted that Hidden Markov Models dominated in prediction performance. They went on to improve predictions up to 9% on 4 aspects they chose ((i) the number of transmembrane segments (TMS) (ii) TMS and position (iii) N-tail location (iv) TM topology) by using a consensus method (majority voting) on their own TMPDB dataset (Ikeda *et al.*, 2000). They also noted that prediction performance was better for prokaryotes when compared with eukaryotes. Both pieces of work were performed on low resolution datasets (which are mainly obtained from the literature and from sequence databases rather than from structural databanks).

It has to be noted that various groups report prediction accuracies on different datasets (of varying quality) and apply different definitions to assess prediction

performance (Chen *et al.*, 2002).

There have been some recent efforts to assess performance using high resolution structural datasets (Cuthbertson *et al.*, 2005; Chen *et al.*, 2002; Jayasinghe *et al.*, 2001b), but these are limited by the small number of available structures. Cuthbertson *et al.* (2005) examined thirteen methods and found that SPLIT4, TMHMM2, HMMTOP2 and TMAP performed best. They also attempted to improve performance by a simple majority-voting procedure.

An HMM-based prediction method, Phobius was presented by Käll *et al.* (2004), for combined prediction of membrane protein topology and signal peptides by using TMHMM and SignalP. A claimed strength is the capability to distinguish signal peptide regions from transmembrane regions. Topology prediction of membrane proteins having signal peptides was improved by assigning the cytoplasmic side to the N-terminus of a mature protein.

Käll *et al.* (2005) have presented PolyPhobius which uses a HMM decoding algorithm (implemented in Java) to include homology information obtained from global multiple sequence alignment which improves the performance in prediction of transmembrane proteins and signal peptides.

Signal peptides are often incorrectly predicted as trans-membrane regions because of their hydrophobic nature. Lao *et al.* (2002b; 2002a) evaluated twelve transmembrane topology prediction methods for the effect of signal peptides in topology prediction and stressed the need for addressing the signal peptide issue. They found that machine learning based prediction methods were less sensitive to this problem than hydropathy based methods.

A consensus prediction by a majority-vote approach was also performed by Nilsson *et al.* (2000) using five prediction methods. They concentrated on the

reliability of predicted topology and found that prediction performance varied strongly with the number of methods that agree with one another.

Arai *et al.* (2004) created ConPred II, a consensus prediction by majority voting method using various combinations of five prediction methods among a set of nine prediction methods — KKD, TMpred, TopPred II, DAS, TMAP, MEMSAT 1.8, SOSUI, TMHMM 2.0, and HMMTOP 2.0 and is available as a web server (`http://bioinfo.si.hirosaki-u.ac.jp/~ConPred2/`).

Taylor *et al.* (2003) have presented a web server, BPROMPT (Bayesian PRediction Of Membrane Protein Topology), for consensus prediction of transmembrane protein topology by combining results from HMMTOP2, DAS, SOSUI, TMpred and TopPred II using a Bayesian Belief Network (`http://www.jenner.ac.uk/BPrompt/`).

To date, none of the work on combining predictors has used Neural Networks as a machine learning approach. Such strategies have been used previously in combining, for example, the output of secondary structure predictors (e.g. JPred (Cuff *et al.*, 1998)). In addition, none of the assessments or consensus methods to date have made use of DAS-TMfilter (Cserzo *et al.*, 2004), an improved version of DAS (Cserzö *et al.*, 1997).

Improving prediction of the transmembrane regions in membrane proteins is useful and significant to both the scientific research community and the pharmaceutical industry because it provides insight into their structure, function and druggability. This work is done as a part of developing a stand-alone tool (for improved transmembrane prediction) for APAT and for subsequent integration into TAPAS.

5.2 Methods

A number of transmembrane predictors and datasets of transmembrane proteins were examined as discussed earlier.

5.2.1 Tools and methods used in this analysis

On the basis of performance as assessed by Möller *et al.* (2001) and the types of outputs returned by these predictors; TMHMM V2.0 (Krogh *et al.*, 2001), MEMSAT 2 (Jones *et al.*, 1994) and DAS-TMfilter (Cserzo *et al.*, 2004) were selected as three of the best prediction methods for combining and improving their prediction values using neural networks.

Although both the HMM-based methods, TMHMM and HMMTOP are known to outperform other methods as evaluated by Ikeda *et al.* (2002), HMMTOP does not provide prediction values for individual residues which is necessary for this project and hence only TMHMM was chosen. MEMSAT has also performed well in various evaluation studies carried out by different groups (Möller *et al.*, 2001; Ikeda *et al.*, 2002). While DAS (Cserzö *et al.*, 1997) has underperformed in various evaluation studies (Möller *et al.*, 2001; Ikeda *et al.*, 2002), DAS-TMfilter (Cserzo *et al.*, 2004) is an improved version of DAS and it was considered interesting to examine its performance. In the end, three tools were chosen that use different methods — HMM (TMHMM), dynamic programming (MEMSAT), and the improved 'Dense Alignment Surface' algorithm (DAS-TMfilter).

As shown by Lao *et al.* (2002b; 2002a), transmembrane predictors find it difficult to distinguish true transmembrane regions from signal peptides owing to the hydrophobic nature of these regions (see Figure 5.3). We therefore assessed

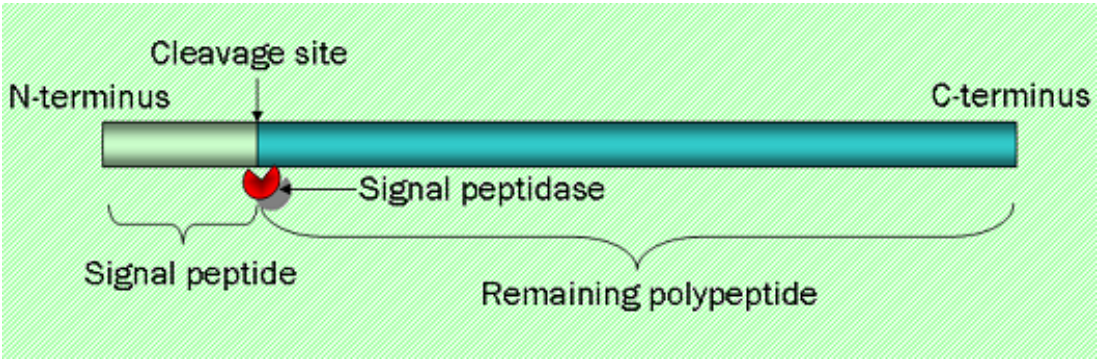


Figure 5.3: Any positive predictions for a residue being in a transmembrane region are masked out (annulled - prediction value set to zero) up to the cleavage site of a signal peptide in a give protein.

the effect of using SignalP 3.0 (Bendtsen *et al.*, 2004; Nielsen *et al.*, 1997) to mask out residues predicted to be part of a signal peptide.

Which machine learning method to use?

Different machine learning methods were discussed in Chapter 2. A neural network was used for the purpose of combining predictions from TMHMM V2.0, MEMSAT 2 and DAS-TMfilter after evaluation of the pros and cons of different machine learning methods.

Although it is straightforward to derive biological meaning out of Bayesian networks, they are still considered to be complex methods. Furthermore, they are not well known for tasks that involve combining methods and thus are not the preferred choice for this work.

Hidden Markov Models (HMMs) are very good at pattern recognition and would have been ideal for actual prediction of transmembrane proteins. However, this study involves combining prediction methods to smooth the output from them. HMMs are not well known for performing such tasks and thus they are

not chosen for this task.

Decision trees are widely used for data mining and classification. They could have been used for this project by preparing proper scenarios for classification of amino acids based on the cutoffs. Although the project involves classifying amino acids, the prediction value for an amino acid is just smoothed by combining the output from other methods. Decision trees are not generally used for such tasks and are reported to have performed badly for combining secondary structure prediction methods (King *et al.*, 2000).

Being binary classifiers, SVMs could not have been the first choice for parts of the related work. For example, while predicting topology of transmembrane proteins, there are more than two outputs - inside, outside and membrane. Although, this project does not involve topology prediction, any future developments on topology prediction would have been relatively difficult, though not impossible.

The reasons for preferring artificial neural networks (ANNs) to SVMs for this project include:

- ANNs being good at non-linear relationships,
- ANNs having a tendency to achieve improved accuracy with an increase in the number of dataset parameters,
- ANNs not being just binary classifiers which would make the implementation relatively straightforward,
- ANNs being well known for tasks which involve combining methods.

King *et al.* (2000) have assessed whether or not it is better to combine prediction methods as opposed to usage of a single secondary structure prediction

method and concluded that it is better to combine predictions. They also observed that Decision trees performed the worst while neural networks performed the best among all the combining methods, both learning and non-learning methods (voting, biased voting, linear discrimination, neural networks and decision trees).

Results from the predictions at the individual residue level were combined using neural networks implemented in the Stuttgart Neural Network Simulator (SNNS V4.2) (Zell *et al.*, 1995) (`http://www-ra.informatik.uni-tuebingen.de/software/snns/welcome_e.html`).

A simple feed-forward neural network (Rumelhart and McClelland, 1986) was employed using supervised training with the Rprop algorithm (Riedmiller and Braun, 1993, 1992).

5.2.2 Datasets

Dataset used at the residue level

Among the various datasets available (Ikeda *et al.*, 2003; Möller *et al.*, 2000; Nilsson *et al.*, 2000; Klein *et al.*, 1985) the larger Möller dataset of integral membrane proteins was selected for training and assessment of residue level predictions. The dataset, downloaded from `ftp://ftp.ebi.ac.uk/pub/databases/testsets/transmembrane/sequences/` consists of subsets A, B, C, C.P and D on the basis of confidence (A being the best and D the worst). Möller suggests that subset D should not be used for training or testing purposes, so the remaining subsets (A, 37 sequences; B, 23 sequences; C, 129 sequences; C.P, 17 sequences) were combined to form dataset 'ABCP' with 206 sequences. However, two sequences from the ABCP dataset could not be processed by MEMSAT

using default parameters and these sequences were excluded to create a final dataset named 'ABCP-2' used for training the neural network and for 5-fold cross-validation. 5-fold cross validation involves splitting the whole dataset into 5 subsets and using 4 of these subsets as training data while using the remaining 1 subset as validation data. The cross-validation process is repeated, with each of the 5 subsets used once as the validation data. The results from the 5 folds then can be averaged to produce a single set of performance statistics.

Datasets used at the protein level

For protein level predictions, independent non-redundant datasets of transmembrane and non-transmembrane proteins were generated from UniprotKB/SwissProt (Wu *et al.*, 2006). A program implemented in Perl was written to look for the term 'TRANSMEM' in 'FT' lines of the UniprotKB/SwissProt data file (see Figure 5.4). If the term was not present, then the sequence was placed in the non-transmembrane dataset. If present, the method looks for the terms 'Potential', 'similarity' or 'Probable'. If any one of these terms is present, the sequence is rejected. If the terms are not present, then the sequence is placed in the transmembrane dataset. Any sequence present in the ABCP-2 training set (described above) was then removed from the transmembrane dataset. In the final phase of data preparation, redundancy was removed from the datasets. Each dataset was treated as an input list of sequences. The first sequence was transferred to an output list. Each remaining sequence in the input list was scanned against the output list and against the Möller training set using FASTA (Pearson, 1990; Pearson and Lipman, 1988). If a significant hit was found, with sequence identity $> 35\%$, the sequence was

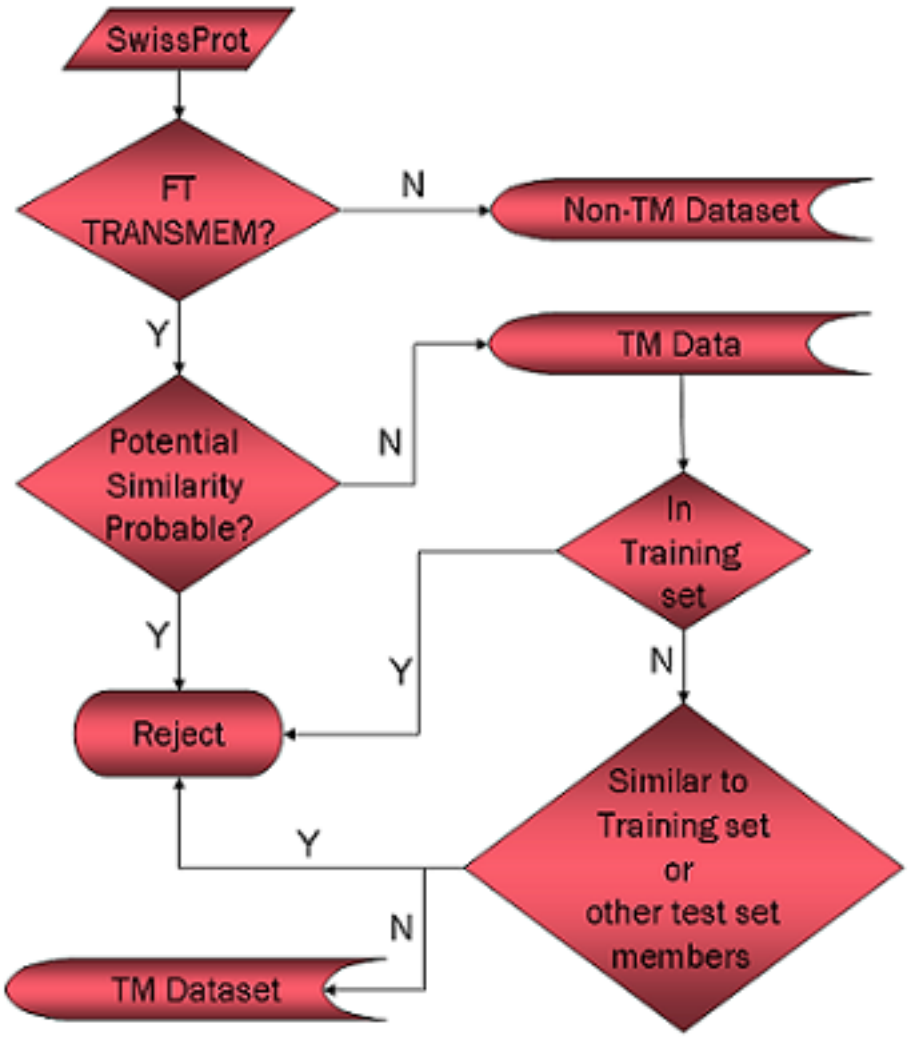


Figure 5.4: Dataset preparation for analysis at the protein level.

rejected; otherwise the sequence was added to the output list. This was repeated for the rest of the dataset or until the output list exceeded 200 sequences. The aim of 35% threshold was not to exclude all homologues, but to exclude all significantly similar sequences. Thus the dataset may contain homologous sequences, but a diverse set of sequence data is guaranteed.

From the initial transmembrane set of 272 sequences, 20 were removed because they were present in the training set, 173 sequences were removed as a result of redundancy within the dataset and a further 29 sequences were removed because of clear homology with the training set. One sequence could not be processed by MEMSAT using default parameters leaving a final transmembrane dataset of 49 sequences (see Figure 5.5). With $< 35\%$ sequence identity to each other or to the Möller data used for training, the non-transmembrane dataset was created in the same way and stopped when it reached a limit of 200 sequences. Five sequences could not be processed by MEMSAT using default parameters leaving a final dataset of 195 sequences. The resulting ratio of transmembrane to non-transmembrane sequences ($\sim$1:4) matches the ratio of transmembrane to non-transmembrane proteins in a typical genome (Krogh *et al.*, 2001; Jones, 1998; Wallin and von Heijne, 1998).

5.2.3 Using APAT and implementation of the neural network

The individual transmembrane predictors (TMHMM, DAS-TMfilter and MEMSAT) and the signal peptide predictor (SignalP) were run using APAT (Deevi and Martin, 2006) (described in Chapter 3).

Input to the neural network consisted of a sliding window of size 5 to combine

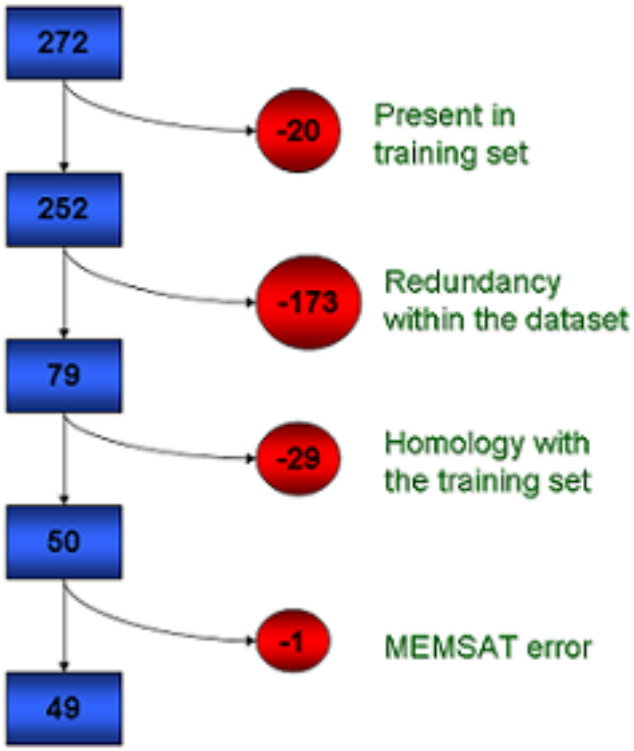


Figure 5.5: Systematic removal of sequences from the transmembrane dataset to improve the quality of dataset.

the outputs from the three prediction methods. TMHMM generates three values ('in', 'mem' and 'out' scores) while single outputs were taken from MEMSAT and DAS-TMfilter. Therefore input to the network consisted of 5×5 input nodes. Networks were trained using both raw and normalized values from the component predictors. Only one hidden layer was used because, in general more than one hidden layer does not provide any advantage, instead more hidden nodes are preferable (Guimarães *et al.*, 2003; Pasquier and Hamodrakas, 1999; Brightwell *et al.*, 1997). Hidden layer sizes of 5, 7, 10, 15 and 20 nodes were examined using a single output node. Summed squared output errors were plotted against the number of training cycles by using early-stopping after 30 cycles. 30 cycles was chosen on evaluation of a number of training sets and showed that improvement in error was levelling off at this point. Stopping at this point avoids over-fitting. Training was performed using resilient back-propagation (Rprop) (Riedmiller and Braun, 1993).

Additional partial validation was performed by using lower hidden layer sizes of 3 and 4 hidden nodes and larger window sizes of 7 and 9.

Dataset preparation, creation of pattern-files for input to the neural network and analysis of results were all performed using scripts written in Perl. Results for individual residue performance (assessed using the Matthews' Correlation Coefficient, MCC (Matthews, 1975)) were averaged over 5-fold cross-validation. Output from the neural networks was a single value between zero and one, and this is used as a threshold (cutoff). The cutoff for a positive prediction was varied in steps of 0.1 to optimize predictions.

Performance was evaluated using:

$$MCC = \frac{(TP * TN) - (FP * FN)}{\sqrt{(TP + FN)(TN + FP)(TP + FP)(TN + FN)}} \tag{5.1}$$

$$Sensitivity\ (TPrate) = \frac{TP}{(TP + FN)} \tag{5.2}$$

$$Specificity = \frac{TN}{(TN + FP)} \tag{5.3}$$

Where TP = True Positive; TN = True Negative; FP = False Positive; FN = False Negative.

5.3 Results and Discussion

Performance of the combined method was assessed at the individual residue and whole protein level. Performance was compared with the individual methods and the effect of masking signal peptide residues as predicted by SignalP was assessed.

5.3.1 Single residue prediction

The performance of each of the individual predictors — DAS-TMfilter, TMHMM, MEMSAT — was compared with the combined predictor, and the effect of masking residues using SignalP was assessed using the ABCP-2 dataset.

Performance of individual predictors

The performance of individual predictors was assessed using their default parameters to find out which among them performs the best. Of the three individual

	Hidden layer size				
Cutoff	5	7	10	15	20
0.1	0.737	0.737	0.733	0.732	0.730
0.2	0.777	0.770	0.769	0.770	0.769
0.3	0.794	0.790	0.790	0.790	0.789
0.4	0.801	0.802	0.803	0.801	0.802
0.5	**0.803**	0.801	0.802	0.801	0.801
0.6	0.795	0.791	0.791	0.790	0.794
0.7	0.775	0.769	0.771	0.770	0.776
0.8	0.720	0.731	0.737	0.736	0.741
0.9	0.558	0.601	0.593	0.571	0.594

Table 5.1: MCC scores from varying the hidden layer size and prediction threshold (cutoff) for the un-masked and un-normalized combined predictor. The best performance is highlighted in bold. If the same performance value is achieved at a lower hidden layer size then that is preferred because a better generalization could be achieved.

predictors used,

- TMHMM performed best with MCC=0.796,
- MEMSAT performed moderately with MCC=0.733, and
- DAS-TMfilter performed worst with MCC=0.702

Performance of the un-masked and un-normalized combined predictor

Initial performance of the combined predictor was assessed using different hidden layer sizes and thresholds and by feeding the raw (un-normalized) results from the predictors into the network (Table 5.1). Optimum performance, resulting in a mean MCC of 0.803, was seen using a prediction threshold of 0.5 with a hidden layer size of 5. The same score is achieved with a cutoff of 0.4 and hidden layer size of 10, but smaller hidden layer sizes are likely to maximize generalization.

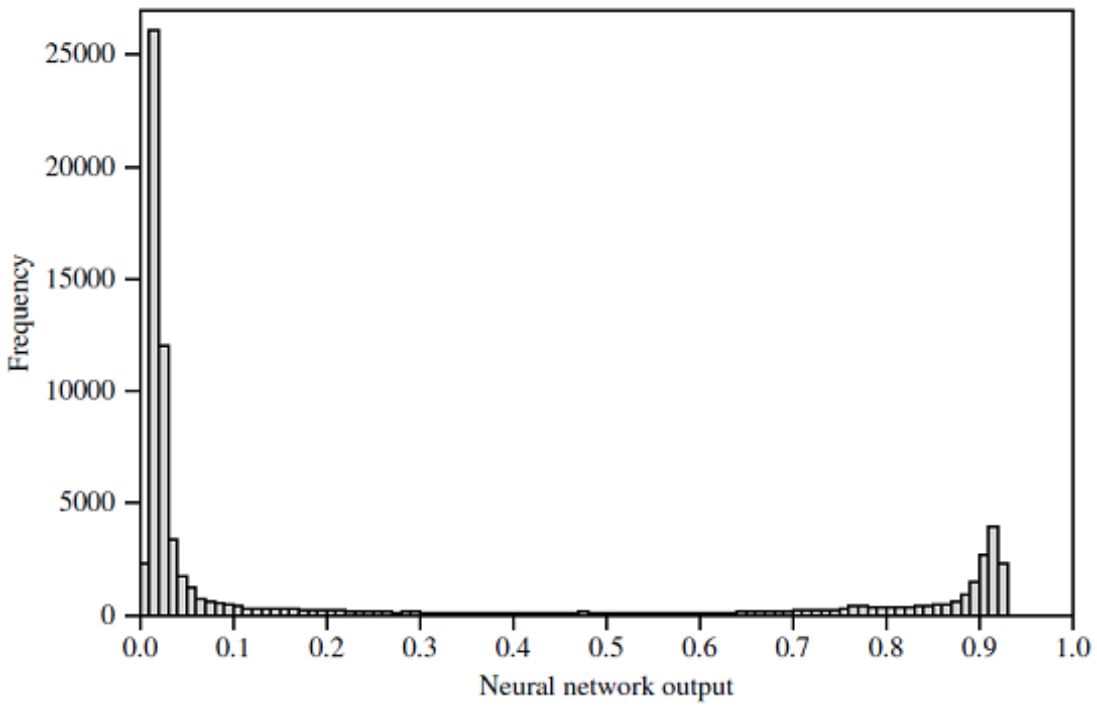


Figure 5.6: Histogram of output values from the neural network for the un-masked un-normalized combined predictor using a hidden layer size of 5. It can be seen that the vast majority of values are < 0.1 or > 0.9 explaining the insensitivity of performance to the cutoff used for positive predictions.

It was interesting to note that the performance of the neural network (as assessed by the MCC) was not very sensitive to the cutoff selected for a positive or negative prediction, even when extreme cutoffs of 0.1 or 0.9 were used. It was therefore assumed that the vast majority of values output by the network were either < 0.1 or > 0.9. This hypothesis was confirmed by plotting a histogram of values output by the network as show in Figure 5.6. Nonetheless, the cutoff was varied for future neural network experiments (although in retrospect it can be seen that all these neural networks are relatively insensitive to the selected cutoff for the same reason).

Performance of the un-masked and normalized combined predictor

The effects of normalizing the results before input to the neural network were then assessed. DAS-TMfilter produces positive output values and a value of > 2.5 is normally recommended as a positive prediction. Values were therefore normalized from the range 0–5 to 0–1 with all values > 5 being treated as five using the equation:

$$S_n = \frac{\max \begin{cases} S_r \\ 5.0 \end{cases}}{5} \tag{5.4}$$

where S_r is the raw score and S_n is the normalized score.

Similarly, MEMSAT generates positive scores for each predicted transmembrane helix. A preliminary examination of output from MEMSAT again suggested that any helix with a score > 5 could be regarded as having very high confidence so normalization was performed in the same way. Surprisingly, normalization did not improve the results with a best MCC of 0.802 being achieved

	Hidden layer size				
Cutoff	5	7	10	15	20
0.1	0.743	0.738	0.740	0.743	0.742
0.2	0.775	0.771	0.774	0.775	0.775
0.3	0.791	0.791	0.792	0.791	0.791
0.4	0.801	**0.802**	0.799	0.801	0.800
0.5	0.800	**0.802**	0.800	0.799	0.801
0.6	0.790	0.791	0.793	0.793	0.792
0.7	0.764	0.771	0.773	0.773	0.773
0.8	0.733	0.736	0.739	0.741	0.734
0.9	0.614	0.592	0.566	0.559	0.571

Table 5.2: MCC score from varying the hidden layer size and prediction threshold (cutoff) for the un-masked and normalized combined predictor. The best performance is highlighted in bold. If the same performance value is achieved at a lower hidden layer size then that is preferred because a better generalization could be achieved.

with a prediction threshold of 0.4 or 0.5 and hidden layer size of 7 (Table 5.2). Raw values were therefore used in future prediction work.

Performance of the un-masked and un-normalized combined predictor using only TMHMM and MEMSAT

The contribution of DAS-TMfilter (the worst individual predictor) was assessed by performing a separate analysis using only TMHMM and MEMSAT. If the prediction performance of the combined predictor (without DAS-TMfilter) is lower than the predictor using all the 3 tools, then it implies that DAS-TMfilter is contributing positively to the combined prediction (Table 5.3) despite its lower individual performance. Optimum performance (MCC=0.801) was obtained using a hidden layer size of 20 and a prediction threshold of 0.4 or 0.5. This was only slightly worse than the combined predictor using all three individual methods, but required a much larger hidden layer size to obtain this level of prediction

	Hidden layer size				
Cutoff	5	7	10	15	20
0.1	0.747	0.738	0.741	0.746	0.743
0.2	0.773	0.768	0.772	0.775	0.772
0.3	0.791	0.791	0.792	0.794	0.790
0.4	0.798	0.799	0.800	0.800	**0.801**
0.5	0.798	0.797	0.799	0.799	**0.801**
0.6	0.787	0.789	0.788	0.791	0.792
0.7	0.770	0.774	0.773	0.771	0.775
0.8	0.733	0.740	0.739	0.735	0.734
0.9	0.611	0.541	0.573	0.591	0.580

Table 5.3: MCC scores from varying the hidden layer size and prediction threshold (cutoff) for the un-masked and un-normalized combined predictor using only TMHMM and MEMSAT. The best performance is highlighted in bold. If the same performance value is achieved at a lower hidden layer size then that is preferred because a better generalization could be achieved.

suggesting that the predictor may be less general. Thus while DAS-TMfilter is the worst individual predictor, it does seem to play a small part in enhancing the overall prediction.

Performance of the masked and un-normalized combined predictor

The effect of masking with SignalP was then assessed. Any residue predicted by SignalP to be part of a signal peptide had its prediction value for being part of a transmembrane segment reset to zero before running the network. In the case of TMHMM which gives three predictions (membrane, inside, outside), the membrane value was set to zero and the inside and outside values both set to 0.5. The best performance was an average MCC of 0.787 obtained with a prediction threshold of 0.5 and a hidden layer size of 15 (Table 5.4). Clearly, for individual residue prediction, masking led to worse performance.

	Hidden layer size				
Cutoff	5	7	10	15	20
0.1	0.734	0.726	0.723	0.719	0.720
0.2	0.766	0.760	0.756	0.752	0.750
0.3	0.779	0.777	0.776	0.763	0.764
0.4	0.784	0.784	0.785	0.785	0.786
0.5	0.785	0.786	0.786	**0.787**	0.786
0.6	0.779	0.778	0.776	0.777	0.780
0.7	0.762	0.755	0.757	0.758	0.764
0.8	0.703	0.720	0.726	0.724	0.729
0.9	0.583	0.613	0.585	0.591	0.601

Table 5.4: MCC scores from varying the hidden layer size and prediction threshold (cutoff) for the masked and un-normalized combined predictor. The best performance is highlighted in bold. If the same performance value is achieved at a lower hidden layer size then that is preferred because a better generalization could be achieved.

Performance of the un-masked and un-normalized combined predictor using lower hidden layer size

In order to examine the effect of using a lower hidden layer size, hidden layer sizes of 3 and 4 nodes were employed (Table 5.5). Best performance with the smaller hidden layer sizes resulted in a mean MCC of 0.801, using a prediction threshold of 0.4 with hidden layer sizes of 3 and 4. This is slightly worse than the optimum performance (a mean MCC of 0.803 seen using a prediction threshold of 0.5 with a hidden layer size of 5). This demonstrates that using hidden layer sizes of 3 and 4 nodes provides no improvement over using 5 hidden nodes.

Input window size 5							
	Hidden layer size						
Cutoff	3	4	5	7	10	15	20
0.1	0.742	0.734	0.737	0.737	0.733	0.732	0.730
0.2	0.776	0.768	0.777	0.770	0.769	0.770	0.769
0.3	0.794	0.786	0.794	0.790	0.790	0.790	0.790
0.4	0.801	0.801	0.801	0.802	**0.803**	0.801	0.802
0.5	0.801	0.798	**0.803**	0.801	0.802	0.801	0.801
0.6	0.789	0.790	0.795	0.791	0.791	0.790	0.794
0.7	0.769	0.773	0.775	0.769	0.771	0.770	0.776
0.8	0.737	0.733	0.720	0.731	0.737	0.736	0.741
0.9	0.596	0.559	0.558	0.601	0.593	0.571	0.594

Table 5.5: MCC scores from varying the hidden layer size and prediction threshold (cutoff) for the un-masked and un-normalized combined predictor while using lower hidden layer size and an input window size of 5. The best performance is highlighted in bold. If the same performance value is achieved at a lower hidden layer size then that is preferred because a better generalization could be achieved.

Performance of the un-masked and un-normalized combined predictor using higher input window size

Input window size is crucial for motif predictions and a larger window size is commonly employed by many secondary structure predictors (Guimarães *et al.*, 2003; Chandonia and Karplus, 1995; Qian and Sejnowski, 1988). Here the aim is to use the neural network to smooth the results from other predictors rather than predicting patterns directly from amino acid sequences. In order to verify that a higher window size did not help, neural networks with window sizes of 7 and 9 were employed. Using a window size of 7, optimum performance resulted in a mean MCC of 0.803 (using a prediction threshold of 0.4 with hidden layer sizes of 10 and 15) (Table 5.6). Using a window size of 9, optimum performance resulted in a mean MCC of 0.803 (using a prediction threshold of 0.4 with hidden layer sizes of 10) (Table 5.7). Using an input window of 5 (Table reftab-ABCP-

Input window size 7							
	Hidden layer size						
Cutoff	3	4	5	7	10	15	20
0.1	0.719	0.739	0.717	0.735	0.733	0.737	0.736
0.2	0.775	0.767	0.774	0.773	0.774	0.773	0.775
0.3	0.789	0.791	0.794	0.793	0.794	0.792	0.794
0.4	0.794	0.798	0.801	0.802	**0.803**	**0.803**	0.801
0.5	0.798	0.800	0.800	0.802	0.801	0.802	0.801
0.6	0.787	0.787	0.794	0.795	0.795	0.794	0.794
0.7	0.771	0.772	0.772	0.772	0.777	0.773	0.779
0.8	0.748	0.731	0.729	0.732	0.746	0.734	0.743
0.9	0.608	0.640	0.618	0.613	0.553	0.605	0.566

Table 5.6: MCC scores from varying the hidden layer size and prediction threshold (cutoff) for the un-masked and un-normalized combined predictor while using an input window size of 7. The best performance is highlighted in bold. If the same performance value is achieved at a lower hidden layer size then that is preferred because a better generalization could be achieved.

2-no-mask), the same score was achieved using a threshold of 0.5 with hidden layer size of 5. Smaller input and hidden layer sizes are likely to maximize generalization and, as expected this demonstrates that using larger window size has not improved the performance.

5.3.2 Whole protein prediction

Often, one would simply like to know whether a protein is a transmembrane protein or not. As described in the Methods, a non-redundant test set of transmembrane and non-transmembrane proteins which showed low sequence identity to the training data used for residue-level predictions was prepared. On the basis of residue-level predictions it was decided whether a protein was a transmembrane protein on the basis of three alternative strategies:

1. the total number of transmembrane residues predicted,

Input window size 9							
	Hidden layer size						
Cutoff	3	4	5	7	10	15	20
0.1	0.724	0.732	0.739	0.719	0.720	0.721	0.730
0.2	0.780	0.771	0.770	0.767	0.769	0.770	0.771
0.3	0.792	0.789	0.785	0.789	0.792	0.792	0.792
0.4	0.798	0.801	0.792	0.801	**0.803**	0.802	0.802
0.5	0.799	0.801	0.798	0.799	0.802	0.801	0.800
0.6	0.786	0.795	0.791	0.793	0.793	0.792	0.791
0.7	0.766	0.774	0.773	0.777	0.777	0.770	0.775
0.8	0.743	0.737	0.725	0.740	0.736	0.737	0.738
0.9	0.641	0.650	0.626	0.635	0.599	0.616	0.589

Table 5.7: MCC scores from varying the hidden layer size and prediction threshold (cutoff) for the un-masked and un-normalized combined predictor while using an input window size of 9. The best performance is highlighted in bold. If the same performance value is achieved at a lower hidden layer size then that is preferred because a better generalization could be achieved.

2. the length of the longest transmembrane segment, and

3. the average length of a transmembrane segment (regions predicted as a single residue were excluded).

Performance of un-masked and un-normalized combined predictor

The best combined residue-level predictor used all three individual predictors without masking or normalization, with a threshold of 0.5 and a hidden layer size of 5. This predictor was used for whole protein level predictions for the non-redundant transmembrane and non-transmembrane datasets (Table 5.8). The best performance was seen using a threshold of 22 residues for the longest transmembrane region (MCC=0.708). Surprisingly this was substantially worse than the performance for individual residue-level predictions (MCC=0.803). A manual examination of the data showed that the reduction in performance came mostly

	Unmasked			Masked		
	Criterion			Criterion		
Cutoff	All	Longest	Mean	All	Longest	Mean
10	0.622	0.622	0.649	0.694	0.694	0.715
12	0.622	0.622	0.663	0.694	0.694	0.715
14	0.642	0.642	0.685	0.704	0.704	0.726
16	0.656	0.677	0.700	0.726	0.738	0.749
18	0.686	0.703	0.697	0.749	**0.762**	0.747
20	0.669	0.704	0.668	0.731	0.729	0.729
22	0.700	**0.708**	0.644	0.701	0.700	0.653
24	0.606	0.556	0.404	0.606	0.556	0.406
26	0.539	0.317	0.223	0.522	0.317	0.223
28	0.539	0.317	0.223	0.522	0.317	0.223
30	0.539	0.289	0.181	0.522	0.289	0.181

Table 5.8: MCC scores from the best residue-level predictor (un-masked, unnormalized) in whole protein level predictions. 'Unmasked' or 'Masked' refers to SignalP masking applied after the residue-level prediction is made. Proteins were predicted as being transmembrane if the number of residues according to a specified criterion was matched or exceeded. 'All': total number of residues predicted as being transmembrane; 'Longest': number of residues in the longest contiguous stretch; 'Mean': mean length of regions predicted as being transmembrane.

from false-positive predictions which appeared to be the result of signal peptides. This suggested that signal masking (according to the prediction from SignalP) might be important in obtaining good protein-level predictions.

Performance of the masked and un-normalized combined predictor

Therefore, the protein-level predictions were re-analyzed using the residue-level predictor, but with SignalP masking after running the network. While the results were still not as good as the residue-level predictions, masking significantly improved the performance of the protein-level predictor (Table 5.8). The best performance was achieved using the length of the longest transmembrane segment ($\geq$18 residues) and performance increased from MCC=0.708 (unmasked) to MCC=0.762 (masked).

Performance of the masked and un-masked best individual predictor

For comparison, the performance of the best individual predictor (TMHMM) in predicting at the protein level was assessed, both unmasked and masked using SignalP (Table 5.9). This resulted in a best MCC of 0.762 masked (longest transmembrane segment $\geq$18 residues) compared with 0.693 unmasked (longest transmembrane segment or total number of transmembrane residues $\geq$18). Thus TMHMM masked by SignalP performs just as well as the combined predictor for protein-level predictions.

5.4 Summary

In summary, combining the outputs of three of the best transmembrane prediction programs (TMHMM, MEMSAT and DAS-TMfilter) using a neural network

	Unmasked			Masked		
	Criterion			Criterion		
Cutoff	All	Longest	Mean	All	Longest	Mean
10	0.640	0.640	0.640	0.715	0.738	0.738
12	0.640	0.640	0.640	0.715	0.738	0.738
14	0.640	0.640	0.640	0.715	0.738	0.738
16	0.647	0.647	0.647	0.715	0.738	0.738
18	**0.693**	**0.693**	0.678	0.749	**0.762**	0.747
20	0.666	0.651	0.651	0.718	0.716	0.716
22	0.668	0.653	0.606	0.715	0.714	0.653
24	0.522	0.361	0.216	0.522	0.361	0.177
26	0.503	0.177	0.181	0.503	0.177	0.181
28	0.503	0.223	0.128	0.503	0.223	0.128
30	0.503	0.222	0.000	0.503	0.223	0.000

Table 5.9: MCC score for TMHMM in whole protein level predictions. 'Unmasked' or 'Masked' refers to SignalP masking applied after the residue-level prediction is made. Proteins were predicted as being transmembrane if the number of residues according to a specified criterion was matched or exceeded. 'All': total number of residues predicted as being transmembrane; 'Longest': number of residues in the longest contiguous stretch; 'Mean': mean length of regions predicted as being transmembrane.

has resulted only in a marginal improvement over the best individual program (MCC=0.803 for combined predictor compared with MCC=0.796 for TMHMM alone). At the whole protein level, when masked to exclude signal peptides using SignalP, the combined predictor did not perform better than TMHMM alone. However masking was seen to be very important and improved the MCC from 0.708 to 0.762 (combined predictor) or 0.693 to 0.762 (TMHMM alone). It is expected that the performance of the combined predictor will improve as enhancements are made to the underlying prediction tools. A new version of MEMSAT (MEMSAT 3) (Jones, 2007) has recently been released and could be included to assess the improvements made to the combined predictor.

Chapter 6

Analysis of the MEP Pathway and Apicoplast Proteins Using TAPAS

The methods developed in the previous chapters were then applied to the analysis of the proteins of the MEP pathway and the apicoplast. As described in the introduction, the MEP pathway of isoprenoid biosynthesis is of great interest to the pharmaceutical sector and the scientific community because it is completely absent in humans, where isoprenoids are synthesized by the Mevalonate pathway. Thus it is a potentially important target for drug intervention in diseases such as malaria, tuberculosis, leprosy, cholera, typhoid, gonorrhea, and syphilis. The apicoplast is a unique organelle present only in apicomplexan protists and is the target site for many nuclear encoded proteins that carry out a wide range of functions, thus making it absolutely indispensable for the survival of these organisms. The MEP pathway occurs inside the apicoplast of apicomplexans,

Disease	Pathogen
Malaria	*Plasmodium falciparum*
Tuberculosis	*Mycobacterium tuberculosis*
Leprosy	*Mycobacterium leprae*
Cholera	*Vibrio cholerae*
Syphilis	*Treponema pallidum*
Bubonic plague	*Yersinia pestis*
Childhood meningitis	*Haemophilus influenza*
Salmonellosis	*Salmonella typhimurium*
Typhoid	*Salmonella typhii*
Meningitis	*Neisseria meningitidis*
Gonorrhea	*Neisseria gonorrhoeae*
Gas gangrene	*Clostridium perfringens*
Botulism	*Clostridium botulinum*
Tetanus	*Clostridium tetani*
Diphtheria	*Corynebacterium diphtheriae*
Chlamydia	*Chlamydia trachomatis*
Toxoplasmosis	*Toxoplasma gondii*
Coccidiosis (poultry and farm animals)	*Eimeria spp*

Table 6.1: Some important diseases associated with the pathogens having the MEP pathway.

but its occurrence is independent of presence or absence of the apicoplast as it is also present in organisms that lack the apicoplast.

Here, my aim was to scan the protein sequences of the apicoplast and the MEP pathway to identify potential drug targets using TAPAS (described in Chapter 4). I decided to look at the apicoplast (∼500 proteins) from *Plasmodium falciparum* and the 8 MEP pathway proteins (enzymes) from some selected species.

6.1 Approach and Methods

6.1.1 Labelling sequence origin

Some of the analysis tools run by APAT require details of the origin of the sequence, for example, eukaryote/prokaryote; gram-positive/gram-negative; etc. Analyzing many sequences from a single organism, as in the case of apicoplast sequences of *Plasmodium falciparum*, is straightforward as it just needs one set of definitions for all sequences, but analyzing a few sequences of many organisms, as in the case of the the MEP pathway proteins requires an automatic specification of these required details. Specifically, the following data are required by specific servers:

1. SubLoc — prokaryotic or eukaryotic,
2. TargetP — plant or non-plant,
3. PSORT — gram-positive bacterium or gram-negative bacterium or yeast or animal or plant.

A perl script was written which looks at the taxonomical classification from 'OC' lines of the UniprotKB/SwissProt data file for each organism, classifies them into the various categories required by different tools, and places the data in a file which can then be used to make a definition file to run APAT through TAPAS (Figure 6.1). Classifying the sequences into prokaryotic or eukaryotic is achieved by looking at the taxonomical domain 'Eukaryota'. The presence or absence of this term in the taxonomical details of an organism classifies it into eukaryote or prokaryote respectively. A sequence is classified into plant or non-plant by looking at the taxonomical kingdom 'Plantae' where the presence of

```
#
DXR_NOCFA : psort origin : Gram-positive bacterium
DXR_NOCFA : species : Nocardia farcinica
DXR_NOCFA : subloc origin : prokaryotic
DXR_NOCFA : targetp origin : non-plant
#
DXR_ORYSA : psort origin : plant
DXR_ORYSA : species : Oryza sativa (Rice)
DXR_ORYSA : subloc origin : eukaryotic
DXR_ORYSA : targetp origin : plant
#
DXR_PARUW : psort origin : Gram-negative bacterium
DXR_PARUW : species : Protochlamydia amoebophila (strain UWE25)
DXR_PARUW : subloc origin : prokaryotic
DXR_PARUW : targetp origin : non-plant
#
```

Figure 6.1: Sample extract of output from origin-finder script which includes specific details required by different tools.

this term groups it into plant and its absence groups it into non-plant. Labelling a sequence as being of animal origin is achieved by looking at the presence of taxonomical kingdom 'Metazoa'. Similarly, yeast sequences are identified by the presence of the taxonomical division 'Ascomycota' or 'Basidiomycota' (Kurtzman and Piskur, 2006). Categorizing bacteria into gram-positive and gram-negative is a more complex task.

Gram-Positive and Gram-Negative

There is no simple computational tool or straightforward way to differentiate bacteria into gram-positive or gram-negative. This classification is based on their ability (gram-positive) or inability (gram-negative) to be stained purple with Gram's staining technique. Based on a literature survey, I prepared a list of phyla/divisions which fall into respective categories (Table 6.2) (Griffiths

Bacterial Phyla/Divisions	
Gram-negative	Gram-Positive
Aquificae	Firmicutes
Bacteroidetes	Actinobacteria
Chlamydiae	Deinococcus-Thermus
Chlorobiaceae	
Chloroflexi	
Crenarchaeota	
Cyanobacteria	
Fusobacteria	
Planctomycetes	
Proteobacteria	
Spirochaetes	
Thermotogae	

Table 6.2: Bacterial phyla/divisions that fall into gram-positive and gram-negative categories.

and Gupta, 2006; Yasin *et al.*, 1996; Belunis *et al.*, 1992; Guiney *et al.*, 1984; Hackman and Wilkins, 1975) (also from websites: `http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/E/Eubacteria.html`, `http://cubic.bioc.columbia.edu/services/proftmb/download/GenomesGramClass`, `http://microbewiki.kenyon.edu/index.php/Pirellula`, `http://www.palaeos.com/Bacteria/default.htm`, `http://highered.mcgraw-hill.com/sites/0072320419/student_view0/chapter21/study_outline.html`).

The origin-finding script then assigns prokaryotes as gram positive or gram negative based on the presence of one of these taxonomical terms (Table 6.2).

```
############################################################

Number of predicted TM residues by TMHMM(after nullifying signal peptide residues) : 111
Length of longest stretch of TM residues : 24
Threshold Value used during current run : 18

Is it a signal peptide according to SignalP? : YES
cleavage site is just before residue : 21
signal peptide is from : 1 - 20

>Prediction : Transmembrane
signal anchor : NO

############################################################
```

Figure 6.2: Output from the script that masks signal peptide residues among TMHMM predictions to improve transmembrane prediction.

6.1.2 Predicting whether a protein is a transmembrane protein

As shown in Chapter 5, masking any transmembrane residues predicted by TMHMM if they fall in a signal peptide region as predicted by SignalP, improved the transmembrane prediction at the whole protein level. Hence, in this analysis, I have used the technique of signal masking TMHMM predictions for predicting whether or not a protein contains transmembrane regions. This procedure uses a threshold of $\geq$18 residues for the length of the longest membrane spanning region because the best performance was obtained using these parameters (Section 5.3.2).

This procedure was incorporated into the TAPAS system for prediction of whether or not a protein is transmembrane.

6.1.3 Analysis of apicoplast proteins

The unpublished sequences of 544 apicoplast proteins from *Plasmodium falciparum* were kindly supplied by Prof. Geoff McFadden (gim@unimelb.edu.au). These sequences were analysed by the TAPAS pipeline followed by manual analysis of the output.

6.1.4 Execution of MEP proteins

In the case of proteins of the MEP pathway, I have selected a list of fifteen important pathogens for which sequences are available. The seven MEP enzymes and the IDI enzyme which is common to both the MEP pathway and the mevalonate pathway are represented by their respective EC numbers along with their SwissProt ACs for the chosen set of pathogenic organisms (Table 6.3). The EC numbers and the corresponding names of the enzymes of the MEP pathway are:

1. 2.2.1.7 $\Longleftrightarrow$ 1-deoxy-D-xylulose-5-phosphate synthase (DXS)
2. 1.1.1.267 $\Longleftrightarrow$ 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXPRI)
3. 2.7.7.60 $\Longleftrightarrow$ 4-diphosphocytidyl-2C-methyl-D-erythritol cytidyltransferase (CDP-ME Synthase)
4. 2.7.1.148 $\Longleftrightarrow$ 4-diphosphocytidyl-2C-methyl-D-erythritol kinase (CDP-ME Kinase)
5. 4.6.1.12 $\Longleftrightarrow$ 2C-methyl-D-erythritol-2,4-cyclodiphosphate synthase (MECDP Synthase)
6. 1.17.4.3 $\Longleftrightarrow$ (E)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase (HMBPP Synthase)

7. 1.17.1.2 $\Longleftrightarrow$ (E)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HMBPP Reductase)

8. 5.3.3.2 $\Longleftrightarrow$ isopentenyl diphosphate isomerase (IPP Isomerase)/isopentenyl diphosphate isomerase:dimethylallyl diphosphate isomerase (IDI)

6.1.5 Analysis of the output

A perl script was written to carry out further analysis of the output from TAPAS. This performs quantitative analysis and also produces a 'target score' for each protein to suggest its ability to be a good drug target for SBDD.

Quantitative analysis

The quantitative analysis was provided in the form of an HTML table which contains the following data:

1. Number of proteins analysed in the current analysis run

2. Number of proteins having human hits

3. Number of proteins having enzyme hits

4. Number of proteins having 3D structure hits

5. Number of proteins having ligand hits

6. Number of proteins that are transmembrane

	EC Number						
Organism	2.2.1.7	1.1.1.267	2.7.7.60	2.7.1.148	4.6.1.12	1.17.4.3	1.17.1.2	5.3.3.2
Chlamydia trachomatis	O84335	O84074	O84468	O84810	O84441	O84060	O84867	
Clostridium perfringens	Q8XJE1	Q8XJR1	Q8XHQ3	Q8XIA9	Q8XI08	P58667	P58675	
Clostridium tetani	Q894H0	Q895K5	Q890M1	Q899A2	Q899E9	Q895K3	Q895G2	
Corynebacterium diphtheriae	Q6NGV3	Q6NGL1	Q6NFC1	Q6NIA1	Q6NFC2	Q6NGL3	Q6NI36	P60923
Haemophilus influenzae	P45205	P44055	O05029	P45271	P44815	P44667	P44976	
Mycobacterium leprae	Q50000	Q9CBU3	Q9CCW6	Q9CD51	Q9CCW5	Q9CBU5	Q9X781	
Mycobacterium tuberculosis	P0A554	P64012	P96864	P65178	P65183	O33350	P0A5I0,P0A5I2	P72002
Neisseria gonorrhoeae (strain ATCC 700825 / FA 1090)	Q5FAI2	Q5F5X0	Q5F829	Q5F9F6	Q5F830	Q5F913	Q5FAF2	
Neisseria meningitides (serogroup A)	Q9JW13	Q9JX33	Q9JTM3	Q9JUX8	Q9JTM4	Q9JU34	P65191	
Neisseria meningitides (serogroup B)	Q9JXV7	Q9K1G8	Q9JYM4	Q9JZW4	Q9JYM5	Q9JZ40	P65192	
Salmonella typhi	Q8Z8X3	Q8Z9A6	Q8Z471	Q8Z699	Q8Z472	P58670	P58678	Q8Z3X9
Salmonella typhimurium	Q8ZRD1	Q8ZRP3	Q8ZMF6	P30753	Q8ZMF7	P58671	P58679	Q8ZM82
Toxoplasma gondii	Q1JSN4							
Treponema palidum	O83796	O83610	O83525	O83386	O83525	O83460	O83558	
Vibrio cholerae	Q9KTL3	Q9KPV8	Q9KUJ2	Q9KQ23	Q9KUJ1	Q9KTX1	Q9KU44	
Yersinia pestis	Q8ZC45	Q8ZH62	Q8ZBP6	Q8ZEY1	Q8ZBP7	P58672	P58680	

Table 6.3: SwissProt accession codes of a few important pathogenic organisms hosting the MEP pathway are chosen for the analysis.

Target score

A target score was predicted for rating each sequence based on the presence or absence of certain parameters. The scoring scheme is shown in Figure 6.3. Weighting different parameters appropriately based on their importance and the value they add to the final outcome is a significant factor in scoring and as simple a scheme as possible was chosen. Here I discuss the rationale behind weighting different factors differently.

1. Structure — This is clearly necessary for SBDD. Thus a protein is given a score of +3, if its structure is known. If the structure is not known, then it is important to check whether it is a transmembrane protein. In order to determine whether or not the structure could be solved with relative ease. If the protein is not a transmembrane protein, then it is assigned a score of +2 because there is higher possibility that the structure could be solved. If the protein is a transmembrane protein, then there are two scenarios:

   (a) Signal Anchor — Although signal anchor proteins are transmembrane proteins, the N-terminal signal anchor region is the only part of the protein that is buried in a membrane. The rest of the protein can be easily cleaved from the signal anchor region which enhances the possibility of solving its structure. Thus such proteins are also assigned a score of +2.

   (b) Not a signal anchor — Being a transmembrane protein and not just a signal anchor diminishes the possibility of solving its structure and therefore it is assigned a score of +1, also considering the fact that approximately 50% of successful drugs are targeted against membrane

proteins (Elofsson and Heijne, 2007; Terstappen and Reggiani, 2001; Flower, 1999; Gudermann *et al.*, 1995).

2. Human homologue — While designing an antimicrobial drug, not having a human homologue for the protein being tested as a drug target is highly desirable because it reduces the chances of any possible cross reactivity and hence it is assigned a score of +2. If the protein has a human homologue, then it is given a score of 0. Although it is not desirable to have a human homologue, it cannot be ruled out from being a good drug target (instead the drug molecule will require precise selectivity).

3. Ligand or EC — Having a known ligand is an advantage either in unbound form, or preferably, in bound form such that precise interactions can be observed. Having both bound and unbound structures allows flexibility of the binding site to be examined. While having an EC number indicates that the protein is an enzyme, here it mainly serves in identifying known ligands of proteins with similar EC numbers. Therefore, a protein having a known ligand or an EC number is given a score of +1 and proteins without these details is are given a score of 0.

The target score ranges from 1 to 6 where a target score of 6 denotes a prediction that the protein will be an excellent target to be taken forward for more detailed structural analysis for SBDD. Any score above 3 indicates that the protein is a potential drug target for SBDD. In fact, many sequences whose scores are 3 or below could also be good drug targets in reality, but may not be currently suitable for SBDD because the structure is not yet available. Some commercially available drugs act on targets that are generally regarded as non-

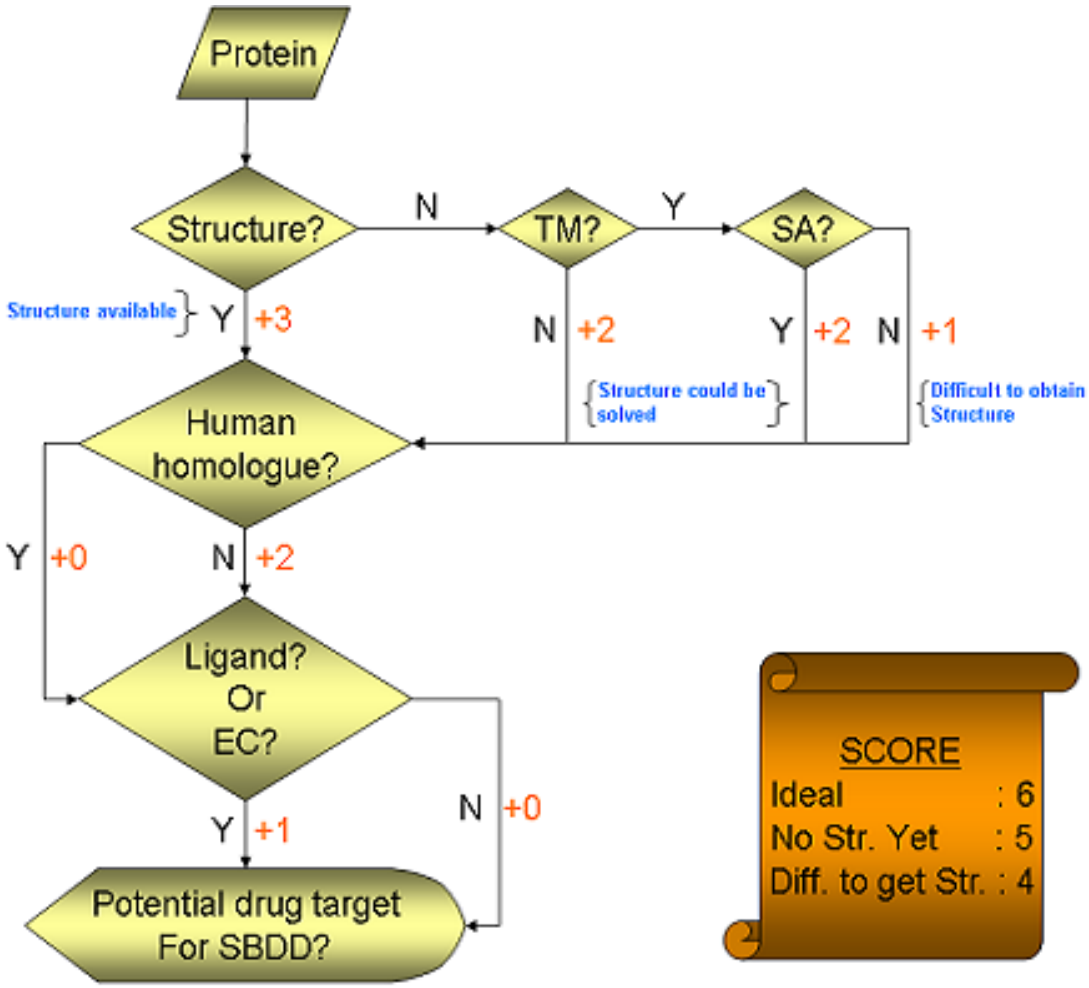


Figure 6.3: Scoring scheme for the target score. TM — transmembrane, SA — Signal Anchor, EC — Enzyme commission number, and SBDD — Structure Based Drug Design

druggable (Owens, 2007). So, it should be noted that the target score is only appropriate for ranking of a protein's suitability for further analysis for SBDD at the sequence level (not at the structure level). The pipeline does not predict druggability of a protein.

6.2 Results and Discussion

Most of the results were provided in the form of HTML tables with click-able links to obtain additional relevant data. These results and sequences are all shown on the CD provided with this thesis. A 'README' file on the CD describes the location of various files, *sequence numbers* used in the following discussion and additional details.

6.2.1 Analysis of the output

The analysis of TAPAS output includes the quantitative analysis of the number of sequences that could be placed in a particular category such as those having human hits, enzyme hits, structure hits, ligand hits, and transmembrane regions.

An extract of the quantitative analysis page for the apicoplast sequences is shown in Figure 6.4. 52% of the 544 apicoplast proteins have human homologues according to TAPAS. Although having human hits is not desirable, a protein can still be a potential drug target. For example, IDI is also present in humans, but still considered as an attractive drug target for designing novel drugs as described in Section 1.1.1 (Steinbacher *et al.*, 2003b).

49% of the 544 apicoplast proteins were predicted to be transmembrane proteins. This large proportion could be explained by the presence of 4 membranes

Description of the quantitative analysis	Value
Number of proteins analysed in the current execution	544
Number of proteins having human hits	284
Number of proteins having enzyme hits	208
Number of proteins having 3D structure hits	187
Number of proteins having ligand hits	130
Number of proteins that are transmembrane	269

Figure 6.4: Quantitative analysis of the TAPAS output for apicoplast sequences.

(owing to secondary endosymbiosis) in an apicoplast which might be hosting these proteins and by the possibility of a few unpredicted signal peptides. In fact, a careful review of the output from SignalP revealed that some of these proteins were predicted to be 'signal anchor proteins' which have uncleaved signal peptides that traverse the membrane once and are considered as valid transmembrane proteins (Gomi and Mitaku, 1999; Wahlberg and Spiess, 1997; High *et al.*, 1991) (Figure 6.5). Prediction of a transmembrane region in a protein is seen as a negative trait when predicting the suitability for SBDD (unless it is a signal anchor which could easily be cleared off) because the structure of these proteins cannot normally be solved. However, these extra-cellular domains may be cleaved and their structures solved in isolation. While membrane bound proteins are not normally suitable for SBDD, it remains true that approximately 50% of successful drug targets are transmembrane proteins (Elofsson and Heijne, 2007; Terstappen and Reggiani, 2001; Flower, 1999; Gudermann *et al.*, 1995).

Quantitative analysis and target scores of MEP proteins of *Corynebacterium*

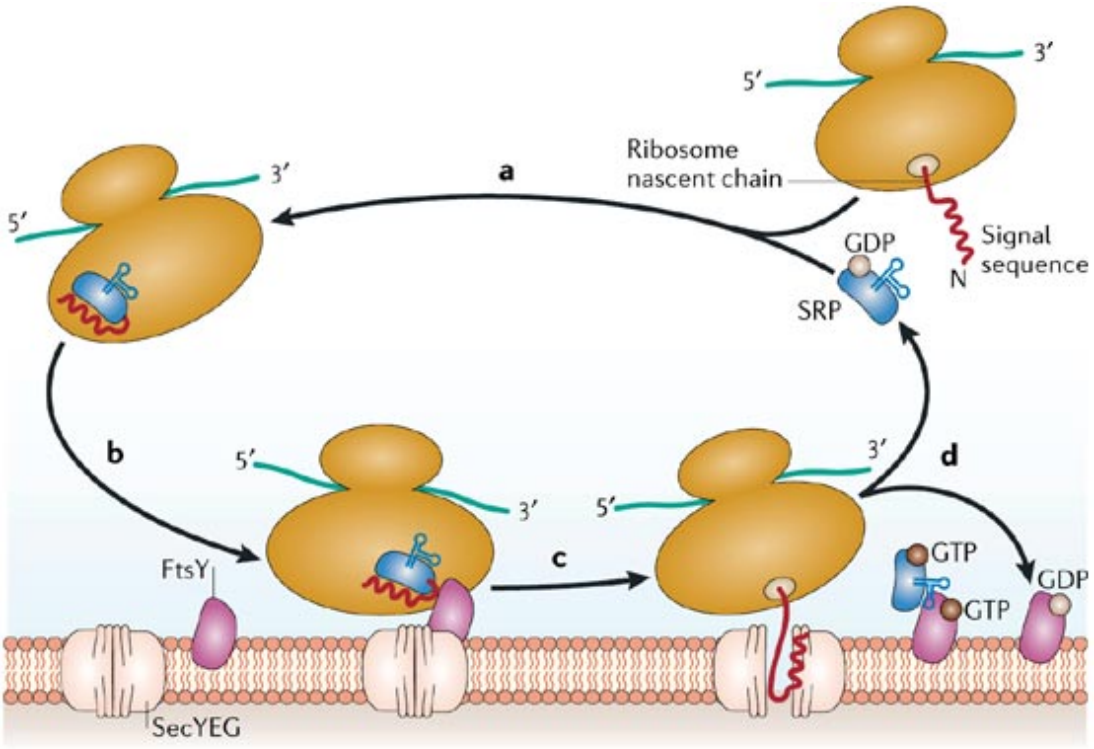


Figure 6.5: a. Preprotein or membrane protein synthesis starts on a free ribosome in the cytosol. The signal-recognition particle (SRP) complex binds to the signal or signal-anchor sequence, which is exposed from the ribosome tunnel exit after approximately 70 amino acids have been synthesized. b. The ribosome nascent chain-SRP complex is subsequently targeted to the protein-conducting channel (PCC) of the Sec translocase by the membrane bound receptor FtsY (or SR in mammals). c. The SRP-FtsY interaction increases the GTP-binding affinity of both proteins, and subsequent GTP binding releases the signal sequence from its association with the SRP, after which the large subunit of the ribosome docks onto the PCC. The signal or signal-anchor sequence opens the PCC in conjunction with the ribosome and initiates the translocation or membrane insertion event. d. Hydrolysis of GTP dissociates the SRP-FtsY complex and recycles the SRP into the cytosol for another round of ribosome membrane targeting. (Figure and caption reproduced from `http://www.nature.com/nrmicro/journal/v4/n7/images/nrmicro1440-f3.jpg`.)

Target Score	Number of sequences	Sequence numbers
6	36	1, 2 ,3 ,6 , 31 , 32 , 33 , 37, 38, 45, 49, 55, 58, 63, 65, 71, 73, 77, 84, 87, 89, 134, 153, 182, 219, 308, 337, 356, 423, 430, 443, 478, 485, 503, 513, 514
5	36	4, 8, 9, 43, 56, 59, 62, 74, 99, 104, 189, 194, 196, 198, 221, 228, 235, 310, 324, 332, 365, 366, 379, 389, 403, 406, 407, 410, 426, 452, 470, 472, 512, 516, 518, 521
4	243	7, 11, 13, 14, 15, 16, 17, 18, 21, 22, 23, 24, 25, 27, 28, 29, 30, 36, 39, 41, 42, 48, 50, 57, 64, 69, 72, 75, 78, 80, 81, 82, 83, 85, 88, 93, 94, 96, 98, 101, 106, 107, 113, 116, 118, 123, 124, 128, 130, 131, 132, 137, 138, 139, 141, 142, 143, 144, 145, 146, 150, 151, 152, 154, 156, 157, 158, 159, 160, 161, 163, 165, 167, 168, 171, 172, 173, 179, 180, 183, 186, 187, 192, 197, 199, 200, 201, 202, 204, 205, 207, 208, 211, 212, 213, 214, 215, 218, 223, 226, 227, 229, 233, 234, 237, 238, 241, 242, 244, 246, 249, 250, 251, 255, 256, 257, 260, 261, 263, 264, 268, 270, 272, 273, 274, 275, 279, 280, 282, 284, 285, 289, 290, 292, 293, 294, 296, 298, 299, 300, 306, 307, 309, 311, 313, 314, 316, 319, 323, 325, 327, 328, 329, 330, 331, 333, 335, 338, 341, 343, 345, 347, 348, 349, 351, 352, 353, 355, 358, 362, 364, 367, 369, 372, 373, 376, 378, 385, 386, 390, 392, 394, 397, 400, 401, 402, 404, 405, 408, 409, 414, 417, 420, 421, 422, 425, 429, 431, 434, 441, 444, 445, 447, 449, 450, 451, 453, 455, 458, 460, 464, 466, 468, 471, 473, 474, 477, 479, 480, 493, 494, 495, 498, 499, 500, 502, 504, 506, 507, 508, 509, 519, 520, 525, 529, 531, 534, 535, 536, 537, 539, 540, 543
3	130	5, 10, 12, 19, 20, 26, 44, 47, 53, 54, 67, 70, 91, 92, 103, 105, 109, 111, 112, 115, 120, 121, 122, 125, 129, 133, 140, 147, 148, 155, 162, 164, 166, 174, 175, 177, 178, 184, 188, 190, 191, 193, 195, 203, 220, 230, 231, 232, 240, 243, 245, 252, 258, 259, 262, 265, 266, 267, 271, 276, 277, 278, 281, 286, 288, 295, 297, 301, 304, 312, 317, 318, 320, 322, 336, 339, 344, 354, 359, 360, 361, 368, 370, 371, 374, 375, 383, 387, 393, 395, 396, 398, 399, 412, 416, 424, 427, 428, 435, 436, 437, 438, 446, 459, 461, 465, 475, 476, 482, 483, 484, 486, 487, 488, 489, 490, 496, 497, 501, 511, 515, 517, 522, 523, 526, 527, 532, 533, 538, 541, 542, 544
2	80	34, 35, 40, 46, 51, 52, 60, 61, 66, 68, 76, 79, 86, 90, 95, 97, 108, 110, 114, 117, 119, 126, 135, 136, 149, 170, 176, 185, 206, 209, 210, 217, 222, 224, 225, 236, 239, 247, 248, 253, 254, 283, 287, 291, 302, 303, 305, 315, 321, 326, 334, 342, 350, 357, 377, 380, 381, 384, 388, 391, 413, 415, 432, 439, 440, 442, 448, 454, 456, 457, 462, 463, 467, 469, 481, 492, 505, 510, 524, 530
1	19	100, 102, 127, 169, 181, 216, 269, 340, 346, 363, 382, 411, 418, 419, 433, 491, 528

Table 6.4: Target scores, number of sequences, and sequence numbers of apicoplast proteins that fall into a particular scoring zone.

diphtheriae are shown in Figure 6.6 as an example of the output for MEP sequences.

Sample extracts of the HTML table containing the SBDD target scores for each apicoplast sequence output from TAPAS are shown in Figure 6.7.

A sample HTML table that shows the number of apicoplast sequences that fall into high and low SBDD target scoring zones is shown in Figure 6.8.

The number of apicoplast sequences that fell into a particular scoring zone of the target score is shown in Figure 6.9. Among the 544 apicoplast sequences, 315 (58%) were predicted to be potential drug targets for SBDD based on target scores being high (Table 6.4). With increased availability of structural information,

Description of the quantitative analysis	Value
Number of proteins analysed in the current execution	8
Number of proteins having human hits	2
Number of proteins having enzyme hits	8
Number of proteins having 3D structure hits	5
Number of proteins having ligand hits	5
Number of proteins that are transmembrane	0

Input	Human	Enzyme	Structure	Ligands	Transmembrane	Signal Anchor	Target Score (1 to 6)
Seq1	NO	YES	YES	YES	NO	NO	6
Seq2	NO	YES	YES	YES	NO	NO	6
Seq3	YES	YES	YES	YES	NO	NO	4
Seq4	NO	YES	NO	NO	NO	NO	5
Seq5	NO	YES	YES	YES	NO	NO	6
Seq6	NO	YES	NO	NO	NO	NO	5
Seq7	NO	YES	NO	NO	NO	NO	5
Seq8	YES	YES	YES	YES	NO	NO	4

Figure 6.6: Quantitative analysis of the TAPAS output for *Corynebacterium diphtheriae* sequences.

Input	Human	Enzyme	Structure	Ligands	Transmembrane	Signal Anchor	Target Score (1 to 6)
Seq1	NO	YES	YES	YES	NO	NO	6
Seq2	NO	YES	YES	YES	YES	YES	6
Seq3	NO	YES	YES	YES	YES	YES	6
Seq4	NO	NO	YES	NO	NO	NO	5
Seq5	YES	NO	YES	NO	NO	NO	3
Seq6	NO	YES	YES	YES	NO	NO	6
Seq7	YES	YES	YES	NO	YES	NO	4
Seq8	NO	YES	NO	NO	YES	YES	5
Seq9	NO	NO	YES	NO	YES	NO	5
Seq10	YES	NO	YES	NO	NO	NO	3
Seq430	NO	NO	YES	YES	YES	YES	6
Seq431	NO	NO	NO	NO	YES	YES	4
Seq432	YES	NO	NO	NO	YES	YES	2
Seq433	YES	NO	NO	NO	YES	NO	1
Seq434	NO	NO	NO	NO	YES	YES	4
Seq435	NO	NO	NO	NO	YES	NO	3
Seq436	YES	YES	NO	NO	NO	NO	3
Seq437	NO	NO	NO	NO	YES	NO	3
Seq438	NO	NO	NO	NO	YES	NO	3
Seq439	YES	YES	NO	NO	YES	NO	2
Seq440	YES	NO	NO	NO	NO	NO	2
Seq441	NO	NO	NO	NO	NO	YES	4
Seq442	YES	NO	NO	NO	NO	NO	2
Seq443	NO	NO	YES	YES	NO	NO	6

Figure 6.7: An extract showing the target score predicted for each apicoplast sequence based on the TAPAS output (figure was edited to accommodate a wide range of target scores — 1-6).

Description of the quantitative analysis	Sequences (total: 544)
Number of proteins with a target score of 6	36
Number of proteins with a target score of 5	36
Number of proteins with a target score of 4	243
Number of proteins with a target score of 3	130
Number of proteins with a target score of 2	80
Number of proteins with a target score of 1	19

Figure 6.8: An extract showing the number of apicoplast sequences that fall into high and low scoring zones for SBDD.

more sequences (especially those with a score of 3) will obtain better scores and thereby move into the potential drug target zone. This higher proportion of drug targets could be attributed to the supposed occurrence of many vital pathways in the apicoplast and also the fact that it is not the percentage of drug targets in the whole organism (*Plasmodium falciparum*), but that of a vital organelle. However, out of the 544 sequences, only 36 (6.6%) were given the highest rank (6 out of 6) and further 36 (6.6%) were given the second highest rank (5 out of 6) (Figure 6.8) because of the distribution of 58% of targets as a result of ranking. These are the sequences which should be taken forward for detailed structural studies immediately. As such, this project deals with druggability for SBDD at a basic stage which is mainly useful for concentrating on some proteins from a large set of unannotated/hypothetical proteins and this percentage is bound to reduce after carrying out extensive structural studies through lack of binding affinity.

Here, I present a review of 13 randomly chosen proteins from the 36 that

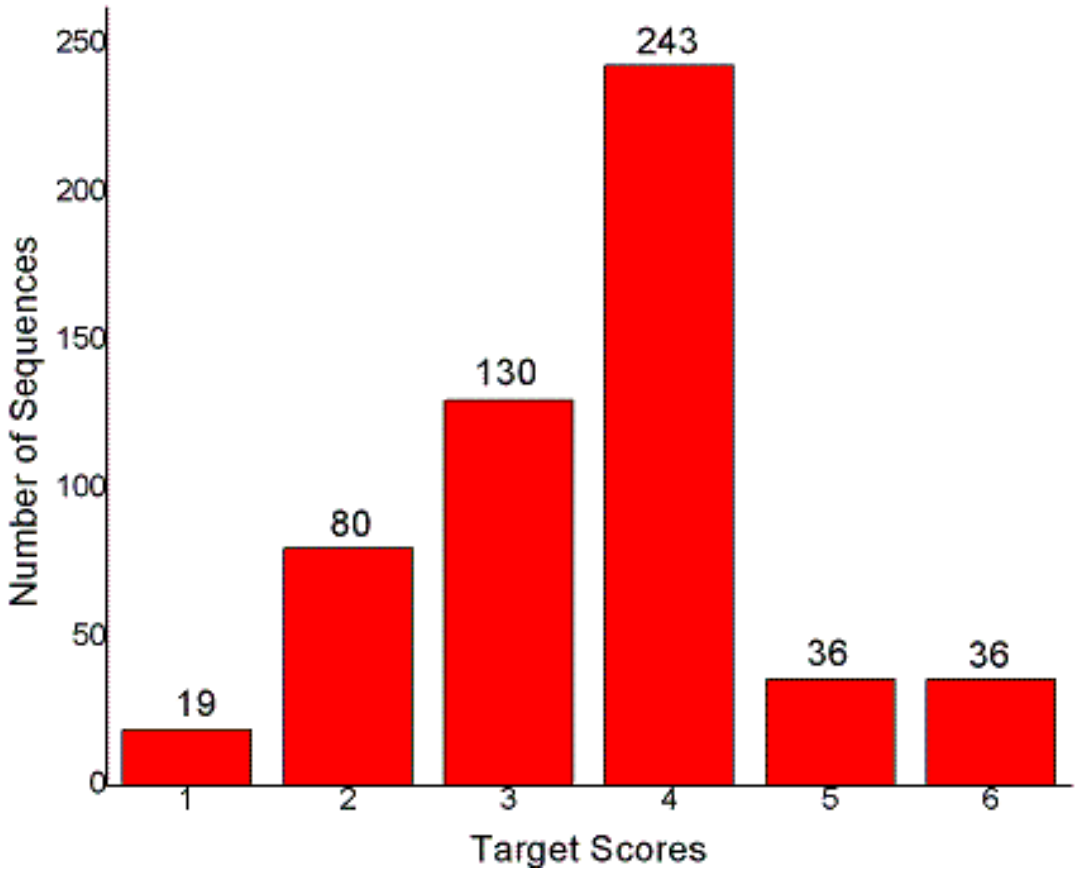


Figure 6.9: Target scores and the number of sequences of apicoplast that fall into a particular scoring zone.

were given a SBDD target score of 6 out of 6. 12 of the 13 reviewed are excellent proven drug targets with known drugs or inhibitors (Table 6.5). The remaining protein is a hypothetical protein which is probably a tRNA nucleotidyltransferase (EC number: 2.7.7.25) based on the output from BLAST. A BLAST hit 'XP_727593' (E-value: 0) is a tRNA nucleotidyltransferase mitochondrial precursor from *Plasmodium yoelii yoelii* str. 17XNL and the best structure hit 'PDB: 1VFG' (E-value: $2e-08$) is a tRNA nucleotidyltransferase from *Aquifex aeolicus*. Currently, there are no known drugs or inhibitors for this protein, but it has a known ligand 'Diphosphomethylphosphonic Acid Adenosyl Ester' (HETNAM=APC, PDB: 1VFG (Tomita *et al.*, 2004)). Following a link (`http://bioinformatics.charite.de/superligands/drug_similarity.php?hetero=APC`) for drug similarity on the RCSB site (`http://www.rcsb.org/pdb/explore/explore.do?structureId=1VFG`), lead to a webpage showing the top 30 drug structures most similar in 2 dimensions to APC which include drugs like Fludarabine, Bucladesine, Cobamamide (all of these have $> 80\%$ 2D similarity). These could be potential lead compounds for designing drugs to act on Sequence 356.

A review of other sequences with positive scores revealed that 8 sequences scored 5 out of 6 because of not having a known ligand. Having a known structure, these proteins could be potential drug targets for SBDD and virtual screening (Nicola *et al.*, 2007). Similarly, 28 sequences scored 5 out of 6 for not having either structure or ligand (but have an EC number). Crystallographers and NMR spectroscopists can concentrate on these proteins for solving their structure. The sequence numbers for these proteins are shown in Table 6.6.

With the increase in structural information, computational techniques like

SBDD, virtual screening, and chemical library design and screening are becoming more popular to cut down cost and time in the drug discovery process (Fauman *et al.*, 2003). Even HTS is expensive because of staffing and compound stock depletion. Recently such techniques have been used on many *P. falciparum* sequences with available structural information as a result of structures recently solved through X-ray crystallography (Nicola *et al.*, 2007; Mehlin, 2005). Even when there are no X-ray crystallographic structures available, alternate computational techniques like comparative modelling are being used where possible, to help in the drug discovery process (Singh *et al.*, 2006a). Currently, there are only very few *P. falciparum* proteins with known structures (Mehlin, 2005), but many of them have bacterial homologues with known structures which provides a great opportunity to model the structures of these proteins to look at the differences in interspecies selectivity (binding affinity) and to use them in the drug discovery process.

6.3 Summary

Among the 544 apicoplast protein sequences 58% (160 sequences) were predicted as potential drug targets for SBDD. These are the sequences with high (> 3) SBDD target scores. However, out of the 544 sequences, only 36 (6.6%) were given the highest rank (6 out of 6) and further 36 (6.6%) were given the second highest rank (5 out of 6) because of the distribution of 58% of targets as a result of ranking. This percentage is bound to increase with the availability of more structural data and bound to decrease after carrying out detailed structural studies (because of lack of binding affinity). 52% of the apicoplast sequences were found to have human homologues which is not desirable, although a protein can still

Sequence number	EC number	Protein name	Known drugs/inhibitors	References
1	1.1.1.267	1-deoxy-D-xylulose 5-phosphate reductoisomerase	Fosmidomycin and it's derivative FR900098	(Yajima *et al.*, 2007; Lell *et al.*, 2003)
2	2.2.1.7	1-deoxy-D-xylulose 5-phosphate synthase	2,3-diphospho-D-glyceric acid, beta-fluoropyruvate, D-3-Phosphoglyceric acid, phosphonoacetohydroxamate, etc.	(Eubanks and Poulter, 2003; Altincicek *et al.*, 2000; Kuzuyama *et al.*, 2000)
3	4.6.1.12	2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase	EDTA	(Rohdich *et al.*, 2001)
6	1.1.1.100	3-oxoacyl-[acyl-carrier protein] reductase, putative	Hexachlorophene	(Wickramasinghe *et al.*, 2006)
38	2.3.1.41	beta-ketoacyl-acyl carrier protein synthase III precursor, putative	Thiolactomycin and it's analogues	(Jones *et al.*, 2005; Waller *et al.*, 2003; Douglas *et al.*, 2002)
45	2.8.1.7	cysteine desulfurase, putative	4-Chloromercuribenzoate, Iodoacetamide, N-Ethylmaleimide, L-Allylglycine and Vinylglycine	(Zheng *et al.*, 1994, 1993)
49	4.2.1.24	delta-aminolevulinic acid dehydratase	Lead, Trichloroethylene, Bromobenzene, and Styrene	(Scinicariello *et al.*, 2007; Rajaraman *et al.*, 2005; Fujita *et al.*, 2002)
58	2.7.7.6	DNA-directed RNA polymerase alpha chain, putative	Rifampicin, Rifabutin, and Rifapentin	(Artsimovitch *et al.*, 2005)
65	1.3.1.9	enoyl-acyl carrier reductase	Triclosan	(Nicola *et al.*, 2007; Surolia and Surolia, 2001)
71		ferredoxin	Metronidazole	(Land *et al.*, 2002; Quon *et al.*, 1992)
73	3.5.1.88	formylmethionine deformylase, putative	Hydroxamic acid derivatives	(Apfel *et al.*, 2000)
219		hypothetical protein (RNA polymerase sigma factor or DNA-directed RNA polymerase, sigma subunit)	Actinomycin, Amanitin, chromomycin, Heparin, aureolic acid, etc.	(Sethi, 1971)
356	2.7.7.25 (probable)	hypothetical protein (Probable tRNA nucleotidyltransferase		

Table 6.5: A review of the drug targets that produced an SBDD target score of 5 out of 5.

Target Score	Criterion	Number of sequences	Sequence numbers
5	No structure	0	-
5	No ligand	8	4, 9, 43, 62, 452, 512, 516, 518
5	No structure and ligand	28	8, 56, 59, 74, 99, 104, 189, 194, 196, 198, 221, 228, 235, 310, 324, 332, 365, 366, 379, 389, 403, 406, 407, 410, 426, 470, 472, 521

Table 6.6: Target scores and the number of sequences of apicoplast that fell into a particular scoring zone.

be a good drug target taking other factors into consideration such as exploiting the differences in interspecies selectivity. 49% of the apicoplast sequences were predicted to have transmembrane regions which is generally not good for SBDD because it is difficult to obtain structural data (except signal anchor proteins whose signal region can be cleaved to determine structure of rest of the protein). 12 out of the 13 (reviewed) proteins whose SBDD target score was 6 out of 6 are well established drug targets having known drugs or inhibitors while the remaining protein is a hypothetical protein thought to be a tRNA nucleotidyltransferase based on results from BLAST. There are no known drugs or inhibitors available for this enzyme, but there is a known ligand 'Diphosphomethylphosphonic Acid Adenosyl Ester' which has many drug hits with more than 80% 2D similarity. 8 sequences scored 5 out of 6 for not having a known ligand. These proteins could be potential drug targets for techniques such as SBDD and virtual screening because they have a known 3D structure. 28 sequences scored 5 out of 6 for not having both structure and ligand. Crystallographers and NMR spectroscopists can try to solve the 3D structure of these proteins.

6.3.1 Evaluation of performance of TAPAS ranking scheme

Ideally, one would have liked to evaluate the performance of TAPAS in ranking drug targets. However, it is virtually impossible to evaluate the performance in any realistic way because, while it is relatively straightforward to obtain sequences of known drug targets, it is virtually impossible to obtain sequences of failed drug targets and the reasons for failing. Even if such data were available, the reasons for failure may not be inherent in the target itself but in the drugs

that were tested. Thus, even if one had collaborated with laboratories such as Structural Proteomics of Rational Targets (SPoRT — a UK Biotechnology and Biological Sciences Research Council (BBSRC) initiative), or the Office of Cancer Genomics (OCG — `http://ocg.cancer.gov/`), and obtained sequences that were not successful, a good negative dataset would not have been obtained since a protein cannot be classified as a "bad" target just because one or two laboratories have not succeeded. Thus, TAPAS is simply trying to define sequences which are worth exploring rather than trying to predict whether SBDD will be successful i.e., it is identifying the characteristics of a target in terms of its suitability rather than trying to predict a successful out come.

Test sets such as those used by Macchiarulo *et al.* (2004) and Glaser *et al.* (2006) are all suitable for extensive structural studies of proteins but would not be suitable for evaluating the performance of TAPAS. They could only test that the program is functioning correctly (rather than any evaluation of performance) but this is already tested by studying sequences of the MEP pathway and the apicoplast. The method is not looking at extensive structural details of proteins in order to define potential ligand binding sites. It is simple ranking targets in view of their biological role rather than specific drug design criteria. This would be the next stage in the process — having selected biologically sensible targets, one would go on to examine their suitability for ligand design.

Chapter 7

Conclusions

The aim of this project was to automate the process of protein annotation further leading to preliminary evaluation of a set of protein sequences for SBDD. This will enable us to concentrate on highly ranked protein sequences in order to further our knowledge of druggability with the help of detailed structural studies. Proteins of the MEP pathway and the apicoplast were chosen for the analysis because they are of interest to the pharmaceutical sector and the scientific community.

The MEP pathway of isoprenoid biosynthesis is totally absent in humans, but present in many pathogens such as *Plasmodium falciparum*, *Mycobacterium tuberculosis*, *Yersinia pestis*, *Vibrio cholerae*, and *Treponema pallidum*. The MEP pathway is indispensable in these organisms which makes it a potential drug target.

The apicoplast is a unique organelle that occurs in apicomplexan protists and hosts approximately 500 nuclear-encoded proteins including proteins of the MEP pathway and the fatty acid biosynthesis pathway. The apicoplast is essential for the survival of apicomplexans which include pathogens such as *Plasmodium falciparum* and *Toxoplasma gondii*. Their absence in humans coupled with their

necessity for the survival of these pathogens, makes proteins of the apicoplast potentially suitable drug targets.

7.1 Automation of annotation

After a review of various bioinformatics tools for mass protein sequence analysis, I concluded that none of these tools was ideal for my project. This resulted in the development of APAT and TAPAS. APAT was designed to execute many tools, where the tools were not serially dependent on the output from other tools, whereas TAPAS was a specialized pipeline for ranking targets for SBDD where the output from one tool would be the input for another.

7.1.1 Development of APAT

The output produced by a variety of annotation tools was analyzed and it was determined that all the annotations can be expressed in one of four ways (per-residue numbers, per-residue strings, per-domain values, per-sequence values). Based on this analysis, the input and output of the APAT system was standardized using XML. The output XML format, was designed to accommodate annotations provided by any prediction server. A display tool and wrappers to a number of annotation/prediction tools running both locally and remotely were designed based on this XML format. The choice of tools is left for users to decide according to their needs.

The APAT system is designed to be downloaded and run locally allowing the user automatically to execute many annotation tools on one or more sequences. Comparative analysis was made straightforward by presenting results in a uni-

form manner. Users can easily choose the annotation wrappers they wish to execute and additional wrappers could be easily written using existing wrappers as examples or alternately, they may want to write a simple wrapper to web-services provided by Taverna (Oinn *et al.*, 2004) or EBI (Labarga *et al.*, 2007) in which case the wrapper will handle the XML output so that it fits into the specified DTD to utilize the display program. The XML output can be directly used for post analysis tasks without using the display program (e.g. the XML output was further processed and used in the work to improve transmembrane protein prediction by combined neural network predictor). The HTML display could be improved in future by having a better choice of graph plotting. Other future developments to the display tool could be — a) to show all the annotations together on the sequence in order to have a holistic picture, b) to provide a direct comparison between similar tools, c) to provide a final summary (and suggestions) of output from various tools for each sequence. Possible enhancements to the software to deal with non-responsive servers instead of just reporting their status would also be useful. Allowing multiple sequence alignment to be provided as input and handling servers which require details such as sequence alignments as input will be another useful enhancement. Similarly, improving APAT to deal with servers which return complex data such as 3D models from comparative modelling would be an advantage.

Source code for all the programs, the DTD for input and output, a detailed description of the DTD, and a guide to implementing service wrappers were made available for download at `http://www.bioinf.org.uk/apat/`.

At the time of writing (June 2008), APAT had been downloaded by >600 users illustrating the value of a system of this type. Frishman (2007) in his

review of protein annotation on a genomic scale has written about tools such as APAT having the advantage of being highly configurable and flexible.

7.1.2 Development of TAPAS

TAPAS is designed to score a set of protein sequences on the basis of suitability for SBDD (at the sequence level devoid of detailed structural studies) by subjecting them to various tasks such as database searches, and annotation and prediction tools. Here, the output from one tool is treated as the input for another and decisions about what tools are run are made on the basis of the output of preceding tools. Additional annotations were obtained from APAT (integrated as a standalone tool).

During the process of annotation, TAPAS looks at some characteristics of a protein which are crucial to providing insight into the ability of a protein to be rated as a potential target for SBDD. These are the presence of human hits, enzyme hits, structure hits, ligand hits, and transmembrane regions. In addition to these, further details about protein sequences such as their association with KEGG pathway maps, and output from BLAST and APAT were presented in the form of an HTML table with hyperlinks to other data sources for additional information.

TAPAS is useful and suitable for executing on a set of protein sequences (especially hypothetical or unannotated) that belong to a particular pathway, an organelle or an organism. The resulting ranking of targets was supported by the fact that 12 of 13 reviewed top-ranked proteins are already being exploited as drug targets. Possible enhancements for TAPAS could include using a relational database for storing and managing results (instead of the current file-based sys-

tem), improving the scoring scheme by adding additional features (at the sequence level) and altering weights to certain parameters (based on additional knowledge gained) and including additional factors (at the structure level) such as knowledge about clefts, active site interactions, and binding affinity. When these extensive structural details are included in TAPAS, the other possible enhancements could include integration of automatic comparative modelling (to obtain 3D models of a protein structure), virtual screening (to obtain some potential lead molecules), and docking modules (to examine the binding affinity). Another useful enhancement could be integration of protein function prediction (by including tools such as ProFunc (Laskowski *et al.*, 2005)). Improving the ranking scheme and performing a review of highly ranked sequences through methods such as text mining, or data mining may also be an advantage.

The ranking scheme of TAPAS, which is currently suitable for handling microbial sequences, could be used for other higher organisms by making slight modifications to the weights attached to various factors.

It would be interesting to try to perform the same analysis using Taverna and/or ICENI (both described in Chapter 2) to compare the performance and flexibility in implementing and extending the pipelines.

7.1.3 Improving prediction of transmembrane proteins

Prediction of transmembrane regions in a protein was one of the most important annotations applied by APAT and TAPAS. In an effort to improve transmembrane predictions, a combined neural network predictor was developed. This combined predictor used the output from three of the best transmembrane predictors that are currently available — TMHMM (Krogh *et al.*, 2001), MEMSAT

(Jones *et al.*, 1994), and DAS-TMfilter (Cserzo *et al.*, 2004). Performance of the combined predictor was evaluated at both the residue level and the whole protein level and was compared with the individual predictors used. The effect of masking signal peptide residues as predicted by SignalP was also evaluated. APAT was used for running all these tools and the XML output from APAT was used for as input post-processing.

Residue level

Among the three individual predictors used, TMHMM performed best with an MCC of 0.796. Optimum performance of the combined predictor was achieved with an un-masked and unnormalized combined predictor, giving an MCC of 0.803 using a prediction threshold of 0.5 and a hidden layer size of 5. Thus, combining the output of the 3 best predictors only marginally improved performance compared with TMHMM used alone. While DAS-TMfilter alone was the worst predictor, it did enhance the quality of the combined prediction. Surprisingly, masking with SignalP reduced the performance of the combined predictor (MCC=0.787).

Whole protein level

In the case of selecting targets for SBDD, one simply wishes to know whether a protein has transmembrane regions. Whole protein level predictions were made based on residue level predictions by examining the total number of transmembrane residues predicted, the length of the longest transmembrane segment, and the average length of a transmembrane segment. The best combined predictor at the residue level was used and compared with the best individual predictor

(TMHMM). The best performance of the combined predictor (MCC=0.708) was obtained using a threshold of 22 residues for the longest transmembrane region. This value is clearly worse than the performance value at the residue-level. Manual examination revealed that the worse performance is caused by signal peptides. This led to masking signal peptide residues at the whole protein level. For the masked and un-normalized combined predictor, the best performance, with an MCC of 0.762, was obtained using a threshold of $\geq$18 residues for the longest transmembrane region.

The performance of the best individual predictor (TMHMM), before and after masking was assessed at the protein level. The best MCC of 0.762 was obtained after masking (longest transmembrane segment $\geq$18 residues) compared with an MCC of 0.693 unmasked (longest transmembrane segment or the total number of transmembrane residues $\geq$18). Thus, it was concluded that at the whole protein level, TMHMM masked by SignalP performs as well as the combined predictor.

The performance of the combined predictor is likely to improve as individual prediction tools are enhanced. A new version of MEMSAT was recently released and could be included in the analysis to assess the improvements made to the combined predictor.

7.1.4 Analysis of the MEP pathway and apicoplast proteins using TAPAS

Various methods developed earlier and described above were then applied to the analysis of the proteins of the MEP pathway and the apicoplast. I decided to look at the apicoplast from *Plasmodium falciparum* and MEP pathway proteins from selected pathogens. Details of the origin of the sequence were required by

some of the analysis tools run by APAT. Labelling for MEP pathway proteins was achieved by looking at the taxonomical classification from the 'OC' lines of the UniprotKB/SwissProt data file for each organism. As mentioned above, transmembrane region prediction is one of the important annotations applied by APAT and TAPAS. As masking signal peptide residues improved the transmembrane prediction at the whole protein level, the technique of signal masking TMHMM predictions for predicting whether or not a protein is transmembrane was employed in TAPAS. This used a threshold of $\geq$18 residues for the length of the longest membrane spanning region.

The sequences of 544 apicoplast proteins from *Plasmodium falciparum*, kindly supplied by Prof. Geoff McFadden (`gim@unimelb.edu.au`), and the sequences of the MEP pathway from selected pathogens, were analysed by TAPAS followed by post analysis which included manual analysis of the final output. Post analysis involved quantitative analysis and SBDD target score prediction for ranking proteins. Quantitative analysis of the apicoplast output provided the percentage of proteins that had human homologues (52%), enzyme hits (38%), 3D structure hits (34%), ligand hits (24%), and transmembrane regions (49%). 58% of apicoplast proteins were given a positive SBDD target score over a scale of 1 to 6. However, out of the 544 sequences, only 36 (6.6%) were given the highest rank (6 out of 6) and further 36 (6.6%) were given the second highest rank (5 out of 6) because of the distribution of 58% of targets as a result of ranking. 12 out of 13 (reviewed) top-ranked (6 out of 6) sequences were already being exploited as drug targets while the remaining protein is a hypothetical protein thought to be a tRNA nucleotidyltransferase based on output from BLAST. There is no known drug or inhibitor associated with it. However 'Diphosphomethylphosphonic Acid

Adenosyl Ester' is a known ligand which shows over 80% 2D similarity to a number of drugs (`http://bioinformatics.charite.de/superligands/drug_similarity.php?hetero=APC`).

There are 8 sequences which scored 5 out of 6 because they did not have a known ligand. Having a solved structure, these could be potential drug targets for SBDD, or virtual screening (Nicola *et al.*, 2007). Similarly there are 28 sequences which scored 5 out of 6 because they neither have a known structure nor a ligand. These are the proteins crystallographers and NMR spectroscopists could concentrate on to solve their 3D structure. This ranking of proteins is useful in guiding one to decide which proteins to focus on among a large set of unannotated protein sequences.

Analysis of the MEP pathway and apicoplast proteins using TAPAS resulted in some significant annotations and clues about suitability of each protein to be a target for SBDD.

7.2 Summary

In summary, this project has been successful in achieving its primary aims. I have developed a new tool (APAT) for automated application of a number of annotation and prediction servers to one or more protein sequences. I have attempted to improve transmembrane protein prediction using a combined neural network predictor and found that masking the prediction values of signal peptide residues would improve the predictions from TMHMM alone (the same performance is achieved by the combined predictor). I have developed a specialized pipeline (TAPAS), which makes use of APAT, for ranking potential targets for structure-based drug design (SBDD). This has then been applied to protein sequences of

the MEP pathway from a number of selected organisms and to proteins of the apicoplast from *Plasmodium falciparum*. The results justified the ranking scheme used in TAPAS, as 12 of the 13 (reviewed) top-ranked hits are already being exploited as drug targets. A number of other proteins, for which structures are known, were suggested as potential targets and a further group of sequences were suggested as novel targets which should be brought to the attention of protein crystallographers and NMR spectroscopists.

Bibliography

Acharya, K. R., Sturrock, E. D., Riordan, J. F. and Ehlers, M. R. W. (2003) Ace revisited: a new target for structure-based drug design, *Nat Rev Drug Discov*, **2**, 891–902.

Adam, P., Hecht, S., Eisenreich, W., Kaiser, J., Grawert, T., Arigoni, D., Bacher, A. and Rohdich, F. (2002) Biosynthesis of terpenes: Studies on 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase, *Proc Natl Acad Sci U S A*, **99**, 12108–12113.

Agranoff, B. W., Eggerer, H., Henning, U. and Lynen, F. (1959) Isopentenol pyrophosphate isomerase, *J. Am. Chem. Soc.*, **81**, 1254–1255.

Agranoff, B. W., Eggerer, H., Henning, U. and Lynen, F. (1960) Biosynthesis of terpenes. VII. Isopentenyl pyrophosphate isomerase, *J Biol Chem*, **235**, 326–332.

Alderden, R. A., Hall, M. D. and Hambley, T. W. (2006) The discovery and development of cisplatin, *J. Chem. Educ.*, **83**, 728–734.

Almond, J. and Snelling, D. (1998) Unicore: Secure and uniform access to distributed resources via the world wide web, *white paper*.

Aloisio, G., Cafaro, M., Fiore, S. and Mirto, M. (2005) ProGenGrid: a grid-enabled platform for bioinformatics, *Stud Health Technol Inform*, **112**, 113–126.

Altincicek, B., Hintz, M., Sanderbrand, S., Wiesner, J., Beck, E. and Jomaa, H. (2000) Tools for discovery of inhibitors of the 1-deoxy-D-xylulose 5-phosphate (DXP) synthase and DXP reductoisomerase: an approach with enzymes from the pathogenic bacterium Pseudomonas aeruginosa, *FEMS Microbiol Lett*, **190**, 329–333.

Altincicek, B., Duin, E. C., Reichenberg, A., Hedderich, R., Kollas, A.-K., Hintz, M., Wagner, S., Wiesner, J., Beck, E. and Jomaa, H. (2002) LytB protein catalyzes the terminal step of the 2-C-methyl-D-erythritol-4-phosphate pathway of isoprenoid biosynthesis, *FEBS Lett*, **532**, 437–440.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990) Basic local alignment search tool, *J. Mol. Biol.*, **215**, 403–410.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, *Nuc. Ac. Res.*, **25**, 3389–3402.

An, J., Totrov, M. and Abagyan, R. (2004) Comprehensive identification of "druggable" protein ligand binding sites, *Genome Inform*, **15**, 31–41.

Anastasiadis, A. D., Magoulas, G. D. and Vrahatis, M. N., (2003). An efficient improvement of the Rprop algorithm. `http://www.dcs.bbk.ac.uk/$\sim$aris/Camera\_ready\_ANNPR03\_final.pdf`.

Anderson, A. C. (2003) The process of structure-based drug design, *Chem Biol*, **10**, 787–797.

Anderson, M. S., Muehlbacher, M., Street, I. P., Proffitt, J. and Poulter, C. D. (1989) Isopentenyl diphosphate:dimethylallyl diphosphate isomerase. An improved purification of the enzyme and isolation of the gene from Saccharomyces cerevisiae, *J Biol Chem*, **264**, 19169–19175.

Apfel, C., Banner, D. W., Bur, D., Dietz, M., Hirata, T., Hubschwerlen, C., Locher, H., Page, M. G., Pirson, W., Rossé, G. and Specklin, J. L. (2000) Hydroxamic acid derivatives as potent peptide deformylase inhibitors and antibacterial agents, *J Med Chem*, **43**, 2324–2331.

Apweiler, R., Attwood, T. K., Bairoch, A., Bateman, A., Birney, E., Biswas, M., Bucher, P., Cerutti, L., Corpet, F., Croning, M. D., Durbin, R., Falquet, L., Fleischmann, W., Gouzy, J., Hermjakob, H., Hulo, N., Jonassen, I., Kahn, D., Kanapin, A., Karavidopoulou, Y., Lopez, R., Marx, B., Mulder, N. J., Oinn, T. M., Pagni, M., Servant, F., Sigrist, C. J. and Zdobnov, E. M. (2001) The InterPro database, an integrated documentation resource for protein families, domains and functional sites, *Nuc. Ac. Res.*, **29**, 37–40.

Arai, M., Ikeda, M. and Shimizu, T. (2003) Comprehensive analysis of transmembrane topologies in prokaryotic genomes, *Gene*, **304**, 77–86.

Arai, M., Mitsuke, H., Ikeda, M., Xia, J.-X., Kikuchi, T., Satake, M. and Shimizu, T. (2004) ConPred II: a consensus prediction method for obtaining transmembrane topology models with high reliability, *Nucleic Acids Res*, **32**, W390–W393.

Arora, A., Abildgaard, F., Bushweller, J. H. and Tamm, L. K. (2001) Structure of outer membrane protein A transmembrane domain by NMR spectroscopy, *Nat Struct Biol*, **8**, 334–338.

Artsimovitch, I., Vassylyeva, M. N., Svetlov, D., Svetlov, V., Perederina, A., Igarashi, N., Matsugaki, N., Wakatsuki, S., Tahirov, T. H. and Vassylyev, D. G. (2005) Allosteric modulation of the RNA polymerase catalytic reaction is an essential component of transcription control by rifamycins, *Cell*, **122**, 351–363.

Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., Davis, A. P., Dolinski, K., Dwight, S. S., Eppig, J. T., Harris, M. A., Hill, D. P., Issel-Tarver, L., Kasarskis, A., Lewis, S., Matese, J. C., Richardson, J. E., Ringwald, M., Rubin, G. M. and Sherlock, G. (2000) Gene ontology: tool for the unification of biology. The Gene Ontology Consortium, *Nature: Genetics*, **25**, 25–29.

Bach, T. J., Boronat, A., Campos, N., Ferrer, A. and Vollack, K. U. (1999) Mevalonate biosynthesis in plants, *Crit Rev Biochem Mol Biol*, **34**, 107–122.

Bagos, P. G., Liakopoulos, T. D. and Hamodrakas, S. J. (2005) Evaluation of methods for predicting the topology of beta-barrel outer membrane proteins and a consensus prediction method, *BMC Bioinformatics*, **6**, 7–7.

Bagos, P. G., Liakopoulos, T. D., Spyropoulos, I. C. and Hamodrakas, S. J. (2004a) A Hidden Markov Model method, capable of predicting and discriminating beta-barrel outer membrane proteins, *BMC Bioinformatics*, **5**, 29–29.

Bagos, P. G., Liakopoulos, T. D., Spyropoulos, I. C. and Hamodrakas, S. J.

(2004b) PRED-TMBB: a web server for predicting the topology of beta-barrel outer membrane proteins, *Nucleic Acids Res*, **32**, W400–W404.

Bairoch, A. (2000) The ENZYME database in 2000, *Nucleic Acids Res*, **28**, 304–305.

Bairoch, A. and Apweiler, R. (2000) The SWISS-PROT protein sequence database and its supplement TrEMBL in 2000, *Nuc. Ac. Res.*, **28**, 45–48.

Baker, D. and Sali, A. (2001) Protein structure prediction and structural genomics, *Science*, **294**, 93–96.

Bayes, T. (1763) An essay towards solving a problem in the doctrine of chances., *Philosophical Transactions of the Royal Society*, **53**, 370–418.

Begley, M., Gahan, C. G. M., Kollas, A.-K., Hintz, M., Hill, C., Jomaa, H. and Eberl, M. (2004) The interplay between classical and alternative isoprenoid biosynthesis controls gammadelta T cell bioactivity of Listeria monocytogenes, *FEBS Lett*, **561**, 99–9104.

Belunis, C. J., Mdluli, K. E., Raetz, C. R. and Nano, F. E. (1992) A novel 3-deoxy-D-manno-octulosonic acid transferase from Chlamydia trachomatis required for expression of the genus-specific epitope, *J Biol Chem*, **267**, 18702–18707.

Bender, A., van Dooren, G. G., Ralph, S. A., McFadden, G. I. and Schneider, G. (2003) Properties and prediction of mitochondrial transit peptides from Plasmodium falciparum, *Mol Biochem Parasitol*, **132**, 59–66.

Bendtsen, J. D., Nielsen, H., von Heijne, G. and Brunak, S. (2004) Improved prediction of signal peptides: SignalP 3.0, *J Mol Biol*, **340**, 783–795.

Benson, D. A., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J. and Wheeler, D. L. (2007) GenBank, *Nucleic Acids Res*, **35**, D21–D25.

Berman, F., Chien, A., Cooper, K., Dongarra, J., Foster, I., Gannon, D., Johnsson, L., Kennedy, K., Kesselman, C., Mellor-Crummey, J., Reed, D., Torczon, L. and Wolski, R. (2001) The GrADS Project: Software support for high-level Grid application development, *The International Journal of High Performance Computing Applications*, **15**, 327–344.

Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., Shindyalov, I. N. and Bourne, P. E. (2000) The Protein Data Bank, *Nucleic Acids Res*, **28**, 235–242.

Beytía, E. D. and Porter, J. W. (1976) Biochemistry of polyisoprenoid biosynthesis, *Annu Rev Biochem*, **45**, 113–142.

Bigelow, H. and Rost, B. (2006) PROFtmb: a web server for predicting bacterial transmembrane beta barrel proteins, *Nucleic Acids Res*, **34**, W186–W188.

Binda, C., Newton-Vinson, P., Hubálek, F., Edmondson, D. E. and Mattevi, A. (2002) Structure of human monoamine oxidase B, a drug target for the treatment of neurological disorders, *Nat Struct Biol*, **9**, 22–26.

Blom, N., Gammeltoft, S. and Brunak, S. (1999) Sequence and structure-based prediction of eukaryotic protein phosphorylation sites, *J. Mol. Biol.*, **294**, 1351–1362.

Boeckmann, B., Bairoch, A., Apweiler, R., Blatter, M.-C., Estreicher, A., Gasteiger, E., Martin, M. J., Michoud, K., O'Donovan, C., Phan, I., Pilbout, S.

and Schneider, M. (2003) The SWISS-PROT protein knowledgebase and its supplement TrEMBL in 2003, *Nuc. Ac. Res.*, **31**, 365–370.

Bonanno, J. B., Edo, C., Eswar, N., Pieper, U., Romanowski, M. J., Ilyin, V., Gerchman, S. E., Kycia, H., Studier, F. W., Sali, A. and Burley, S. K. (2001) Structural genomics of enzymes involved in sterol/isoprenoid biosynthesis, *Proc Natl Acad Sci U S A*, **98**, 12896–12901.

Boucher, Y. and Doolittle, W. F. (2000) The role of lateral gene transfer in the evolution of isoprenoid biosynthesis pathways, *Mol Microbiol*, **37**, 703–716.

Bracey, M. H., Hanson, M. A., Masuda, K. R., Stevens, R. C. and Cravatt, B. F. (2002) Structural adaptations in a membrane enzyme that terminates endocannabinoid signaling, *Science*, **298**, 1793–1796.

Breukink, E. (2006) A lesson in efficient killing from two-component lantibiotics, *Mol Microbiol*, **61**, 271–273.

Brightwell, G., Kenyon, C. and Paugam-Moisy, H., (1997). Multilayer neural networks: One or two hidden layers? In Mozer, M. C., Jordan, M. I. and Petsche, T. (eds.), *Advances in Neural Information Processing Systems*, volume 9, page 148. The MIT Press.

Brocks, J. J., Logan, G. A., Buick, R. and Summons, R. E. (1999) Archean molecular fossils and the early rise of eukaryotes, *Science*, **285**, 1033–1036.

Brown, D. and Superti-Furga, G. (2003) Rediscovering the sweet spot in drug discovery, *Drug Discov Today*, **8**, 1067–1077.

Brunak, S., (1993). Doing sequence analysis by inspecting the order in which neural networks learn. In *Proceedings of Computation of Biomolecular Structures*

- *Achievements, Problems and Perspectives, Springer Verlag, Berlin*, pages 43–54.

Buhaescu, I. and Izzedine, H. (2007) Mevalonate pathway: a review of clinical and therapeutical implications, *Clin Biochem*, **40**, 575–584.

Burgarella, S., Cattaneo, D., Pinciroli, F. and Masseroli, M. (2005) MicroGen: a MIAME compliant web system for microarray experiment information and workflow management, *BMC Bioinformatics*, **6 Suppl 4**, S6–S6.

Campbell, M., Hahn, F. M., Poulter, C. D. and Leustek, T. (1998) Analysis of the isopentenyl diphosphate isomerase gene family from Arabidopsis thaliana, *Plant Mol Biol*, **36**, 323–328.

Campbell, S. J., Gold, N. D., Jackson, R. M. and Westhead, D. R. (2003) Ligand binding: Functional site location, similarity and docking, *Curr Opin Struct Biol*, **13**, 389–395.

Cantor, C. R. and Little, D. P. (1998) Massive attack on high-throughput biology, *Nat Genet*, **20**, 5–6.

Cao, J., Jarvis, S. A., Saini, S. and Nudd, G. R., (2003). Gridflow: Workflow management for grid computing. In *CCGRID '03: Proceedings of the 3st International Symposium on Cluster Computing and the Grid*, page 198, Washington, DC, USA. IEEE Computer Society.

Cassera, M. B., Merino, E. F., Peres, V. J., Kimura, E. A., Wunderlich, G. and Katzin, A. M. (2007) Effect of fosmidomycin on metabolic and transcript profiles of the methylerythritol phosphate pathway in Plasmodium falciparum, *Mem Inst Oswaldo Cruz*, **102**, 377–383.

Cavalier-Smith, T. (1982) The evolutionary origin and phylogeny of eukaryote flagella, *Symp Soc Exp Biol*, **35**, 465–493.

Cavalier-Smith, T. (2000) Membrane heredity and early chloroplast evolution, *Trends Plant Sci*, **5**, 174–182.

Chan, A. W. E. and Weir, M. P. (2001) Using chemistry to target treatments, *Chemical Innovation*, **31**, 12–17.

Chandonia, J. M. and Karplus, M. (1995) Neural networks for secondary structure and structural class predictions, *Protein Sci*, **4**, 275–285.

Chatterjee, C., Paul, M., Xie, L. and van der Donk, W. A. (2005) Biosynthesis and mode of action of lantibiotics, *Chem Rev*, **105**, 633–684.

Chen, C. P., Kernytsky, A. and Rost, B. (2002) Transmembrane helix predictions revisited, *Protein Sci*, **11**, 2774–2791.

Chen, H., Huang, N. and Sun, Z. (2006) SubLoc: a server/client suite for protein subcellular location based on SOAP, *Bioinformatics*, **22**, 376–377.

Chen, Y.-P. P. and Chen, F. (2008) Identifying targets for drug discovery using bioinformatics, *Expert Opin Ther Targets*, **12**, 383–389.

Cheng, A. C., Coleman, R. G., Smyth, K. T., Cao, Q., Soulard, P., Caffrey, D. R., Salzberg, A. C. and Huang, E. S. (2007) Structure-based maximal affinity model predicts small-molecule druggability, *Nat Biotechnol*, **25**, 71–75.

Cho, W. and Stahelin, R. V. (2005) Membrane-protein interactions in cell signaling and membrane trafficking, *Annu Rev Biophys Biomol Struct*, **34**, 119–151.

Chugh, J. K. and Wallace, B. A. (2001) Peptaibols: models for ion channels, *Biochem Soc Trans*, **29**, 565–570.

Claros, M. G. and von Heijne, G. (1994) TopPred II: an improved software for membrane protein structure predictions, *Comput Appl Biosci*, **10**, 685–686.

Collins, F. S., Green, E. D., Guttmacher, A. E. and Guyer, M. S. (2003) A vision for the future of genomics research, *Nature*, **422**, 835–847.

Crane, C. M., Kaiser, J., Ramsden, N. L., Lauw, S., Rohdich, F., Eisenreich, W., Hunter, W. N., Bacher, A. and Diederich, F. (2006) Fluorescent inhibitors for IspF, an enzyme in the non-mevalonate pathway for isoprenoid biosynthesis and a potential target for antimalarial therapy, *Angew Chem Int Ed Engl*, **45**, 1069–1074.

Cserzö, M., Wallin, E., Simon, I., von Heijne, G. and Elofsson, A. (1997) Prediction of transmembrane alpha-helices in prokaryotic membrane proteins: the dense alignment surface method, *Protein Eng*, **10**, 673–676.

Cserzo, M., Eisenhaber, F., Eisenhaber, B. and Simon, I. (2004) TM or not TM: transmembrane protein prediction with low false positive rate using DAS-TMfilter, *Bioinformatics*, **20**, 136–137.

Cuff, J. A. and Barton, G. J. (1999) Evaluation and improvement of multiple sequence methods for protein secondary structure prediction, *Proteins*, **34**, 508–519.

Cuff, J. A., Clamp, M. E., Siddiqui, A. S., Finlay, M. and Barton, G. J. (1998) JPred: a consensus secondary structure prediction server, *Bioinformatics*, **14**, 892–893.

Cuthbertson, J. M., Doyle, D. A. and Sansom, M. S. P. (2005) Transmembrane helix prediction: a comparative evaluation and analysis, *Protein Eng Des Sel*, **18**, 295–308.

Dahl, E. L., Shock, J. L., Shenai, B. R., Gut, J., DeRisi, J. L. and Rosenthal, P. J. (2006) Tetracyclines specifically target the apicoplast of the malaria parasite Plasmodium falciparum, *Antimicrob Agents Chemother*, **50**, 3124–3131.

Davey, R., Savva, G., Dicks, J. and Roberts, I. N. (2007) MPP: a microarray-to-phylogeny pipeline for analysis of gene and marker content datasets, *Bioinformatics*, **23**, 1023–1025.

de Ruyck, J. and Wouters, J. (2008) Structure-based drug design targeting biosynthesis of isoprenoids: a crystallographic state of the art of the involved enzymes, *Curr Protein Pept Sci*, **9**, 117–137.

de Ruyck, J., Durisotti, V., Oudjama, Y. and Wouters, J. (2006) Structural role for Tyr-104 in Escherichia coli isopentenyl-diphosphate isomerase: Site-directed mutagenesis, enzymology, and protein crystallography, *J Biol Chem*, **281**, 17864–17869.

de Ruyck, J., Rothman, S. C., Poulter, C. D. and Wouters, J. (2005) Structure of Thermus thermophilus type 2 isopentenyl diphosphate isomerase inferred from crystallography and molecular dynamics, *Biochem Biophys Res Commun*, **338**, 1515–1518.

Deber, C. M., Wang, C., Liu, L. P., Prior, A. S., Agrawal, S., Muskat, B. L. and Cuticchia, A. J. (2001) TM Finder: a prediction program for transmembrane

protein segments using a combination of hydrophobicity and nonpolar phase helicity scales, *Protein Sci*, **10**, 212–219.

Deelman, E., Blythe, J., Gil, Y. and Kesselman, C., (2004). *Grid resource management: state of the art and future trends*, chapter Workflow management in GriPhyN, pages 99–116. Kluwer Academic Publishers, Norwell, MA, USA.

Deelman, E., Blythe, J., Gil, Y., Kesselman, C., Mehta, G., Vahi, K., Blackburn, K., Lazzarini, A., Arbree, A., Cavanaugh, R. and Koranda, S. (March 2003) Mapping abstract complex workflows onto grid environments, *Journal of Grid Computing*, **V1**, 25–39.

Deevi, S. V. V. and Martin, A. C. R. (2006) An extensible automated protein annotation tool: Standardizing input and output using validated XML, *Bioinformatics*, **22**, 291–296.

Dewick, P. M. (2002) The biosynthesis of C5-C25 terpenoid compounds, *Nat Prod Rep*, **19**, 181–222.

Ding, C. H. and Dubchak, I. (2001) Multi-class protein fold recognition using support vector machines and neural networks, *Bioinformatics*, **17**, 349–358.

Disney, M. D., Childs, J. L. and Turner, D. H. (2004) Hoechst 33258 selectively inhibits group I intron self-splicing by affecting RNA folding, *Chembiochem*, **5**, 1647–1652.

Douglas, J. D., Senior, S. J., Morehouse, C., Phetsukiri, B., Campbell, I. B., Besra, G. S. and Minnikin, D. E. (2002) Analogues of thiolactomycin: Potential drugs with enhanced anti-mycobacterial activity, *Microbiology*, **148**, 3101–3109.

Dowell, R. D., Jokerst, R. M., Day, A., Eddy, S. R. and Stein, L. (2001) The distributed annotation system, *BMC Bioinformatics*, **2**, 7–7.

Drăghici, S. and Potter, R. B. (2003) Predicting HIV drug resistance with neural networks, *Bioinformatics*, **19**, 98–9107.

Drews, J. (1996) Genomic sciences and the medicine of tomorrow, *Nat Biotechnol*, **14**, 1516–1518.

Drews, J. and Ryser, S. (1997a) Classic drug targets, *Nat Biotechnol*, **15**, 1350. Pullout.

Drews, J. and Ryser, S. (1997b) The role of innovation in drug development, *Nat Biotechnol*, **15**, 1318–1319.

Dubey, J. P. and Welcome, F. L. (1988) Toxoplasma gondii-induced abortion in sheep, *J Am Vet Med Assoc*, **193**, 697–700.

Dubey, V. S., Bhalla, R. and Luthra, R. (2003) An overview of the non-mevalonate pathway for terpenoid biosynthesis in plants, *J Biosci*, **28**, 637–646.

Durbecq, V., Sainz, G., Oudjama, Y., Clantin, B., Bompard-Gilles, C., Tricot, C., Caillet, J., Stalon, V., Droogmans, L. and Villeret, V. (2001) Crystal structure of isopentenyl diphosphate:dimethylallyl diphosphate isomerase, *EMBO J*, **20**, 1530–1537.

Durinck, S., Moreau, Y., Kasprzyk, A., Davis, S., De Moor, B., Brazma, A. and Huber, W. (2005) BioMart and Bioconductor: a powerful link between biological databases and microarray data analysis, *Bioinformatics*, **21**, 3439–3440.

Eberl, M., Hintz, M., Jamba, Z., Beck, E., Jomaa, H. and Christiansen, G. (2004) Mycoplasma penetrans is capable of activating V gamma 9/V delta 2 T cells while other human pathogenic mycoplasmas fail to do so, *Infect Immun*, **72**, 4881–4883.

Eckart, J. D. and Sobral, B. W. S. (2003) A life scientist's gateway to distributed data management and computing: the PathPort/ToolBus framework, *OMICS*, **7**, 79–88.

Ecker, D. and Griffey, R. (1999) RNA as a small-molecule drug target: Doubling the value of genomics, *Drug Discov Today*, **4**, 420–429.

Eisenreich, W., Bacher, A., Arigoni, D. and Rohdich, F. (2004) Biosynthesis of isoprenoids via the non-mevalonate pathway, *Cell Mol Life Sci*, **61**, 1401–1426.

Eisenreich, W., Schwarz, M., Cartayrade, A., Arigoni, D., Zenk, M. H. and Bacher, A. (1998) The deoxyxylulose phosphate pathway of terpenoid biosynthesis in plants and microorganisms, *Chem Biol*, **5**, R221–R233.

Elofsson, A. and Heijne, G. v. (2007) Membrane Protein Structure: Prediction versus Reality, *Annu Rev Biochem*, **76**, 125–140.

Emanuelsson, O., Nielsen, H., Brunak, S. and von Heijne, G. (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence, *J. Mol. Biol.*, **300**, 1005–1016.

Emanuelsson, O., Nielsen, H. and von Heijne, G. (1999) ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites, *Protein Sci*, **8**, 978–984.

Eubanks, L. M. and Poulter, C. D. (2003) Rhodobacter capsulatus 1-deoxy-D-xylulose 5-phosphate synthase: Steady-state kinetics and substrate binding, *Biochemistry*, **42**, 1140–1149.

Fagan, R. and Swindells, M. (2000) Bioinformatics, target discovery and the pharmaceutical/biotechnology industry, *Curr Opin Mol Ther*, **2**, 655–661.

Fagan, R., Swindells, M., Overington, J. and Weir, M. (2001) Nicastrin, a presenilin-interacting protein, contains an aminopeptidase/transferrin receptor superfamily domain, *Trends Biochem Sci*, **26**, 213–214.

Fahringer, T., Jugravu, A., Pllana, S., Prodan, R., Clovis Seragiotto, J. and Truong, H.-L. (2005) Askalon: a tool set for cluster and grid computing: Research articles, *Concurr. Comput. : Pract. Exper.*, **17**, 143–169.

Fauman, E. B., Hopkins, A. L. and Groom, C. R., (2003). *Structural Bioinformatics*, chapter 23. Structural Bioinformatics in Drug Discovery, pages 477–497. Wiley-Liss Inc., Hoboken, New Jersey.

Finkelstein, R. and Rock, C., (2002). *In C.R. Somerville, E.M. Meyerowitz, eds, The Arabidopsis Book.*, chapter Abscisic acid biosynthesis and response. American Society of Plant Biologists, Rockville, MD. Also available as `www.aspb.org/publications/arabidopsis/`.

Flower, D. R. (1999) Modelling G-protein-coupled receptors for drug design, *Biochim Biophys Acta*, **1422**, 207–234.

Foster, I. and Kesselman, C. (eds.), (1999). *The grid: blueprint for a new computing infrastructure*. Morgan Kaufmann Publishers Inc., San Francisco, CA, USA.

Fowler, P. W. and Coveney, P. V. (2006) A computational protocol for the integration of the monotopic protein prostaglandin H2 synthase into a phospholipid bilayer, *Biophys J*, **91**, 401–410.

Fox, T., Brennan, D., Austen, D. A., Swalley, S. E., Coll, J. T., Raybuck, S. A. and Chambers, S. P. (2007) Development of a protease production platform for structure-based drug design, *Curr Protein Pept Sci*, **8**, 439–445.

Frishman, D., Albermann, K., Hani, J., Heumann, K., Metanomski, A., Zollner, A. and Mewes, H. W. (2001) Functional and structural genomics using PEDANT, *Bioinformatics*, **17**, 44–57.

Frishman, D. (2007) Protein annotation at genomic scale: the current status, *Chem Rev*, **107**, 3448–3466.

Fujita, A., Massirer, K. B., Durham, A. M., Ferreira, C. E. and Sogayar, M. C. (2005) The GATO gene annotation tool for research laboratories, *Braz J Med Biol Res*, **38**, 1571–1574.

Fujita, H., Nishitani, C. and Ogawa, K. (2002) Lead, chemical porphyria, and heme as a biological mediator, *Tohoku J Exp Med*, **196**, 53–64.

Funes, S., Davidson, E., Reyes-Prieto, A., Magallón, S., Herion, P., King, M. P. and González-Halphen, D. (2002) A green algal apicoplast ancestor, *Science*, **298**, 2155–2155.

Furmento, N., Lee, W., Mayer, A., Newhouse, S. and Darlington, J., (2002). Iceni: an open grid service architecture implemented with jini. In *Supercomputing '02: Proceedings of the 2002 ACM/IEEE conference on Supercomputing*, pages 1–10, Los Alamitos, CA, USA. IEEE Computer Society Press.

Gabrielsen, M., Bond, C. S., Hallyburton, I., Hecht, S., Bacher, A., Eisenreich, W., Rohdich, F. and Hunter, W. N. (2004) Hexameric assembly of the bifunctional methylerythritol 2,4-cyclodiphosphate synthase and protein-protein associations in the deoxy-xylulose-dependent pathway of isoprenoid precursor biosynthesis, *J Biol Chem*, **279**, 52753–52761.

Gabrielsen, M., Kaiser, J., Rohdich, F., Eisenreich, W., Laupitz, R., Bacher, A., Bond, C. S. and Hunter, W. N. (2006) The crystal structure of a plant 2C-methyl-D-erythritol 4-phosphate cytidylyltransferase exhibits a distinct quaternary structure compared to bacterial homologues and a possible role in feedback regulation for cytidine monophosphate, *FEBS J*, **273**, 1065–1073.

Gambari, R., Feriotto, G., Rutigliano, C., Bianchi, N. and Mischiati, C. (2000) Biospecific interaction analysis (BIA) of low-molecular weight DNA-binding drugs, *J Pharmacol Exp Ther*, **294**, 370–377.

Garcia Castro, A., Thoraval, S., Garcia, L. J. and Ragan, M. A. (2005) Workflows in bioinformatics: Meta-analysis and prototype implementation of a workflow generator, *BMC Bioinformatics*, **6**, 87–87.

Garrow, A. G., Agnew, A. and Westhead, D. R. (2005) TMB-Hunt: an amino acid composition based method to screen proteomes for beta-barrel transmembrane proteins, *BMC Bioinformatics*, **6**, 56–56.

Gattiker, A., Gasteiger, E. and Bairoch, A. (2002) ScanProsite: a reference implementation of a PROSITE scanning tool, *Appl. Bioinformatics*, **1**, 107–108.

Geppert, A., Kradolfer, M. and Tombros, D., (1998). Market-based workflow management. In *TREC '98: Proceedings of the International IFIP/GI Working*

Conference on Trends in Distributed Systems for Electronic Commerce, pages 179–191, London, UK. Springer-Verlag.

Glaser, F., Morris, R. J., Najmanovich, R. J., Laskowski, R. A. and Thornton, J. M. (2006) A method for localizing ligand binding pockets in protein structures, *Proteins*, **62**, 479–488.

Goldstein, J. L. and Brown, M. S. (1990) Regulation of the mevalonate pathway, *Nature*, **343**, 425–430.

Gomi, M. and Mitaku, S. (1999) Sequence analysis for discrimination of signal peptides and signal anchor sequences, *Genome Informatics*, **10**, 340–341.

Goñi, F. M. (2002) Non-permanent proteins in membranes: when proteins come as visitors (Review), *Mol Membr Biol*, **19**, 237–245.

Gough, J., (2002). Hidden markov models. `http://www.cs.bris.ac.uk/~gough/book/`.

Gräwert, T., Kaiser, J., Zepeck, F., Laupitz, R., Hecht, S., Amslinger, S., Schramek, N., Schleicher, E., Weber, S., Haslbeck, M., Buchner, J., Rieder, C., Arigoni, D., Bacher, A., Eisenreich, W. and Rohdich, F. (2004) IspH protein of Escherichia coli: Studies on iron-sulfur cluster implementation and catalysis, *J Am Chem Soc*, **126**, 12847–12855.

Gray, M. W., Burger, G. and Lang, B. F. (1999) Mitochondrial evolution, *Science*, **283**, 1476–1481.

Griffiths, E. and Gupta, R. S. (2006) Molecular signatures in protein sequences that are characteristics of the phylum Aquificae, *Int J Syst Evol Microbiol*, **56**, 99–9107.

Gromiha, M. M., Ahmad, S. and Suwa, M. (2004) Neural network-based prediction of transmembrane beta-strand segments in outer membrane proteins, *J Comput Chem*, **25**, 762–767.

Gudermann, T., Nürnberg, B. and Schultz, G. (1995) Receptors and G proteins as primary components of transmembrane signal transduction. Part 1. G-protein-coupled receptors: Structure and function, *J Mol Med*, **73**, 51–63.

Guimarães, K. S., Melo, J. C. B. and Cavalcanti, G. D. C., (2003). Combining few neural networks for effective secondary structure prediction. In *BIBE '03: Proceedings of the 3rd IEEE Symposium on BioInformatics and BioEngineering*, page 415, Washington, DC, USA. IEEE Computer Society.

Guiney, D. G., Hasegawa, P. and Davis, C. E. (1984) Plasmid transfer from Escherichia coli to Bacteroides fragilis: Differential expression of antibiotic resistance phenotypes, *Proc Natl Acad Sci U S A*, **81**, 7203–7206.

Guo, J.-t., Ellrott, K., Chung, W. J., Xu, D., Passovets, S. and Xu, Y. (2004) PROSPECT-PSPP: an automatic computational pipeline for protein structure prediction, *Nucleic Acids Res*, **32**, W522–W525.

Hackman, A. S. and Wilkins, T. D. (1975) Comparison of cefoxitin and cephalothin therapy of a mixed Bacteroides fragilis and Fusobacterius necrophorum infection in mice, *Antimicrob Agents Chemother*, **8**, 224–225.

Hahn, F. M., Hurlburt, A. P. and Poulter, C. D. (1999) Escherichia coli open reading frame 696 is idi, a nonessential gene encoding isopentenyl diphosphate isomerase, *J Bacteriol*, **181**, 4499–4504.

Hahn, F. M., Xuan, J. W., Chambers, A. F. and Poulter, C. D. (1996) Human isopentenyl diphosphate: Dimethylallyl diphosphate isomerase: Overproduction, purification, and characterization, *Arch Biochem Biophys*, **332**, 30–34.

Hajduk, P. J., Huth, J. R. and Fesik, S. W. (2005a) Druggability indices for protein targets derived from NMR-based screening data, *J Med Chem*, **48**, 2518–2525.

Hajduk, P. J., Huth, J. R. and Tse, C. (2005b) Predicting protein druggability, *Drug Discov Today*, **10**, 1675–1682.

Hall, L. O., Chawla, N., Bowyer, K. W. and Kegelmeyer, W. P., (1999). Learning rules from distributed data. In *Large-Scale Parallel Data Mining*, pages 211–220.

Hamscher, V., Schwiegelshohn, U., Streit, A. and Yahyapour, R., (2000). Evaluation of job-scheduling strategies for grid computing. In *GRID '00: Proceedings of the First IEEE/ACM International Workshop on Grid Computing*, pages 191–202, London, UK. Springer-Verlag.

Harb, O. S., Chatterjee, B., Fraunholz, M. J., Crawford, M. J., Nishi, M. and Roos, D. S. (2004) Multiple functionally redundant signals mediate targeting to the apicoplast in the apicomplexan parasite Toxoplasma gondii, *Eukaryot Cell*, **3**, 663–674.

He, C. Y., Striepen, B., Pletcher, C. H., Murray, J. M. and Roos, D. S. (2001) Targeting and processing of nuclear-encoded apicoplast proteins in plastid segregation mutants of Toxoplasma gondii, *J Biol Chem*, **276**, 28436–28442.

Hearst, M. A., Scholkapf, B., Dumais, S., Osuna, E. and Platt, J. (1998) Trends and Constroversies - Support Vector Machines, *IEEEIS*, **13**, 18–28.

Henriksson, L. M., Unge, T., Carlsson, J., Aqvist, J., Mowbray, S. L. and Jones, T. A. (2007) Structures of Mycobacterium tuberculosis 1-deoxy-D-xylulose-5-phosphate reductoisomerase provide new insights into catalysis, *J Biol Chem*, **282**, 19905–19916.

High, S., Flint, N. and Dobberstein, B. (1991) Requirements for the membrane insertion of signal-anchor type proteins, *J Cell Biol*, **113**, 25–34.

Hirokawa, T., Boon-Chieng, S. and Mitaku, S. (1998) SOSUI: classification and secondary structure prediction system for membrane proteins, *Bioinformatics*, **14**, 378–379.

Hofmann, K. and Stoffel, W. (1993) TMbase a database of membrane spanning proteins segments, *Biol Chem Hoppe-Seyler*, **347**, 166.

Hollingsworth, D., (1995). The workflow reference model. Handbook, The Workflow Management Coalition.

Hoon, S., Ratnapu, K. K., Chia, J.-M., Kumarasamy, B., Juguang, X., Clamp, M., Stabenau, A., Potter, S., Clarke, L. and Stupka, E. (2003) Biopipe: a flexible framework for protocol-based bioinformatics analysis, *Genome Res*, **13**, 1904–1915.

Hopkins, A. L. and Groom, C. R. (2002) The druggable genome, *Nat Rev Drug Discov*, **1**, 727–730.

Hopkins, A. L., Mason, J. S. and Overington, J. P. (2006) Can we rationally design promiscuous drugs?, *Curr Opin Struct Biol*, **16**, 127–136.

Hsieh, M.-H. and Goodman, H. M. (2006) Functional evidence for the involvement of Arabidopsis IspF homolog in the nonmevalonate pathway of plastid isoprenoid biosynthesis, *Planta*, **223**, 779–784.

Hubbard, T., Barker, D., Birney, E., Cameron, G., Chen, Y., Clark, L., Cox, T., Cuff, J., Curwen, V., Down, T., Durbin, R., Eyras, E., Gilbert, J., Hammond, M., Huminiecki, L., Kasprzyk, A., Lehvaslaiho, H., Lijnzaad, P., Melsopp, C., Mongin, E., Pettett, R., Pocock, M., Potter, S., Rust, A., Schmidt, E., Searle, S., Slater, G., Smith, J., Spooner, W., Stabenau, A., Stalker, J., Stupka, E., Ureta-Vidal, A., Vastrik, I. and Clamp, M. (2002) The Ensembl genome database project, *Nuc. Ac. Res.*, **30**, 38–41.

Hulo, N., Sigrist, C. J. A., Le Saux, V., Langendijk-Genevaux, P. S., Bordoli, L., Gattiker, A., De Castro, E., Bucher, P. and Bairoch, A. (2004) Recent improvements to the PROSITE database, *Nuc. Ac. Res.*, **32**, D134–D137.

Hunter, W. N. (2007) The Non-mevalonate Pathway of Isoprenoid Precursor Biosynthesis, *J Biol Chem*, **282**, 21573–21577.

Hwang, S. and Kesselman, C., (2003). Grid workflow: A flexible failure handling framework for the grid. In *twelfth IEEE International Symposium on High Performance Distributed Computing (HPDC-12)*, pages 126–137, Los Alamitos, CA, USA. IEEE Computer Society Press.

Ikeda, M., Arai, M., Lao, D. M. and Shimizu, T. (2002) Transmembrane topology prediction methods: a re-assessment and improvement by a consensus method using a dataset of experimentally-characterized transmembrane topologies, *In Silico Biol*, **2**, 19–33.

Ikeda, M., Arai, M., Okuno, T. and Shimizu, T. (2003) TMPDB: a database of experimentally-characterized transmembrane topologies, *Nucleic Acids Res*, **31**, 406–409.

Ikeda, M., Arai, M. and Shimizu, T. (2000) Evaluation of transmembrane topology prediction methods by using an experimentally characterized topology dataset, *Genome Informatics*, **11**, 420–427.

Jacoboni, I., Martelli, P. L., Fariselli, P., De Pinto, V. and Casadio, R. (2001) Prediction of the transmembrane regions of beta-barrel membrane proteins with a neural network-based predictor, *Protein Sci*, **10**, 779–787.

Jayasinghe, S., Hristova, K. and White, S. H. (2001a) Energetics, stability, and prediction of transmembrane helices, *J Mol Biol*, **312**, 927–934.

Jayasinghe, S., Hristova, K. and White, S. H. (2001b) MPtopo: A database of membrane protein topology, *Protein Sci*, **10**, 455–458.

Jenkins, M. C. (2001) Advances and prospects for subunit vaccines against protozoa of veterinary importance, *Vet Parasitol*, **101**, 291–310.

Johnson, J. E. and Cornell, R. B. (1999) Amphitropic proteins: Regulation by reversible membrane interactions (review), *Mol Membr Biol*, **16**, 217–235.

Jones, D. T. (1998) Do transmembrane protein superfolds exist?, *FEBS Lett*, **423**, 281–285.

Jones, D. T., Taylor, W. R. and Thornton, J. M. (1994) A model recognition approach to the prediction of all-helical membrane protein structure and topology, *Biochemistry*, **33**, 3038–3049.

Jones, D. T. (2007) Improving the accuracy of transmembrane protein topology prediction using evolutionary information, *Bioinformatics*, **23**, 538–544.

Jones, E. and Mongin-Bulewski, C. (2002) Drug discovery technology 2002. Start-up showcase and structure-based drug design, *IDrugs*, **5**, 894–895.

Jones, S. M., Urch, J. E., Kaiser, M., Brun, R., Harwood, J. L., Berry, C. and Gilbert, I. H. (2005) Analogues of thiolactomycin as potential antimalarial agents, *J Med Chem*, **48**, 5932–5941.

Julenius, K., Mølgaard, A., Gupta, R. and Brunak, S. (2004) Prediction, conservation analysis and structural characterization of mammalian mucin-type O-glycosylation sites, *Glycobiology*.

Junier, T. and Bucher, P. (1998) SEView: a Java applet for browsing molecular sequence data, *In Silico Biol*, **1**, 13–20.

Juretić, D., Lee, B., Trinajstić, N. and Williams, R. W. (1993) Conformational preference functions for predicting helices in membrane proteins, *Biopolymers*, **33**, 255–273.

Juretić, D., Zoranić, L. and Zucić, D. (2002) Basic charge clusters and predictions of membrane protein topology, *J Chem Inf Comput Sci*, **42**, 620–632.

Käll, L., Krogh, A. and Sonnhammer, E. L. L. (2004) A combined transmembrane topology and signal peptide prediction method, *J Mol Biol*, **338**, 1027–1036.

Käll, L., Krogh, A. and Sonnhammer, E. L. L. (2005) An HMM posterior decoder for sequence feature prediction that includes homology information, *Bioinformatics*, **21 Suppl 1**, i251–i257.

Kaneda, K., Kuzuyama, T., Takagi, M., Hayakawa, Y. and Seto, H. (2001) An unusual isopentenyl diphosphate isomerase found in the mevalonate pathway gene cluster from Streptomyces sp. strain CL190, *Proc Natl Acad Sci U S A*, **98**, 932–937.

Kanehisa, M., Goto, S., Kawashima, S. and Nakaya, A. (2002) The KEGG databases at GenomeNet, *Nucleic Acids Res*, **30**, 42–46.

Karkola, S., Höltje, H.-D. and Wähälä, K. (2007) A three-dimensional model of CYP19 aromatase for structure-based drug design, *J Steroid Biochem Mol Biol*, **105**, 63–70.

Kemp, L. E., Bond, C. S. and Hunter, W. N. (2002) Structure of 2C-methyl-D-erythritol 2,4- cyclodiphosphate synthase: an essential enzyme for isoprenoid biosynthesis and target for antimicrobial drug development, *Proc Natl Acad Sci U S A*, **99**, 6591–6596.

Kennard, o. (1993) Dna-drug interactions, *Pure & Appl. Chem.*, **65**, 1213–1222.

Kim, D., Xu, D., Guo, J.-t., Ellrott, K. and Xu, Y. (2003) PROSPECT II: protein structure prediction program for genome-scale applications, *Protein Eng*, **16**, 641–650.

King, R. D., Ouali, M., Strong, A. T., Aly, A., Elmaghraby, A., Kantardzic, M. and Page, D. (2000) Is it better to combine predictions?, *Protein Eng*, **13**, 15–19.

Kinoshita, T. (2007) [Application and development of structure-based drug design using X-ray analysis], *Nippon Yakurigaku Zasshi*, **129**, 186–190.

Kishida, H., Wada, T., Unzai, S., Kuzuyama, T., Takagi, M., Terada, T., Shirouzu, M., Yokoyama, S., Tame, J. R. H. and Park, S.-Y. (2003) Structure and catalytic mechanism of 2-C-methyl-D-erythritol 2,4-cyclodiphosphate (MECDP) synthase, an enzyme in the non-mevalonate pathway of isoprenoid synthesis, *Acta Crystallogr D Biol Crystallogr*, **59**, 23–31.

Klein, P., Kanehisa, M. and DeLisi, C. (1985) The detection and classification of membrane-spanning proteins, *Biochim Biophys Acta*, **815**, 468–476.

Klinck, R., Westhof, E., Walker, S., Afshar, M., Collier, A. and Aboul-Ela, F. (2000) A potential RNA drug target in the hepatitis C virus internal ribosomal entry site, *RNA*, **6**, 1423–1431.

Kobayashi, K., Suzuki, M., Tang, J., Nagata, N., Ohyama, K., Seki, H., Kiuchi, R., Kaneko, Y., Nakazawa, M., Matsui, M., Matsumoto, S., Yoshida, S. and Muranaka, T. (2007) Lovastatin insensitive 1, a Novel pentatricopeptide repeat protein, is a potential regulatory factor of isoprenoid biosynthesis in Arabidopsis, *Plant Cell Physiol*, **48**, 322–331.

Köhler, S., Delwiche, C. F., Denny, P. W., Tilney, L. G., Webster, P., Wilson, R. J., Palmer, J. D. and Roos, D. S. (1997) A plastid of probable green algal origin in Apicomplexan parasites, *Science*, **275**, 1485–1489.

Kollas, A.-K., Duin, E. C., Eberl, M., Altincicek, B., Hintz, M., Reichenberg, A., Henschker, D., Henne, A., Steinbrecher, I., Ostrovsky, D. N., Hedderich, R., Beck, E., Jomaa, H. and Wiesner, J. (2002) Functional characterization of GcpE, an essential enzyme of the non-mevalonate pathway of isoprenoid biosynthesis, *FEBS Lett*, **532**, 432–436.

Kroemer, R. T. (2007) Structure-based drug design: Docking and scoring, *Curr Protein Pept Sci*, **8**, 312–328.

Krogh, A., Larsson, B., von Heijne, G. and Sonnhammer, E. L. (2001) Predicting transmembrane protein topology with a Hidden Markov Model: Application to complete genomes, *J. Mol. Biol.*, **305**, 567–580.

Kurtzman, C. P. and Piskur, J., (2006). *Comparative Genomics: Using Fungi as Models*, volume 15 of *Topics in Current Genetics*, chapter Taxonomy and phylogenetic diversity among the yeasts, pages 29–46. Springer-Verlag, Berlin.

Kuzuyama, T., Takagi, M., Takahashi, S. and Seto, H. (2000) Cloning and characterization of 1-deoxy-D-xylulose 5-phosphate synthase from Streptomyces sp. Strain CL190, which uses both the mevalonate and nonmevalonate pathways for isopentenyl diphosphate biosynthesis, *J Bacteriol*, **182**, 891–897.

Kyte, J. and Doolittle, R. F. (1982) A simple method for displaying the hydropathic character of a protein, *J Mol Biol*, **157**, 105–132.

Labarga, A., Valentin, F., Anderson, M. and Lopez, R. (2007) Web services at the European bioinformatics institute, *Nucleic Acids Res*, **35**, W6–11.

Lacroix, Z., Legendre, C., Raschid, L. and Snyder, B. (2007) BIPASS: BioInformatics Pipeline Alternative Splicing Services, *Nucleic Acids Res.*

Land, K. M., Delgadillo, M. G. and Johnson, P. J. (2002) In vivo expression of ferredoxin in a drug resistant trichomonad increases metronidazole susceptibility, *Mol Biochem Parasitol*, **121**, 153–157.

Lange, B. M., Rujan, T., Martin, W. and Croteau, R. (2000) Isoprenoid biosynthesis: the evolution of two ancient and distinct pathways across genomes, *Proc Natl Acad Sci U S A*, **97**, 13172–13177.

Lao, D. M., Arai, M., Ikeda, M. and Shimizu, T. (2002a) The presence of signal peptide significantly affects transmembrane topology prediction, *Bioinformatics*, **18**, 1562–1566.

Lao, D. M., Okuno, T. and Shimizu, T. (2002b) Evaluating transmembrane topology prediction methods for the effect of signal peptide in topology prediction, *In Silico Biol*, **2**, 485–494.

Laskowski, R. A. (2001) PDBsum: summaries and analyses of PDB structures, *Nuc. Ac. Res.*, **29**, 221–222.

Laskowski, R. A., Luscombe, N. M., Swindells, M. B. and Thornton, J. M. (1996) Protein clefts in molecular recognition and function, *Protein Sci*, **5**, 2438–2452.

Laskowski, R. A., Watson, J. D. and Thornton, J. M. (2005) ProFunc: a server for predicting protein function from 3D structure, *Nucleic Acids Res*, **33**, W89–W93.

Laszewski, G. v., (2005). Java cog kit workflow concepts for scientific experiments. Technical report, Argonne National Laboratory, Argonne, IL, USA.

Le Pecq, J. B., Le Bret, M., Barbet, J. and Roques, B. (1975) DNA polyintercalating drugs: DNA binding of diacridine derivatives, *Proc Natl Acad Sci U S A*, **72**, 2915–2919.

Lell, B., Ruangweerayut, R., Wiesner, J., Missinou, M. A., Schindler, A., Baranek, T., Hintz, M., Hutchinson, D., Jomaa, H. and Kremsner, P. G. (2003)

Fosmidomycin, a novel chemotherapeutic agent for malaria, *Antimicrob Agents Chemother*, **47**, 735–738.

Letondal, C. (2001) A Web interface generator for molecular biology programs in Unix, *Bioinformatics*, **17**, 73–82.

Lewis, R. A. (1991) Clefts and binding sites in protein receptors, *Methods Enzymol*, **202**, 126–156.

Li, X.-h., Zou, H.-j., Wu, A.-h., Ye, Y.-l. and Shen, J.-h. (2007) Structure-based drug design of a novel family of chalcones as PPARalpha agonists: Virtual screening, synthesis, and biological activities in vitro, *Acta Pharmacol Sin*, **28**, 2040–2052.

Lian, C. C., Tang, F., Issac, P. and Krishnan, A. (2005) Gel: grid execution language, *J. Parallel Distrib. Comput.*, **65**, 857–869.

Lipinski, C. A., Lombardo, F., Dominy, B. W. and Feeney, P. J. (2001) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings, *Adv Drug Deliv Rev*, **46**, 3–26.

Liu, E. D., Yang, G. L., Tian, B. J., Li, Z. W. and Chen, Y. (2002) Application of resilient backpropagation neural network in predicting hydrophobic parameters of alkylbenzenes, *SP*, **20**, 216–218.

Lohmann, R., Schneider, G., Behrens, D. and Wrede, P. (1994) A neural network model for the prediction of membrane-spanning amino acid sequences, *Protein Sci*, **3**, 1597–1601.

Lomize, A. L., Pogozheva, I. D., Lomize, M. A. and Mosberg, H. I. (2007) The role

of hydrophobic interactions in positioning of peripheral proteins in membranes, *BMC Struct Biol*, **7**, 44–44.

Lu, J., Wu, W., Cao, S., Zhao, H., Zeng, H., Lin, L., Sun, X. and Tang, K. (2007) Molecular cloning and characterization of 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase gene from Ginkgo biloba, *Mol Biol Rep*.

Ludscher, B., Altintas, I., Berkley, C., Higgins, D., Jaeger, E., Jones, M., Lee, E., Tao, J. and Zhao, Y. (2005) Scientific workflow management and the kepler system, *Concurrency and Computation: Practice & Experience*, **36**.

Luft, B. J., Hafner, R., Korzun, A. H., Leport, C., Antoniskis, D., Bosler, E. M., Bourland, D. D., Uttamchandani, R., Fuhrer, J. and Jacobson, J. (1993) Toxoplasmic encephalitis in patients with the acquired immunodeficiency syndrome. Members of the ACTG 077p/ANRS 009 Study Team, *N Engl J Med*, **329**, 995–991000.

Mac Sweeney, A., Lange, R., Fernandes, R. P. M., Schulz, H., Dale, G. E., Douangamath, A., Proteau, P. J. and Oefner, C. (2005) The crystal structure of E.coli 1-deoxy-D-xylulose-5-phosphate reductoisomerase in a ternary complex with the antimalarial compound fosmidomycin and NADPH reveals a tight-binding closed enzyme conformation, *J Mol Biol*, **345**, 115–127.

Macchiarulo, A., Nobeli, I. and Thornton, J. M. (2004) Ligand selectivity and competition between enzymes in silico, *Nat Biotechnol*, **22**, 1039–1045.

Maggio, E. T. and Ramnarayan, K. (2001) Recent developments in computational proteomics, *Trends Biotechnol*, **19**, 266–272.

Maggio, E. T. (2002) Want novel drugs in a hurry? You had better do the math!, *Drug Discov Today*, **7**, 855–856.

Mahmoud, S. S. and Croteau, R. B. (2002) Strategies for transgenic manipulation of monoterpene biosynthesis in plants, *Trends Plant Sci*, **7**, 366–373.

Margulis, L., (1981). *Symbiosis in Cell Evolution*. W. H. Freeman and Company, San Francisco.

Margulis, L. and Bermudes, D. (1985) Symbiosis as a mechanism of evolution: Status of cell symbiosis theory, *Symbiosis*, **1**, 101–124.

Martelli, P. L., Fariselli, P., Krogh, A. and Casadio, R. (2002) A sequence-profile-based HMM for predicting and discriminating beta barrel membrane proteins, *Bioinformatics*, **18 Suppl 1**, S46–S53.

Martin, A. C. R. (2004) PDBSprotEC: a Web-accessible database linking PDB chains to EC numbers via SwissProt, *Bioinformatics*, **20**, 986–988.

Martin, A. C. R. (2005) Mapping PDB chains to UniProtKB entries, *Bioinformatics*, **21**, 4297–4301.

Massagué, J. and Pandiella, A. (1993) Membrane-anchored growth factors, *Annu Rev Biochem*, **62**, 515–541.

Matthews, B. W. (1975) Comparison of the predicted and observed secondary structure of T4 phage lysozyme, *Biochim Biophys Acta*, **405**, 442–451.

McFadden, G. (1999) Chloroplasts. Ever decreasing circles, *Nature*, **400**, 119–120.

McFadden, G. I. (2001) Primary and secondary endosymbiosis and the origin of plastids, *J Phycol*, **37**, 951–959.

Mcgough, S., Young, L., Afzal, A., Newhouse, S. and Darlington, J., (2004). Workflow enactment in iceni. In *In UK e-Science All Hands Meeting*, pages 894–900.

McGuffin, L. J., Bryson, K. and Jones, D. T. (2000) The PSIPRED protein structure prediction server, *Bioinformatics*, **16**, 404–405.

Mehlin, C. (2005) Structure-based drug discovery for Plasmodium falciparum, *Comb Chem High Throughput Screen*, **8**, 5–14.

Metzker, M. L. (2005) Emerging technologies in DNA sequencing, *Genome Res*, **15**, 1767–1776.

Meyer, O., Grosdemange-Billiard, C., Tritsch, D. and Rohmer, M. (2003) Isoprenoid biosynthesis via the MEP pathway. Synthesis of (3,4)-3,4-dihydroxy-5-oxohexylphosphonic acid, an isosteric analogue of 1-deoxy-D-xylulose 5-phosphate, the substrate of the 1-deoxy-D-xylulose 5-phosphate reductoisomerase, *Org Biomol Chem*, **1**, 4367–4372.

Miallau, L., Alphey, M. S., Kemp, L. E., Leonard, G. A., McSweeney, S. M., Hecht, S., Bacher, A., Eisenreich, W., Rohdich, F. and Hunter, W. N. (2003) Biosynthesis of isoprenoids: Crystal structure of 4-diphosphocytidyl-2C-methyl-D-erythritol kinase, *Proc Natl Acad Sci U S A*, **100**, 9173–9178.

Milburn, D., Laskowski, R. A. and Thornton, J. M. (1998) Sequences annotated by structure: a tool to facilitate the use of structural information in sequence analysis, *Protein Eng.*, **11**, 855–859.

Missinou, M. A., Borrmann, S., Schindler, A., Issifou, S., Adegnika, A. A., Mat-

siegui, P.-B., Binder, R., Lell, B., Wiesner, J., Baranek, T., Jomaa, H. and Kremsner, P. G. (2002) Fosmidomycin for malaria, *Lancet*, **360**, 1941–1942.

Mitts, M. R., Grant, D. B. and Heideman, W. (1990) Adenylate cyclase in Saccharomyces cerevisiae is a peripheral membrane protein, *Mol Cell Biol*, **10**, 3873–3883.

Möller, S., Croning, M. D. and Apweiler, R. (2001) Evaluation of methods for the prediction of membrane spanning regions, *Bioinformatics*, **17**, 646–653.

Möller, S., Kriventseva, E. V. and Apweiler, R. (2000) A collection of well characterised integral membrane proteins, *Bioinformatics*, **16**, 1159–1160.

Moreira, D., Le Guyader, H. and Philippe, H. (2000) The origin of red algae and the evolution of chloroplasts, *Nature*, **405**, 69–72.

Na-Bangchang, K., Ruengweerayut, R., Karbwang, J., Chauemung, A. and Hutchinson, D. (2007) Pharmacokinetics and pharmacodynamics of fosmidomycin monotherapy and combination therapy with clindamycin in the treatment of multidrug resistant falciparum malaria, *Malar J*, **6**, 70–70.

Nair, R. and Rost, B. (2004) LOCnet and LOCtarget: sub-cellular localization for structural genomics targets, *Nuc. Ac. Res.*, **32**, W517–W521.

Nakai, K. and Horton, P. (1999) PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization, *Trends Biochem Sci*, **24**, 34–36.

Nakai, K. and Kanehisa, M. (1992) A knowledge base for predicting protein localization sites in eukaryotic cells, *Genomics*, **14**, 897–911.

Nayal, M., Hitz, B. C. and Honig, B. (1999) GRASS: a server for the graphical representation and analysis of structures, *Protein Sci.*, **8**, 676–679.

Neshich, G., Togawa, R. C., Mancini, A. L., Kuser, P. R., Yamagishi, M. E. B., Pappas, G., Torres, W. V., Fonseca e Campos, T., Ferreira, L. L., Luna, F. M., Oliveira, A. G., Miura, R. T., Inoue, M. K., Horita, L. G., de Souza, D. F., Dominiquini, F., Alvaro, A., Lima, C. S., Ogawa, F. O., Gomes, G. B., Palandrani, J. F., dos Santos, G. F., de Freitas, E. M., Mattiuz, A. R., Costa, I. C., de Almeida, C. L., Souza, S., Baudet, C. and Higa, R. H. (2003) STING Millennium: A web-based suite of programs for comprehensive and simultaneous analysis of protein structure and sequence, *Nuc. Ac. Res.*, **31**, 3386–3392.

Ni, S., Robinson, H., Marsing, G. C., Bussiere, D. E. and Kennedy, M. A. (2004) Structure of 2C-methyl-D-erythritol-2,4-cyclodiphosphate synthase from Shewanella oneidensis at 1.6 A: identification of farnesyl pyrophosphate trapped in a hydrophobic cavity, *Acta Crystallogr D Biol Crystallogr*, **60**, 1949–1957.

Nicola, G., Smith, C. A., Lucumi, E., Kuo, M. R., Karagyozov, L., Fidock, D. A., Sacchettini, J. C. and Abagyan, R. (2007) Discovery of novel inhibitors targeting enoyl-acyl carrier protein reductase in Plasmodium falciparum by structure-based virtual screening, *Biochem Biophys Res Commun*, **358**, 686–691.

Niedermayer, D., (1998). An introduction to bayesian networks and their contemporary applications. `http://www.niedermayer.ca/papers/bayesian/`.

Nielsen, H., Engelbrecht, J., Brunak, S. and von Heijne, G. (1997) Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites, *Protein Eng*, **10**, 1–6.

Nilsson, J., Persson, B. and von Heijne, G. (2000) Consensus predictions of membrane protein topology, *FEBS Lett*, **486**, 267–269.

Oinn, T. M. (2003) Talisman–rapid application development for the grid, *Bioinformatics*, **19 Suppl 1**, i212–i214.

Oinn, T., Addis, M., Ferris, J., Marvin, D., Senger, M., Greenwood, M., Carver, T., Glover, K., Pocock, M. R., Wipat, A. and Li, P. (2004) Taverna: a tool for the composition and enactment of bioinformatics workflows, *Bioinformatics*, **20**, 3045–3054.

Okuhara, M., Kuroda, Y., Goto, T., Okamoto, M., Terano, H., Kohsaka, M., Aoki, H. and Imanaka, H. (1980) Studies on new phosphonic acid antibiotics. III. Isolation and characterization of FR-31564, FR-32863 and FR-33289, *J Antibiot (Tokyo)*, **33**, 24–28.

Oliveira, L., Hulsen, T., Lutje Hulsik, D., Paiva, A. C. M. and Vriend, G. (2004) Heavier-than-air flying machines are impossible, *FEBS Lett*, **564**, 269–273.

Oppenheim, J. J., Biragyn, A., Kwak, L. W. and Yang, D. (2003) Roles of antimicrobial peptides such as defensins in innate and adaptive immunity, *Ann Rheum Dis*, **62 Suppl 2**, ii17–ii21.

Oudjama, Y., Durbecq, V., Sainz, G., Clantin, B., Tricot, C., Stalon, V., Villeret, V. and Droogmans, L. (2001) Preliminary structural studies of Escherichia coli isopentenyl diphosphate isomerase, *Acta Crystallogr D Biol Crystallogr*, **57**, 287–288.

Overington, J. P., Al-Lazikani, B. and Hopkins, A. L. (2006) How many drug targets are there?, *Nat Rev Drug Discov*, **5**, 993–996.

Owens, J. (2007) Target validation: Determining druggability, *Nat Rev Drug Discovery*, **6**, 187.

Palmer, J. D. (2003) The symbiotic birth and spread of plastids: How many times and whodunit?, *J Phycol*, **39**, 4–12.

Pasquier, C. and Hamodrakas, S. J. (1999) An hierarchical artificial neural network system for the classification of transmembrane proteins, *Protein Eng*, **12**, 631–634.

Pasquier, C., Promponas, V. J., Palaios, G. A., Hamodrakas, J. S. and Hamodrakas, S. J. (1999) A novel method for predicting transmembrane segments in proteins based on a statistical analysis of the SwissProt database: the PRED-TMR algorithm, *Protein Eng*, **12**, 381–385.

Pearl, J., (August 1985). Bayesian networks: A model of self-activated memory for evidential reasoning. In *Proceedings of the 7th Conference of the Cognitive Science Society, University of California, Irvine*, pages 329–334.

Pearl, J., (1988). *Probabilistic Reasoning in Intelligent Systems: Networks of Plausible Inference*. Morgan Kaufmann Publishers Inc., San Francisco, CA, USA.

Pearl, J., (March 2000). *Causality : Models, Reasoning, and Inference*. Cambridge University Press.

Pearson, W. R. (1990) Rapid and sensitive sequence comparison with FASTP and FASTA, *Methods Enzymol*, **183**, 63–98.

Pearson, W. R. and Lipman, D. J. (1988) Improved tools for biological sequence comparison, *Proc Natl Acad Sci U S A*, **85**, 2444–2448.

Persson, B. and Argos, P. (1997) Prediction of membrane protein topology utilizing multiple sequence alignments, *J Protein Chem*, **16**, 453–457.

Peters, M. B., Raha, K. and Merz, K. M. (2006) Quantum mechanics in structure-based drug design, *Curr Opin Drug Discov Devel*, **9**, 370–379.

Peyrone, M. (1845) Ueber die einwirkung des ammoniaks auf platinchlorür, *Ann Chemi Pharm*, **51**, 1–29.

Picot, D., Loll, P. J. and Garavito, R. M. (1994) The X-ray crystal structure of the membrane protein prostaglandin H2 synthase-1, *Nature*, **367**, 243–249.

Pruess, M., Kersey, P. and Apweiler, R. (2005) The Integr8 project–a resource for genomic and proteomic data, *In Silico Biol*, **5**, 179–185.

Qian, N. and Sejnowski, T. J. (1988) Predicting the secondary structure of globular proteins using neural network models, *J Mol Biol*, **202**, 865–884.

Quintrell, N., Lebo, R., Varmus, H., Bishop, J. M., Pettenati, M. J., Le Beau, M. M., Diaz, M. O. and Rowley, J. D. (1987) Identification of a human gene (HCK) that encodes a protein-tyrosine kinase and is expressed in hemopoietic cells, *Mol Cell Biol*, **7**, 2267–2275.

Quon, D. V., d'Oliveira, C. E. and Johnson, P. J. (1992) Reduced transcription of the ferredoxin gene in metronidazole-resistant Trichomonas vaginalis, *Proc Natl Acad Sci U S A*, **89**, 4402–4406.

Rabiner, L. R., (1990). *Readings in speech recognition*, chapter A tutorial on hidden Markov models and selected applications in speech recognition, pages 267–296. Morgan Kaufmann Publishers Inc., San Francisco, CA, USA.

Raha, K., Peters, M. B., Wang, B., Yu, N., Wollacott, A. M., Westerhoff, L. M. and Merz, K. M. (2007) The role of quantum mechanics in structure-based drug design, *Drug Discov Today*, **12**, 725–731.

Rajaraman, P., Schwartz, B. S., Rothman, N., Yeager, M., Fine, H. A., Shapiro, W. R., Selker, R. G., Black, P. M. and Inskip, P. D. (2005) Delta-aminolevulinic acid dehydratase polymorphism and risk of brain tumors in adults, *Environ Health Perspect*, **113**, 1209–1211.

Ralph, S. A., D'Ombrain, M. C. and McFadden, G. I. (2001) The apicoplast as an antimalarial drug target, *Drug Resist Updat*, **4**, 145–151.

Ralph, S. A., Foth, B. J., Hall, N. and McFadden, G. I. (2004a) Evolutionary pressures on apicoplast transit peptides, *Mol Biol Evol*, **21**, 2183–2194.

Ralph, S. A., van Dooren, G. G., Waller, R. F., Crawford, M. J., Fraunholz, M. J., Foth, B. J., Tonkin, C. J., Roos, D. S. and McFadden, G. I. (2004b) Tropical infectious diseases: Metabolic maps and functions of the Plasmodium falciparum apicoplast, *Nat Rev Microbiol*, **2**, 203–216.

Ricagno, S., Grolle, S., Bringer-Meyer, S., Sahm, H., Lindqvist, Y. and Schneider, G. (2004) Crystal structure of 1-deoxy-d-xylulose-5-phosphate reductoisomerase from Zymomonas mobilis at 1.9-A resolution, *Biochim Biophys Acta*, **1698**, 37–44.

Rice, P., (2007). Grid and e-science r&d. Embl-ebi annual scientific report 2007, EMBL-EBI.

Richard, S. B., Bowman, M. E., Kwiatkowski, W., Kang, I., Chow, C., Lillo, A. M., Cane, D. E. and Noel, J. P. (2001) Structure of 4-diphosphocytidyl-

2-C- methylerythritol synthetase involved in mevalonate- independent isoprenoid biosynthesis, *Nat Struct Biol*, **8**, 641–648.

Richard, S. B., Ferrer, J.-L., Bowman, M. E., Lillo, A. M., Tetzlaff, C. N., Cane, D. E. and Noel, J. P. (2002) Structure and mechanism of 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase. An enzyme in the mevalonate-independent isoprenoid biosynthetic pathway, *J Biol Chem*, **277**, 8667–8672.

Riedmiller, M. and Braun, H., (1992). Rprop- a fast adaptive learning algorithm. Technical Report (Also Proc. of ISCIS VII), Universitat Karlsruhe.

Riedmiller, M. and Braun, H., (1993). A direct adaptive method for faster back-propagation learning: the Rprop algorithm. In *Proceedings of the International Conference on Neural Networks*, pages 586–591.

Rindler, M. J. (1998) Carboxypeptidase E, a peripheral membrane protein implicated in the targeting of hormones to secretory granules, co-aggregates with granule content proteins at acidic pH, *J Biol Chem*, **273**, 31180–31185.

Rochet, H. and Martin-Eauclaire, M.-F. (eds.), (2000). *Animal Toxins: Facts and Protocols.* Birkhäuser, Basel, Switzerland.

Rodríguez-Concepción, M. and Boronat, A. (2002) Elucidation of the methylerythritol phosphate pathway for isoprenoid biosynthesis in bacteria and plastids. A metabolic milestone achieved through genomics, *Plant Physiol*, **130**, 1079–1089.

Rohdich, F., Eisenreich, W., Wungsintaweekul, J., Hecht, S., Schuhr, C. A. and Bacher, A. (2001) Biosynthesis of terpenoids. 2C-Methyl-D-erythritol

2,4-cyclodiphosphate synthase (IspF) from Plasmodium falciparum, *Eur J Biochem*, **268**, 3190–3197.

Rohdich, F., Bacher, A. and Eisenreich, W. (2004) Perspectives in anti-infective drug design. The late steps in the biosynthesis of the universal terpenoid precursors, isopentenyl diphosphate and dimethylallyl diphosphate, *Bioorg Chem*, **32**, 292–308.

Rohmer, M. (1999) The discovery of a mevalonate-independent pathway for isoprenoid biosynthesis in bacteria, algae and higher plants, *Nat Prod Rep*, **16**, 565–574.

Roizen, N., Swisher, C. N., Stein, M. A., Hopkins, J., Boyer, K. M., Holfels, E., Mets, M. B., Stein, L., Patel, D. and Meier, P. (1995) Neurologic and developmental outcome in treated congenital toxoplasmosis, *Pediatrics*, **95**, 11–20.

Rosenberg, B., Vancamp, L. and Krigas, T. (1965) Inhibition of cell division in Escherichia Coli by electrolysis products from a platinum electrode, *Nature*, **205**, 698–699.

Rost, B. (1996) PHD: predicting one-dimensional protein structure by profile-based neural networks, *Meth. Enzymol.*, **266**, 525–539.

Rost, B., Yachdav, G. and Liu, J. (2004) The PredictProtein server, *Nucleic Acids Res*, **32**, W321–W326.

Rumelhart, D. E. and McClelland, J. L. (1986) On learning the past tenses of english verbs, *Parallel distributed processing: explorations in the microstructure of cognition, vol. 2: psychological and biological models*, pages 216–271.

Russell, S. J. and Norvig, P., (2003). *Artificial Intelligence: A Modern Approach*. Pearson Education.

Russell, S. J., Norvig, P., Candy, J. F., Malik, J. M. and Edwards, D. D., (1996). *Artificial intelligence: a modern approach*. Prentice-Hall, Inc., Upper Saddle River, NJ, USA.

Ryan, C. A., Pearce, G., Scheer, J. and Moura, D. S. (2002) Polypeptide hormones, *Plant Cell*, **14 Suppl**, S251–S264.

Sacchettini, J. C. and Poulter, C. D. (1997) Creating isoprenoid diversity, *Science*, **277**, 1788–1789.

Samuelsen, O., Haukland, H. H., Jenssen, H., Krämer, M., Sandvik, K., Ulvatne, H. and Vorland, L. H. (2005) Induced resistance to the antimicrobial peptide lactoferricin B in Staphylococcus aureus, *FEBS Lett*, **579**, 3421–3426.

Sanger, F., Nicklen, S. and Coulson, A. R. (1977) DNA sequencing with chain-terminating inhibitors, *Proc Natl Acad Sci U S A*, **74**, 5463–5467.

Saphire, E. O. (2002) Structure-based drug design, *IDrugs*, **5**, 658–661.

Sauret-Güeto, S., Urós, E. M., Ibáñez, E., Boronat, A. and Rodríguez-Concepción, M. (2006) A mutant pyruvate dehydrogenase E1 subunit allows survival of Escherichia coli strains defective in 1-deoxy-D-xylulose 5-phosphate synthase, *FEBS Lett*, **580**, 736–740.

Schiffmann, W., Joost, M. and Werner, R., (1993). Comparison of optimized backpropagation algorithms. In *Proc. ESANN 93*.

Schmitt, C. K., Meysick, K. C. and O'Brien, A. D. (1999) Bacterial toxins: Friends or foes?, *Emerg Infect Dis*, **5**, 224–234.

Schultz, J., Milpetz, F., Bork, P. and Ponting, C. P. (1998) SMART, a simple modular architecture research tool: Identification of signaling domains, *Proc. Natl. Acad. Sci. USA*, **95**, 5857–5864.

Scinicariello, F., Murray, H. E., Moffett, D. B., Abadin, H. G., Sexton, M. J. and Fowler, B. A. (2007) Lead and delta-aminolevulinic acid dehydratase polymorphism: Where does it lead? A meta-analysis, *Environ Health Perspect*, **115**, 35–41.

Scordis, P., Flower, D. R. and Attwood, T. K. (1999) FingerPRINTScan: intelligent searching of the PRINTS motif database, *Bioinformatics*, **15**, 799–806.

Seemann, M., Bui, B. T. S., Wolff, M., Tritsch, D., Campos, N., Boronat, A., Marquet, A. and Rohmer, M. (2002) Isoprenoid biosynthesis through the methylerythritol phosphate pathway: the (E)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase (GcpE) is a [4Fe-4S] protein, *Angew Chem Int Ed Engl*, **41**, 4337–4339.

Seemann, M., Tse Sum Bui, B., Wolff, M., Miginiac-Maslow, M. and Rohmer, M. (2006) Isoprenoid biosynthesis in plant chloroplasts via the MEP pathway: Direct thylakoid/ferredoxin-dependent photoreduction of GcpE/IspG, *FEBS Lett*, **580**, 1547–1552.

Seemann, M., Wegner, P., Schünemann, V., Bui, B. T. S., Wolff, M., Marquet, A., Trautwein, A. X. and Rohmer, M. (2005) Isoprenoid biosynthesis in chloroplasts via the methylerythritol phosphate pathway: the (E)-4-hydroxy-3-methylbut-

2-enyl diphosphate synthase (GcpE) from Arabidopsis thaliana is a [4Fe-4S] protein, *J Biol Inorg Chem*, **10**, 131–137.

Senger, M., Rice, P. and Oinn, T., (2003). SoapLab — a unified sesame door to analysis tools. In *Proceedings of the UK e-Science All Hands Meeting 2003*.

Sethi, V. S. (1971) Structure and function of DNA-dependent RNA-polymerase, *Prog Biophys Mol Biol*, **23**, 67–6101.

Sgraja, T., Kemp, L. E., Ramsden, N. and Hunter, W. N. (2005) A double mutation of Escherichia coli2C-methyl-D-erythritol-2,4-cyclodiphosphate synthase disrupts six hydrogen bonds with, yet fails to prevent binding of, an isoprenoid diphosphate, *Acta Crystallograph Sect F Struct Biol Cryst Commun*, **61**, 625–629.

Shah, S. P., He, D. Y. M., Sawkins, J. N., Druce, J. C., Quon, G., Lett, D., Zheng, G. X. Y., Xu, T. and Ouellette, B. F. F. (2004) Pegasys: software for executing and integrating analyses of biological sequences, *BMC Bioinformatics*, **5**, 40–40.

Shaikh, S. A., Ahmed, S. R. and Jayaram, B. (2004) A molecular thermodynamic view of DNA-drug interactions: a case study of 25 minor-groove binders, *Arch Biochem Biophys*, **429**, 81–99.

Shigi, Y. (1989) Inhibition of bacterial isoprenoid synthesis by fosmidomycin, a phosphonic acid-containing antibiotic, *J Antimicrob Chemother*, **24**, 131–145.

Siepel, A., Farmer, A., Tolopko, A., Zhuang, M., Mendes, P., Beavis, W. and Sobral, B. (2001) ISYS: a decentralized, component-based approach to the

integration of heterogeneous bioinformatics resources, *Bioinformatics*, **17**, 83–94.

Singh, N., Chevé, G., Avery, M. A. and McCurdy, C. R. (2006a) Comparative protein modeling of 1-deoxy-D-xylulose-5-phosphate reductoisomerase enzyme from Plasmodium falciparum: a potential target for antimalarial drug discovery, *J Chem Inf Model*, **46**, 1360–1370.

Singh, S., Malik, B. K. and Sharma, D. K. (2006b) Molecular drug targets and structure based drug design: A holistic approach, *Bioinformation*, **1**, 314–320.

Sirois, S., Hatzakis, G., Wei, D., Du, Q. and Chou, K.-C. (2005) Assessment of chemical libraries for their druggability, *Comput Biol Chem*, **29**, 55–67.

Small, I., Peeters, N., Legeai, F. and Lurin, C. (2004) Predotar: A tool for rapidly screening proteomes for N-terminal targeting sequences, *Proteomics*, **4**, 1581–1590.

Song, S. S., Kwok, Y. K. and Hwang, K., (2005). Trusted job scheduling in open computational grids: Security-driven heuristics and a fast genetic algorithm. In *19th IEEE International Parallel & Distributed Processing Symposium (IPDPS'05)*, Los Alamitos, CA, USA. IEEE Computer Society Press.

Spinosa, E. J. and de Carvalho, A. C. P. L. F. (2005) Support vector machines for novel class detection in Bioinformatics, *GMR*, **4**, 608–615.

Spooner, D. P., Cao2, J., Jarvis, S. A., He, L. and Nudd, G. R. (2004) Performance-aware workflow management for grid computing, *The Computer Journal*, **48**, 347–357.

Spurgeon, S. L. and Porter, J. W., (1981). *in Biosynthesis of Isoprenoid Compounds*. John Wiley, New York.

Steinbacher, S., Kaiser, J., Eisenreich, W., Huber, R., Bacher, A. and Rohdich, F. (2003a) Structural basis of fosmidomycin action revealed by the complex with 2-C-methyl-D-erythritol 4-phosphate synthase (IspC). Implications for the catalytic mechanism and anti-malaria drug development, *J Biol Chem*, **278**, 18401–18407.

Steinbacher, S., Kaiser, J., Gerhardt, S., Eisenreich, W., Huber, R., Bacher, A. and Rohdich, F. (2003b) Crystal structure of the type II isopentenyl diphosphate:dimethylallyl diphosphate isomerase from Bacillus subtilis, *J Mol Biol*, **329**, 973–982.

Steinbacher, S., Kaiser, J., Wungsintaweekul, J., Hecht, S., Eisenreich, W., Gerhardt, S., Bacher, A. and Rohdich, F. (2002) Structure of 2C-methyl-d-erythritol-2,4-cyclodiphosphate synthase involved in mevalonate-independent biosynthesis of isoprenoids, *J Mol Biol*, **316**, 79–88.

Sugiyama, Y. (2005) Druggability: selecting optimized drug candidates, *Drug Discov Today*, **10**, 1577–1579.

Summons, R. (1999) Molecular probing of deep secrets, *Nature*, **398**, 752–753.

Surolia, N. and Surolia, A. (2001) Triclosan offers protection against blood stages of malaria by inhibiting enoyl-ACP reductase of Plasmodium falciparum, *Nat Med*, **7**, 167–173.

Swindells, M. and Fagan, R. (2001) Target discovery using bioinformatics, *Chemical Innovation*, **31**, 24–28.

Swindells, M. B. and Overington, J. P. (2002) Prioritizing the proteome: Identifying pharmaceutically relevant targets, *Drug Discov Today*, **7**, 516–521.

Tang, F., Chua, C. L., Ho, L.-Y., Lim, Y. P., Issac, P. and Krishnan, A. (2005) Wildfire: distributed, Grid-enabled workflow construction and execution, *BMC Bioinformatics*, **6**, 69–69.

Tannenbaum, T., Wright, D., Miller, K. and Livny, M., (2002). *Condor: a distributed job scheduler*. MIT Press, Cambridge, MA, USA.

Taylor, I., Shields, M. and Wang, I., (2004). *Resource management for the Triana peer-to-peer services*. Kluwer Academic Publishers, Norwell, MA, USA.

Taylor, P. D., Attwood, T. K. and Flower, D. R. (2003) BPROMPT: A consensus server for membrane protein prediction, *Nucleic Acids Res*, **31**, 3698–3700.

Terstappen, G. C. and Reggiani, A. (2001) In silico research in drug discovery, *Trends Pharmacol Sci*, **22**, 23–26.

Tintelnot-Blomley, M. and Lewis, R. A. (2006) A critical appraisal of structure-based drug design, *IDrugs*, **9**, 114–118.

Todd, A. E., Orengo, C. A. and Thornton, J. M. (1999) Evolution of protein function, from a structural perspective, *Curr Opin Chem Biol*, **3**, 548–556.

Tomita, K., Fukai, S., Ishitani, R., Ueda, T., Takeuchi, N., Vassylyev, D. G. and Nureki, O. (2004) Structural basis for template-independent RNA polymerization, *Nature*, **430**, 700–704.

Tusnády, G. E. and Simon, I. (1998) Principles governing amino acid composition

of integral membrane proteins: Application to topology prediction, *J Mol Biol*, **283**, 489–506.

Tusnády, G. E. and Simon, I. (2001) The HMMTOP transmembrane topology prediction server, *Bioinformatics*, **17**, 849–850.

van Dooren, G. G., Su, V., D'Ombrain, M. C. and McFadden, G. I. (2002) Processing of an apicoplast leader sequence in Plasmodium falciparum and the identification of a putative leader cleavage enzyme, *J Biol Chem*, **277**, 23612–23619.

van Kraaij, C., de Vos, W. M., Siezen, R. J. and Kuipers, O. P. (1999) Lantibiotics: biosynthesis, mode of action and applications, *Nat Prod Rep*, **16**, 575–587.

Vapnik, V. N., (1995). *The nature of statistical learning theory.* Springer-Verlag New York, Inc., New York, NY, USA.

Vellai, T., Takács, K. and Vida, G. (1998) A new aspect to the origin and evolution of eukaryotes, *J Mol Evol*, **46**, 499–507.

Vogtherr, M. and Fiebig, K. (2003) NMR-based screening methods for lead discovery, *EXS*, pages 183–202.

Volarath, P., Harrison, R. W. and Weber, I. T. (2007) Structure based drug design for HIV protease: from molecular modeling to cheminformatics, *Curr Top Med Chem*, **7**, 1030–1038.

von Heijne, G. (1992) Membrane protein structure prediction. Hydrophobicity analysis and the positive-inside rule, *J Mol Biol*, **225**, 487–494.

von Heijne, G. (1999) Recent advances in the understanding of membrane protein assembly and structure, *Q Rev Biophys*, **32**, 285–307.

Vorland, L. H., Ulvatne, H., Andersen, J., Haukland, H. H., Rekdal, O., Svendsen, J. S. and Gutteberg, T. J. (1999) Antibacterial effects of lactoferricin B, *Scand J Infect Dis*, **31**, 179–184.

Wada, T., Kuzuyama, T., Satoh, S., Kuramitsu, S., Yokoyama, S., Unzai, S., Tame, J. R. H. and Park, S.-Y. (2003) Crystal structure of 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol kinase, an enzyme in the non-mevalonate pathway of isoprenoid synthesis, *J Biol Chem*, **278**, 30022–30027.

Wahlberg, J. M. and Spiess, M. (1997) Multiple determinants direct the orientation of signal-anchor proteins: the topogenic role of the hydrophobic signal domain, *J Cell Biol*, **137**, 555–562.

Waller, R. F., Keeling, P. J., Donald, R. G., Striepen, B., Handman, E., Lang-Unnasch, N., Cowman, A. F., Besra, G. S., Roos, D. S. and McFadden, G. I. (1998) Nuclear-encoded proteins target to the plastid in Toxoplasma gondii and Plasmodium falciparum, *Proc Natl Acad Sci U S A*, **95**, 12352–12357.

Waller, R. F., Reed, M. B., Cowman, A. F. and McFadden, G. I. (2000) Protein trafficking to the plastid of Plasmodium falciparum is via the secretory pathway, *EMBO J*, **19**, 1794–1802.

Waller, R. F., Keeling, P. J., van Dooren, G. G. and McFadden, G. I. (2003) Comment on A green algal apicoplast ancestor, *Science*, **301**, 49; author reply 49–49; author reply 49.

Waller, R. F. and McFadden, G. I. (2005) The apicoplast: a review of the derived plastid of apicomplexan parasites, *Curr Issues Mol Biol*, **7**, 57–79.

Waller, R. F., Ralph, S. A., Reed, M. B., Su, V., Douglas, J. D., Minnikin, D. E., Cowman, A. F., Besra, G. S. and McFadden, G. I. (2003) A type II pathway for fatty acid biosynthesis presents drug targets in Plasmodium falciparum, *Antimicrob Agents Chemother*, **47**, 297–301.

Wallin, E. and von Heijne, G. (1998) Genome-wide analysis of integral membrane proteins from eubacterial, archaean, and eukaryotic organisms, *Protein Sci*, **7**, 1029–1038.

Wanke, M., Skorupinska-Tudek, K. and Swiezewska, E. (2001) Isoprenoid biosynthesis via 1-deoxy-D-xylulose 5-phosphate/2-C-methyl-D-erythritol 4-phosphate (DOXP/MEP) pathway, *Acta Biochim Pol*, **48**, 663–672.

Weir, M., Swindells, M. and Overington, J. (2001) Insights into protein function through large-scale computational analysis of sequence and structure, *Trends Biotechnol*, **19**, S61–S66.

Wendt, K. U., Poralla, K. and Schulz, G. E. (1997) Structure and function of a squalene cyclase, *Science*, **277**, 1811–1815.

White, S. H. and Wimley, W. C. (1999) Membrane protein folding and stability: Physical principles, *Annu Rev Biophys Biomol Struct*, **28**, 319–365.

White, S. H. (2004) The progress of membrane protein structure determination, *Protein Sci*, **13**, 1948–1949.

W.H.O., (1999). World health report - 1999. Periodical, World Health Organization, Geneva.

Wickramasinghe, S. R., Inglis, K. A., Urch, J. E., Müller, S., van Aalten, D. M. F. and Fairlamb, A. H. (2006) Kinetic, inhibition and structural studies on 3-oxoacyl-ACP reductase from Plasmodium falciparum, a key enzyme in fatty acid biosynthesis, *Biochem J*, **393**, 447–457.

Wiesner, J., Borrmann, S. and Jomaa, H. (2003) Fosmidomycin for the treatment of malaria, *Parasitol Res*, **90 Suppl 2**, S71–S76.

Wiesner, J., Henschker, D., Hutchinson, D. B., Beck, E. and Jomaa, H. (2002) In vitro and in vivo synergy of fosmidomycin, a novel antimalarial drug, with clindamycin, *Antimicrob Agents Chemother*, **46**, 2889–2894.

Wilson, R. J., Denny, P. W., Preiser, P. R., Rangachari, K., Roberts, K., Roy, A., Whyte, A., Strath, M., Moore, D. J., Moore, P. W. and Williamson, D. H. (1996) Complete gene map of the plastid-like DNA of the malaria parasite Plasmodium falciparum, *J Mol Biol*, **261**, 155–172.

Wilson, R. J., Gardner, M. J., Feagin, J. E. and Williamson, D. H. (1991) Have malaria parasites three genomes?, *Parasitol Today*, **7**, 134–136.

Wilson, R. J. and Williamson, D. H. (1997) Extrachromosomal DNA in the Apicomplexa, *Microbiol Mol Biol Rev*, **61**, 1–16.

Wilson, R. J. M. I. (2005) Parasite plastids: Approaching the endgame, *Biol Rev Camb Philos Soc*, **80**, 129–153.

Wolff, M., Seemann, M., Tse Sum Bui, B., Frapart, Y., Tritsch, D., Garcia Estrabot, A., Rodríguez-Concepción, M., Boronat, A., Marquet, A. and Rohmer, M. (2003) Isoprenoid biosynthesis via the methylerythritol phosphate pathway: the (E)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase

(LytB/IspH) from Escherichia coli is a [4Fe-4S] protein, *FEBS Lett*, **541**, 115–120.

Wouters, J., Oudjama, Y., Barkley, S. J., Tricot, C., Stalon, V., Droogmans, L. and Poulter, C. D. (2003) Catalytic mechanism of Escherichia coli isopentenyl diphosphate isomerase involves Cys-67, Glu-116, and Tyr-104 as suggested by crystal structures of complexes with transition state analogues and irreversible inhibitors, *J Biol Chem*, **278**, 11903–11908.

Wouters, J., Oudjama, Y., Stalon, V., Droogmans, L. and Poulter, C. D. (2004) Crystal structure of the C67A mutant of isopentenyl diphosphate isomerase complexed with a mechanism-based irreversible inhibitor, *Proteins*, **54**, 216–221.

Wouters, J., Oudjama, Y., Ghosh, S., Stalon, V., Droogmans, L. and Oldfield, E. (2003) Structure and mechanism of action of isopentenylpyrophosphate-dimethylallylpyrophosphate isomerase, *J Am Chem Soc*, **125**, 3198–3199.

Wouters, J., Yin, F., Song, Y., Zhang, Y., Oudjama, Y., Stalon, V., Droogmans, L., Morita, C. T. and Oldfield, E. (2005) A crystallographic investigation of phosphoantigen binding to isopentenyl pyrophosphate/dimethylallyl pyrophosphate isomerase, *J Am Chem Soc*, **127**, 536–537.

Wu, C. H., Apweiler, R., Bairoch, A., Natale, D. A., Barker, W. C., Boeckmann, B., Ferro, S., Gasteiger, E., Huang, H., Lopez, R., Magrane, M., Martin, M. J., Mazumder, R., O'Donovan, C., Redaschi, N. and Suzek, B. (2006) The Universal Protein Resource (UniProt): an expanding universe of protein information, *Nucleic Acids Res*, **34**, D187–D191.

Xiang, S., Usunow, G., Lange, G., Busch, M. and Tong, L. (2007) Crystal structure of 1-deoxy-D-xylulose 5-phosphate synthase, a crucial enzyme for isoprenoids biosynthesis, *J Biol Chem*, **282**, 2676–2682.

Xu, Y. and Xu, D. (2000) Protein threading using PROSPECT: design and evaluation, *Proteins*, **40**, 343–354.

Yajima, S., Hara, K., Iino, D., Sasaki, Y., Kuzuyama, T., Ohsawa, K. and Seto, H. (2007) Structure of 1-deoxy-D-xylulose 5-phosphate reductoisomerase in a quaternary complex with a magnesium ion, NADPH and the antimalarial drug fosmidomycin, *Acta Crystallograph Sect F Struct Biol Cryst Commun*, **63**, 466–470.

Yajima, S., Nonaka, T., Kuzuyama, T., Seto, H. and Ohsawa, K. (2002) Crystal structure of 1-deoxy-D-xylulose 5-phosphate reductoisomerase complexed with cofactors: Implications of a flexible loop movement upon substrate binding, *J Biochem (Tokyo)*, **131**, 313–317.

Yan, S., Appleby, T., Larson, G., Wu, J. Z., Hamatake, R. K., Hong, Z. and Yao, N. (2007) Thiazolone-acylsulfonamides as novel HCV NS5B polymerase allosteric inhibitors: Convergence of structure-based drug design and X-ray crystallographic study, *Bioorg Med Chem Lett*, **17**, 1991–1995.

Yasin, B., Harwig, S. S., Lehrer, R. I. and Wagar, E. A. (1996) Susceptibility of Chlamydia trachomatis to protegrins and defensins, *Infect Immun*, **64**, 709–713.

Yu, J. and Buyya, R., (2004). A novel architecture for realizing grid workflow using tuple spaces. In *GRID '04: Proceedings of the Fifth IEEE/ACM Interna-*

tional Workshop on Grid Computing, pages 119–128, Washington, DC, USA. IEEE Computer Society.

Yu, J. and Buyya, R. (2005a) A taxonomy of scientific workflow systems for grid computing, *SIGMOD Rec.*, **34**, 44–49.

Yu, J. and Buyya, R., (Apr 2005b). A taxonomy of workflow management systems for grid computing. `http://arxiv.org/abs/cs.DC/0503025`.

Z., W. A. (1893) Beitrag zur konstitution anorganischer verbindungen, *Anorg. Chem.*, **3**, 267.

Zanchetti, G., Colombi, P., Manzoni, M., Anastasia, L., Caimi, L., Borsani, G., Venerando, B., Tettamanti, G., Preti, A., Monti, E. and Bresciani, R. (2007) Sialidase NEU3 is a peripheral membrane protein localized on the cell surface and in endosomal structures, *Biochem J.*

Zdobnov, E. M. and Apweiler, R. (2001) InterProScan–an integration platform for the signature-recognition methods in InterPro, *Bioinformatics*, **17**, 847–848.

Zell, A., Mamier, G., Vogt, M., Mache, N., Hubner, R., Döring, S., Herrmann, K.-U., Soyez, T., Schmalzl, M., Sommer, T., Hatzigeorgiou, A., Posselt, D., Schreiner, T., Kett, B., Clemente, G. and Wieland, J., (1995). Stuttgart neural network simulator. `http://www-ra.informatik.uni-tuebingen.de/SNNS/`.

Zhai, Y. and Saier, M. H. (2002) The beta-barrel finder (BBF) program, allowing identification of outer membrane beta-barrel proteins encoded within prokaryotic genomes, *Protein Sci*, **11**, 2196–2207.

Zhang, C., Liu, L., Xu, H., Wei, Z., Wang, Y., Lin, Y. and Gong, W. (2007)

Crystal structures of human IPP isomerase: new insights into the catalytic mechanism, *J Mol Biol*, **366**, 1437–1446.

Zhang, Z., Carriero, N., Zheng, D., Karro, J., Harrison, P. M. and Gerstein, M. (2006) PseudoPipe: an automated pseudogene identification pipeline, *Bioinformatics*, **22**, 1437–1439.

Zhang, Z., Carriero, N. and Gerstein, M. (2004) Comparative analysis of processed pseudogenes in the mouse and human genomes, *Trends Genet*, **20**, 62–67.

Zhang, Z., Harrison, P. M., Liu, Y. and Gerstein, M. (2003) Millions of years of evolution preserved: a comprehensive catalog of the processed pseudogenes in the human genome, *Genome Res*, **13**, 2541–2558.

Zheng, L., White, R. H., Cash, V. L. and Dean, D. R. (1994) Mechanism for the desulfurization of L-cysteine catalyzed by the nifS gene product, *Biochemistry*, **33**, 4714–4720.

Zheng, L., White, R. H., Cash, V. L., Jack, R. F. and Dean, D. R. (1993) Cysteine desulfurase activity indicates a role for NIFS in metallocluster biosynthesis, *Proc Natl Acad Sci U S A*, **90**, 2754–2758.

Zheng, W., Sun, F., Bartlam, M., Li, X., Li, R. and Rao, Z. (2007) The crystal structure of human isopentenyl diphosphate isomerase at 1.7 A resolution reveals its catalytic mechanism in isoprenoid biosynthesis, *J Mol Biol*, **366**, 1447–1458.

Zuegge, J., Ralph, S., Schmuker, M., McFadden, G. I. and Schneider, G. (2001) Deciphering apicoplast targeting signals–feature extraction from nuclear-

encoded precursors of *Plasmodium falciparum* apicoplast proteins, *Gene*, **280**, 19–26.