Fatty Acid Biosynthesis as a Drug Target in Apicomplexan Parasites

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Abstract: Apicomplexan parasitic diseases impose devastating impacts on much of the world's population. The increasing prevalence of drug resistant parasites and the growing number of immuno-compromised individuals are exacerbating the problem to the point that the need for novel, inexpensive drugs is greater now than ever. Discovery of a prokaryotic, Type II fatty acid synthesis (FAS) pathway associated with the plastid-like organelle (apicoplast) of *Plasmodium* and *Toxoplasma* has provided a wealth of novel drug targets. Since this pathway is both essential and fundamentally different from the cytosolic Type I pathway of the human host, apicoplast FAS has tremendous potential for the development of parasite-specific inhibitors. Many components of this pathway are already the target for existing antibiotics and herbicides, which should significantly reduce the time and cost of drug development. Continuing interest - both in the pharmaceutical and herbicide industries – in fatty acid synthesis inhibitors proffers an ongoing stream of potential new anti-parasitic compounds.

It has now emerged that not all apicomplexan parasites have retained the Type II fatty acid biosynthesis pathway. No fatty acid biosynthesis enzymes are encoded in the genome of *Theileria annulata* or *T. parva*, suggesting that fatty acid synthesis is lacking in these parasites. The human intestinal parasite *Cryptosporidium parvum* appears to have lost the apicoplast entirely; instead relying on an unusual cytosolic Type I FAS. Nevertheless, newly developed anti-cancer and anti-obesity drugs targeting human Type I FAS may yet prove efficacious against *Cryptosporidium* and other apicomplexans that rely on this Type I FAS pathway.

INTRODUCTION

Apicomplexan parasites are the cause of enormous disease burden worldwide. Malaria, the most widespread and lethal of these diseases, is estimated to sicken 500 million people and kill as many as 5 million each year [1]. Other, previously unimportant apicomplexan diseases are on the rise, spread by the increase in international travel and finding fertile ground in immuno-compromised individuals who are unable to resist infection by otherwise non-lethal parasites such as *Toxoplasma gondii* and *Cryptosporidium parvum*. To date, the most effective methods for controlling these diseases remains prevention and drug treatment, but the rise in drug-resistance in *Plasmodium spp.*, the causative agents of malaria, and the lack of effective treatment for emerging parasites such as *Cryptosporidium* highlights the need to develop novel, effective and inexpensive drugs to combat these diseases.

The discovery of a relict plastid in apicomplexan parasites [2, 3] and the subsequent elucidation of the prokaryotic nature of the metabolic pathways that it contained, revealed a new range of potential drug targets for the treatment of apicomplexan diseases [4-7]. The plastid (usually referred to as the apicoplast) is the evolutionary homologue of plant and algal plastids and hence derives ultimately from an endosymbiotic cyanobacterium. Although the apicoplast is no longer photosynthetic, it retains a range of prokaryotic metabolic pathways. Inhibitors targeting these pathways typically have little or no effect on human enzymes and are, therefore, much less likely to have toxic side effects.

The similarity between apicoplast and bacterial and plant pathways presents a further advantage in terms of drug development costs. The market for novel anti-apicomplexan drugs is far from commercially viable. The millions of malaria sufferers are the poorest of the poor, often unable to pay for any health care whatsoever. Emerging diseases such as *Cryptosporidium* have been the subject of some highly publicized outbreaks in the Western world [8], but the immuno-compromised individuals most at risk are, again, the poorest populations in the developing world. The lack of a viable market precludes commercial development of drugs specifically designed to treat apicomplexan parasitic diseases. There is, however, a robust market for both anti-bacterial drugs and herbicides and many of these compounds target enzymes found in the apicoplast. Therefore, the development of anti-parasite drugs could benefit from advances in other, more commercially viable enterprises. In fact, many common anti-bacterial drugs are effective against *P. falciparum* and *T. gondii in vitro* [9]. Some anti-bacterial drugs have already proven effective in their existing form and are even being used for treatment and prophylaxis [4, 10-13]. Many more have limited efficacy *in vivo* but are important launching points for the development of related compounds. This "piggyback" approach places the initial development costs of novel anti-parasite drugs within the affordable realm for non-commercial drug development programs.

An unusual characteristic of some apicoplast-targeted drugs is the slow rate of parasite death under drug treatment. This phenomenon, termed "delayed death", was recognized prior to the discovery of the apicomplexan plastid [14] and is a characteristic of some drugs that interfere with the "housekeeping" processes of the apicoplast. "Delayed death" has been primarily studied in *T. gondii*, where it was shown that macrolide antibiotics and the DNA gyrase inhibitor ciprofloxacin were effective anti-parasitics *in vitro* but that parasite death only occurred after daughter tachyzoites had burst out of their first host and established a new infection [15, 16]. A similar phenomenon has been reported in *P. falciparum* [17, 18]. This delayed effect, where the parasite remains viable for a prolonged period of time after drug exposure, has implications for the treatment of acute infections and may impact the development of drug resistance.

Much has been made of "delayed death" as it relates to the use of inhibitors targeting apicoplast metabolism and this has generated a misconception that all drugs targeting the apicoplast show, or should show, the "delayed death" effect. In this context, it must be strongly emphasized that inhibitors of apicoplast fatty acid synthesis do not show any delay in their effect. Indeed, the timing of parasite death under treatment with fatty acid synthesis inhibitors is similar to that seen with many commonly used anti-parasitic compounds [6, 19]. Therefore, compounds targeting apicoplast fatty acid synthesis should be useful for both prophylaxis and treatment of acute disease and present no greater risk for the development of drug resistance than anti-parasitic drugs with more traditional targets.

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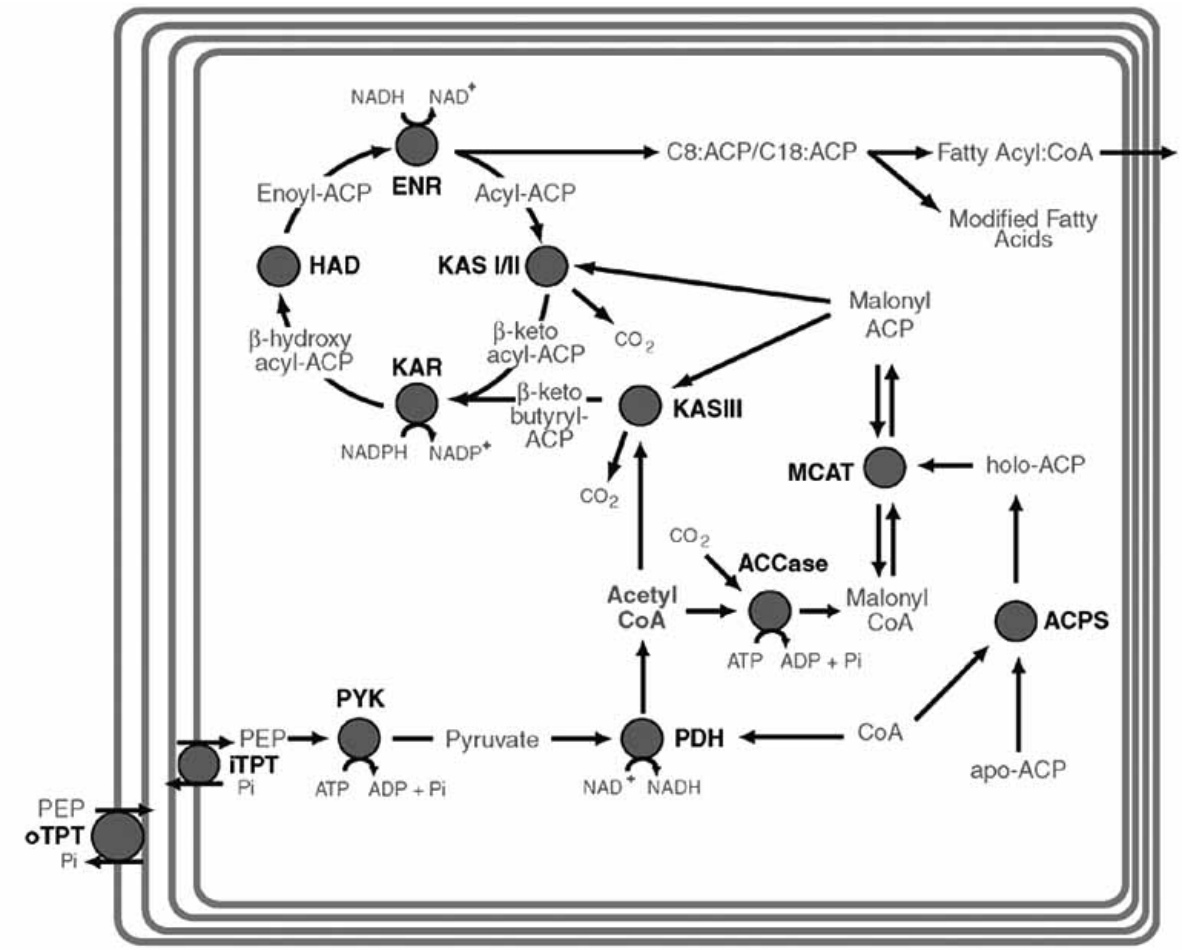


Fig. (1). Type II fatty acid synthesis in the apicoplast of *P. falciparum*.

Abbreviations: oTPT – outer membrane triose phosphate transporter, iTPT – inner membrane triose phosphate transporter, PYK – pyruvate kinase, PDH – pyruvate dehydrogenase, ACCase – acetyl-CoA carboxylase, ACP – acyl carrier protein, ACPS – ACP synthase, MCAT - malonyl-CoA:ACP transacylase, KASI/II/III - ß-ketoacyl-ACP synthase I/II/III, KAR - ß-ketoacyl-ACP reductase, HAD - ß-hydroxyacyl-ACP dehydratase, ENR - Enoyl-ACP reductase.

The interest in fatty acid biosynthesis as a drug target is reflected in the number of excellent reviews on the subject. These include both general reviews [20-24] as well as those more directly targeted at apicomplexan fatty acid biosynthesis and its inhibitors [5, 17, 22, 25, 26]. These reviews thoroughly describe the biochemistry and molecular biology of the fatty acid biosynthesis pathways and of the inhibitors that have been developed to treat apicomplexan diseases. Consequently, we will provide only a brief summary of the basic biology of fatty acid synthesis, instead focusing on the recent progress in existing drug development efforts, unexploited and under-exploited drug targets, and the implications that recent studies in human fatty acid synthesis have for the development of drugs for emerging apicomplexan diseases. This review will also discuss the impact that recent genome initiatives have had on our understanding of the role played by *de novo* fatty acid synthesis in the biology of apicomplexan parasites.

TWO TYPES OF FATTY ACID BIOSYNTHESIS

Fatty acid biosynthesis is commonly defined as the metabolic process by which acetyl-CoA precursors are converted to long fatty acyl chains, usually containing 8 or more carbon atoms. The basic reaction mechanisms of fatty acid synthesis are the same for all organisms and can be separated into an initiation phase and an elongation cycle. Initiation starts with conversion of acetyl-CoA to malonyl-CoA. Malonyl-CoA is then transferred to acyl-carrier protein (ACP), which acts as anchor, binding the growing acyl chain throughout all the subsequent reactions. Initiation is completed when a second acetyl-CoA moiety is added to the malonyl-ACP to form β-ketobutyryl-ACP.

The elongation cycle is an iterative process involving four steps. The product of initiation, β-ketobutyryl-ACP, is reduced to β-hydroxyl-ACP, hydrolyzed to enoyl-ACP and further reduced to form acyl-ACP. Acyl-ACP is extended by the addition of malonyl-ACP in a condensation reaction, forming β-ketoacyl-ACP and starting the cycle again. This iterative process continues, adding two carbons with each cycle, until the full length acyl-chain is created. Termination of the reaction occurs by hydrolysis or by the transesterification of the fatty acyl chain from ACP to a new receptor molecule. A simplified schematic of the fatty acid synthesis reactions from *P. falciparum* is presented in "Fig. (**1**)"

While they share a common reaction mechanism, the fatty acid synthesis enzymes of prokaryotes and eukaryotes have very distinct structures "Fig. (**2**)". In most prokaryotes, malonyl-CoA is synthesized by a multi-subunit acetyl-CoA carboxylase (ACCase) and linked to a small acyl-carrier protein. The subsequent synthesis of full-length fatty acid chains is catalyzed by a collection of discrete

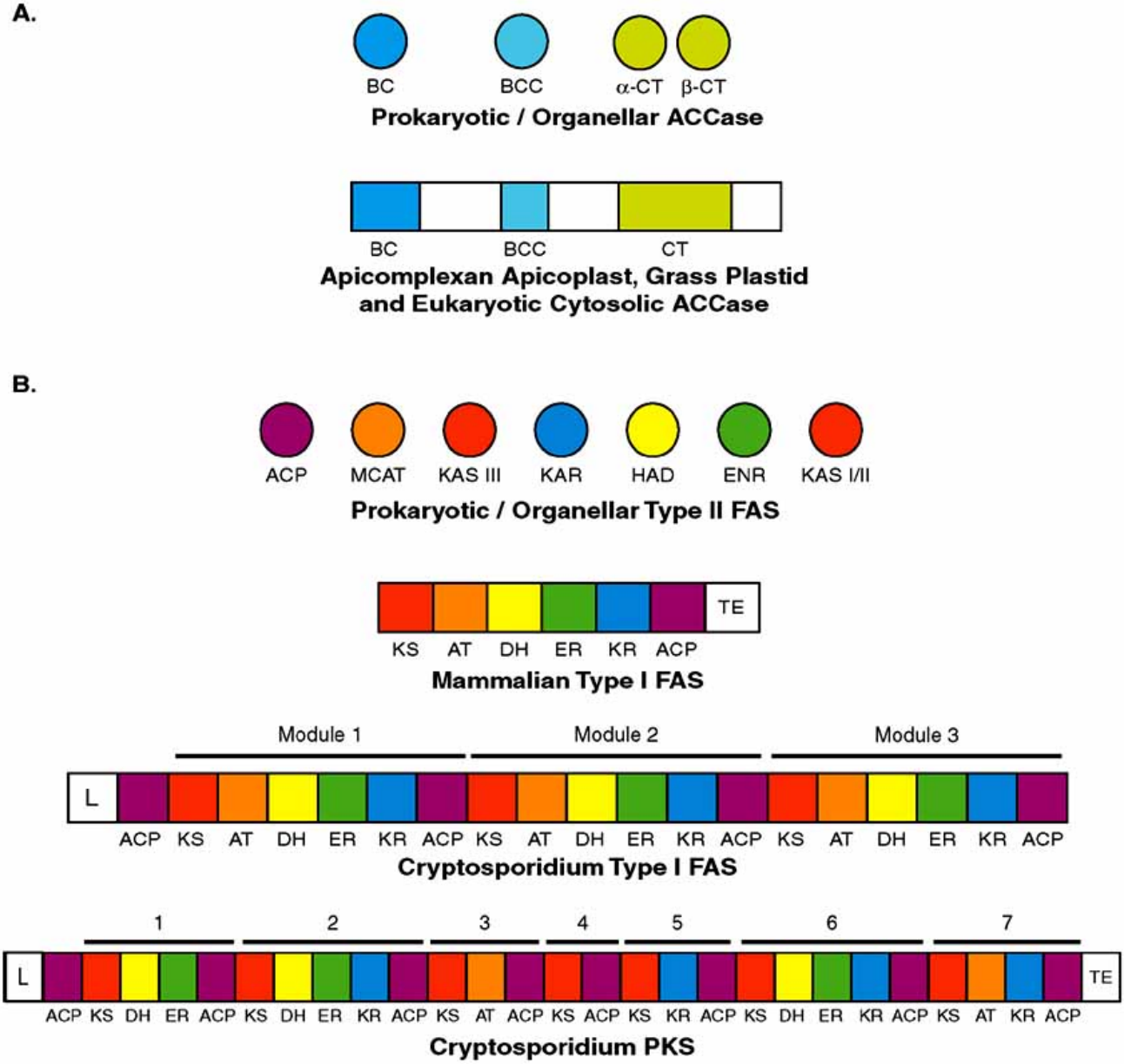


Fig. (2). Structural Characteristic of ACCases and FAS.

A. Schematic representation of the structures of the prokaryotic/ organellar multi-subunit ACCase and the multi-domain ACCase found in the eukaryotic cytosol, and plastid of grasses and apicomplexans. Abbreviations: BC - biotin carboxylase, BCC – biotin carboxy carrier, CT – carboxyltransferase. Adapted from [25].

B. Schematic representation of Type II FAS from prokaryotes and organelles. Type I FAS from mammals and *C. parvum* and polyketide synthase (PKS) from *C. parvum*. Abbreviations: ACCase – acetyl-CoA carboxylase, ACP – acyl carrier protein, ACPS – ACP synthase, MCAT - malonyl-CoA:ACP transacylase. KASI/II/III - ß-ketoacyl-ACP synthase I/II/III, KAR - ß-ketoacyl-ACP Reductase, HAD - ß-hydroxyacyl-ACP dehydratase, ENR - Enoyl-ACP reductase, AT – malonyl/acetyl-transacylase, DH – dehydratase, ER – enoyl reductase, KS – ketoacyl synthase, KR – ketoacyl reductase, L – acyl-CoA ligase, TE – thioesterase. Adapted from [23, 25, 26].

enzymes, known collectively as Type II FAS [24]. In contrast, eukaryotic ACCase is a large, multi-domain protein and malonyl-CoA is linked to the ACP unit of a giant, multi-functional enzyme (Type I FAS), which carries out all the steps of the pathway [27]. Members of the plant kingdom are a hybrid when it comes to fatty acid biosynthesis. The plastid contains Type II FAS, a pathway acquired by the endosymbiotic event that generated this organelle. The plastid-based Type II pathway is the sole source of *de novo* fatty acid synthesis in plants but plastid-derived fatty acids are elongated and undergo other modifications in the cytosol [20]. The plant ACCases present a further twist on the hybrid nature of plant fatty acid synthesis. All plants, like all other eukaryotes, have a multi-domain ACCase in the cytoplasm to provide malonyl-CoA for fatty acid chain elongation and other metabolic pathways [28, 29]. Most plants have a prokaryotic-like multi-subunit ACCase localized to the plastid [28, 29]. The main exception is found among the grasses (family *Poaceae*), which have a eukaryotic-like, multi-domain ACCase in the plastid [30, 31]. This unusual arrangement of fatty acid synthesis enzymes is also found in the apicomplexa.

Apicomplexan parasites such as, *T. gondii* and *P. falciparum*, can potentially exploit two sources of fatty acids. All characterized apicomplexans are able to scavenge long chain fatty acids from their host cells for use in membranes and other lipids [32-34]. These scavenged fatty acids are often extensively modified by the parasite [35] and inhibitors of these modification processes have anti-parasitic properties [36]. The molecular mechanisms underlying the scavenging and downstream modifications, as well as efforts to develop drugs targeting these processes, are reviewed elsewhere [37] and will not be discussed here.

In addition to being able to scavenge fatty acids from the host cell, it has recently emerged that *P. falciparum* and *T. gondii* can synthesize fatty acids *de novo*. Initially it was thought that these parasites relied solely on host-derived fatty acids, but data mining of parasite genomes identified a Type II synthesis pathway in the apicoplast [7] that was subsequently validated by the incorporation of radio-labeled acetate into parasite fatty acids [6]. Importantly, it has also been shown that this apicoplast fatty acid synthesis pathway is a target for known Type II FAS inhibitors [6, 19]. The availability of a complete *P. falciparum* genome has now allowed identification of all the proteins required for Type II FAS in the apicoplast [5].

With the recent completion of several apicomplexan genome sequences, the picture of fatty acid synthesis in the phylum has become more complex, and a pattern linking FAS pathways with host-parasites interactions is emerging. It appears that, similar to the case of plants, certain apicomplexans contain both Type I and Type II FAS. Some lineages have retained only one of the pathways while other parasites have apparently dispensed with fatty acid synthesis entirely [38, 39]. This mosaic distribution of the two pathways throughout the phylum has implications for the development of fatty acid inhibitors as broad-spectrum anti-parasitic drugs and presents interesting evolutionary questions, some of which will be discussed below.

The Type II, apicoplast-localized fatty acid synthesis pathway found in *Plasmodium* and *Toxoplasma* "Fig. (**1**)" has been the most thoroughly studied. All of the proteins involved in this pathway have been identified and most have been characterized. The search for apicomplexan FAS inhibitors has focused almost exclusively on the Type II pathway and several of the constituent enzymes are the subject of ongoing drug development efforts "Table **1**". As such, the enzymology and drug development approaches used to target this pathway will be discussed at length.

APICOPLAST FATTY ACID SYNTHESIS (I) – RAW MATERIALS

One of the less explored, and more intriguing aspects of apicomplexan fatty acid synthesis is how the raw materials for fatty acid synthesis are supplied to the apicoplast. Acetyl-CoA cannot be transported into organelles and, therefore, must be generated within the apicoplast. A source of reducing equivalents must also be supplied to drive the reactions, as the non-photosynthetic apicoplast has no energy generating potential. The provision of precursors for apicoplast fatty acid synthesis appears to follow the mechanism seen in non-photosynthetic plastids of plants. In these plastids, acetyl-CoA for fatty acid synthesis is generated from pyruvate that is

Table 1. FAS Enzymes and Their Inhibitors

Enzyme	Reaction Product	Bacterial Isoforms	Apicomplexan Isoform	Apicomplexan Inhibitors[1]	Enzyme[2] IC_{50} (μM)	Parasite IC_{50} (μM)	Other Untested Inhibitors[3]
ACCase	Malonyl-CoA	Multi-enzyme	Multi-subunit	Clodinafop[4]	Tg 20	Pf 100, Tg 10	CP-640186[5] Soraphen A[6]
ACP synthase	Holo-ACP	ACPS SFP[7]	ACPS SFP	None reported None reported	- -	- -	Sch 538415[8] Anthranilate 4H-oxazol-5-ones[9]
MCAT	Malonyl-CoA-ACP	Fab D	Fab D	None reported	-	-	None reported
KASIII	β-isobutyryl-ACP	Fab H	Fab H	HR19[10]	Pf 5.31	Pf 9	Indole derivatives[11] 4,5-dichloro-1,2-dithiole-3-one[12] benzoylaminobenzoic acid[13]
KAR	β-hydroxyacyl-ACP	FabG	Fab G	None reported	-	-	None reported
HAD	Enoyl-ACP	Fab Z Fab A Fab M	Fab Z	NAS-91[14]	Pf K_i 1.5[15]	Pf 7.4	None reported
ENR	Acyl-ACP	Fab I Fab K Fab L	Fab I	Triclosan[16] Genz 8575[17]	Pf 30 Not reported	Pf 0.7, Tg 0.2 Pf 11.2	1,4-disubstituted imidazoles, aminopyridines, benzodiazepines, indole naphthyridones thiopyridines[18]
KASI/II	β-ketoacyl-ACP	Fab B Fab F	Not Determined	Thiolactomycin & Thiolactomycin analogues[19]	Not reported Not reported	Pf 50, Tg 100 Pf 10	Thiolactomycin analogues[20], platensimycin[21]
Type I FAS	Full-length fatty acids	-	*Cp*FAS	Cerulenin	Not reported	Cp 45	Thiolactomycin analogues[22], C75[23]

[1] When more than one inhibitor of the same class has been characterized, only the most effective is reported; [2] Origin of the enzyme – Pf: Plasmodium falciparum, Tg: Toxoplasma gondii, Cp – Cryptosporidum parvum; [3] These are inhibitors used against other organisms that have not yet been tested in apicomplexans; [4] [19], [58]; [5] [175]; [6] [176]; [7] In some bacterial species SFP activity can substitute for ACPS activity; [8] A natural product derived from an unidentified bacteria [80]; [9] [81]; [10] One of several thiolactomycin derivatives reported in [71]; [11] [89]; [12] [90]; [13] [91]; [14] [112]; [15] The IC_{50} of enzymatic activity was not reported; [16] [6], [122], [124]; [17] [136]; [18] [138-141]; [19] [7, 9, 19, 93]; [20] Thiolactomycin substituted in thiolactone ring [155]; [21] [156]; [22] Derivatives synthesized using (5R)-thiolactomycin [110]; [23] C75 is a synthetic malonyl-CoA mimetic [167].

imported into the plastid as phospho*enol*pyruvate (PEP) by a PEP/phosphate translocator [40]. A plastidic pyruvate kinase converts the imported PEP into pyruvate, which is then converted to acetyl-CoA by a multi-subunit pyruvate dehydrogenase complex (PDH) [41, 42]. This reaction also produces NADH; a source of reducing equivalents required for fatty acid synthesis in non-photosynthetic plastids.

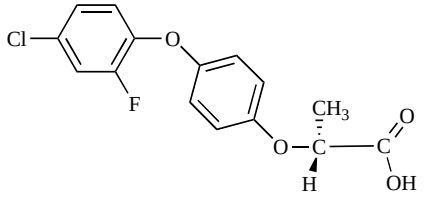
Analysis of the *P. falciparum* genome has identified all the necessary components for this pathway of acetyl-CoA formation. These genes are all nuclear encoded, with the protein products predicted to be apicoplast localized [5]. Two putative triose phosphate transporters (TPTs) are present in the *P. falciparum genome* and both localize to the apicoplast. These TPTs are situated in different apicoplast membranes and are thought to work sequentially to move PEP into the apicoplast, where an apicoplast targeted pyruvate kinase converts the PEP to pyruvate [5]. The *P. falciparum* nuclear genome encodes a single complete PDH complex [43]. GFP fusions show that all of the PDH proteins are targeted to the apicoplast and biochemical evidence suggests that this complex is functional in the conversion of pyruvate to acetyl-CoA [44, 45]. The combination of importers, pyruvate kinase and pyruvate dehydrogenase complex can thus supply carbon and reducing equivalents to the apicoplast FAS system.

As a drug development strategy, blocking the supply of fatty acid synthesis substrates remains largely unexplored. There are no reports of active drug development programs focusing on molecules involved in these processes in either plants or bacteria. This suggests that, as feasible drug targets, these processes will be costly to pursue in the short term. There are, however, characteristics of these molecules that make them possible targets for the future.

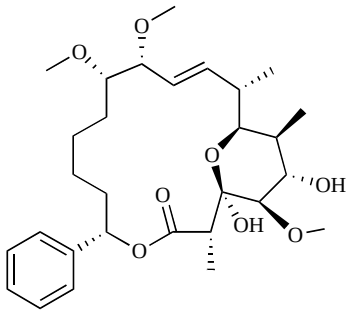
The use of PEP analogues as inhibitors is one approach that has a history in both anti-bacterial and herbicide development, albeit involving enzymes not associated with the transport of PEP nor with the conversion of PEP to pyruvate. Fosfomycin, an anti-bacterial drug used to treat urinary infections, is a PEP analog that interferes with bacterial cell wall synthesis [46]. The herbicide glyphosate, known commercially as RoundUp™, is thought to act as a transition-state analog of PEP [47] that inhibits the shikimate acid pathway in plants and *Plasmodium* [48]. Exploiting PEP analogues as inhibitors of the PEP transporters and/or pyruvate kinase may become a feasible approach once the molecular characteristics of the apicomplexan versions of these proteins are more completely understood.

The use of analogues, and in particular transition-state analogues, is being pursued in the development of pyruvate kinase inhibitors as drugs against *Leishmania* [49]. Phylogenetic and structural studies indicate that *Leishmania mexicana* pyruvate kinase is quite dissimilar to the four human pyruvate kinase isozymes [49, 50] making the development of selective inhibitors feasible. A similar approach may be useful in targeting apicomplexan pyruvate kinase. However, the molecular characterization of the apicoplast pyruvate kinases in *Plasmodium* and *Toxoplasma,* which have not been cloned, lags far behind that of *L. mexicana*, for which a crystal structure has been determined [50]. Clearly, a more detailed understanding of the molecular biology of apicomplexan pyruvate kinases is required before inhibitors of this enzyme can be developed as anti-apicomplexan drugs.

Inhibitors of both triose phosphate transporters and PDH are commonly used in the functional analysis of these molecules. Is it possible that these compounds could be used as a starting point for drug development? Disulfonic stilbene derivatives such as diisothiocyanostilbene-2',2'-disulfonic acid (DIDS) are known inhibitors of plant triose phosphate transporters [51] but are also used as inhibitors of a broad spectrum of other transporters, including ion transporters in human erythrocytes [52]. Coenzyme analogs used as inhibitors to probe the molecular mechanism of PDH also appear to lack specificity [53, 54]. Given that both the triose phosphate transporters and PDH are members of large families of proteins, generating sufficient selectivity will be the main hurdle in developing any of these compounds as useful drugs. With the ongoing molecular characterization of both PDH and the triose transporters from *P. falciparum* [44, 45] it is possible that novel approaches to inhibiting these molecules may be uncovered in the near future.



Clodinafop



Soraphen A

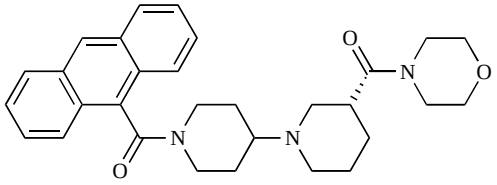


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Fig. (3). Chemical structures of inhibitors targeting plant and mammalian ACCase.

APICOPLAST FATTY ACID SYNTHESIS (II) – INITIATION

Synthesis of malonyl-CoA is the first committed step in fatty acid synthesis and inhibiting malonyl-CoA production significantly impacts metabolic flux through the pathway [42]. The conversion of acetyl-CoA and bicarbonate to malonyl-CoA by ACCase is a two-step process. First, the biotin moiety is carboxylated by the biotin carboxylase subunit. Then the carboxyl group is transferred to acetyl-CoA by carboxyltransferase (CT) subunit to form malonyl CoA [55]. ACCase is one of the two enzymes in fatty acid biosynthesis that is subject to cellular regulation, being activated by its precursors and inhibited by the products of fatty acid biosynthesis [56]. The control that ACCase exerts over the entire pathway makes this enzyme one of the most promising targets for drugs inhibiting this pathway in apicomplexan parasites.

The parallels between ACCases in the apicomplexans and the grasses led to the investigation of the anti-parasitic activity of the aryloxyphenoxypropionates (fops) and cyclohexanediones (dims), which selectively target the plastidic, multi-domain ACCase in plants. The plastidic ACCase of *T. gondii* is inhibited by several

fops; with IC_{50}s in the 10-100μM range. This enzymatic inhibition is mirrored in the effect these compounds have on the growth of the whole organism. The herbicide Clodinafop is the most effective of these compounds, with an IC_{50} of 10μM for *T. gondii* grown in human foreskin fibroblasts (HFF) [57, 58]. In keeping with the observation that these herbicides are specific for the plastidic multi-domain ACCase, the fops had little effect on HFF cells even when the inhibitors were applied at concentrations of 400μM [58]. In contrast to the grasses, *T. gondii* is completely resistant to dims [58]. *P. falciparum* is only weakly inhibited by existing dims and fops, with *in vitro* cultures showing IC_{50}s of 100-200μM [19] which is 2-10 fold higher than *T. gondii* [58] and up to 100-fold higher than susceptible plant species [59].

Multi-domain ACCases function as dimers and the active site is situated at the dimer interface. The fops and dims target the carboxyltransferase function [60] binding at the CT active site and causing a significant conformational change in the enzyme. Fop or dim binding rearranges the dimer, creating a hydrophobic pocket to accommodate the herbicide [61]. This rearrangement effectively precludes the binding of the acetyl-CoA substrate and competitively inhibits the enzyme.

The extensive use of fops and dims as herbicides has led to the emergence of several mutations that confer resistance among the grass ACCases. Studies of these resistance mutants are providing insights into the structure-function relationships of all eukaryotic, cytosolic ACCases and highlight key structural differences that may explain the relative insensitivity of the apicomplexan ACCases to these inhibitors. Two isoleucine residues in the CT domain of ACCase from grasses are the most commonly involved in the development of resistance. These residues are not directly involved with natural substrate binding but become important in the hydrophobic pocket created by herbicide binding [61]. Conversion of the more N-terminal of these isoleucines to a leucine confers resistance to fops and dims in some grasses [59, 62, 63]. The similarly positioned amino acid in cytoplasmic ACCases and in apicomplexans (amino acid 2093 in *T. gondii* and 2709 in P. falciparum) is a leucine and this may be a factor in the resistance of the apicomplexan enzymes to dims. Oddly, *T. gondii* becomes resistant to the fops if the native leucine in this position is mutated to an isoleucine [59]. This suggests that other amino acids contribute to the conformation of the ACCase active site and that differences in these residues have important functional implications in terms of herbicide sensitivity.

The more C-terminal isoleucine residue is conserved in grasses and apicomplexa (amino acid 2357 in *T. gondii* and 3086 in *P. falciparum*), but is a valine in yeast and humans "Fig. (**3**)". Substituting valine or asparagine in this position in the grass ACCases confers varying degrees of resistance to the fops but not the dims [63, 64]. However, converting this valine to an isoleucine in the yeast cytosolic ACCase does not make this enzyme sensitive to fops or dims [64]. Even yeast ACCases with both resistance conferring amino acids mutated to isoleucine remain resistant to fops and dims, supporting the conclusion that other amino acids are involved in the sensitivity of the plant plastid multi-domain ACCases to the fops and dims.

It has been suggested that the significant variation in the residues making up the dimer interface, the site of herbicide binding, may account for differing sensitivities to these herbicides [64]. In much of this region the apicomplexan ACCases have greater similarity to the cytosolic ACCases of yeast and mammals than the plastidic ACCases of grasses "Fig. (**3**)". This may provide a partial explanation of the relative insensitivity of apicomplexan ACCases to existing ACCase inhibiting herbicides. *P. falciparum* and *P. yoelii* also share several amino acids in the dimer interface region that are divergent from all other ACCases, while *T. gondii* apicoplast and the *C. parvum* cytosolic ACCases each have one amino acid differing from the consensus in this area "Fig. (**3**)". Many of these residues are in positions directly involved in dimer formation or in ACCase inhibitor binding [64], and determining if these amino acid differences are important factors in the unique characteristics of apicomplexan ACCases could provide vital data in the design of inhibitors that specifically target apicomplexan ACCases.

A frequently overlooked characteristic of ACCase inhibitors, particularly the herbicides, is that they are cheap to make. The estimated retail price of one gram of clodinafop, the most effective inhibitor of *T. gondii* ACCase, was less than 15 cents (US) in 2005.[1] This has important implications in meeting the cost guidelines set out by the agencies involved in developing and distributing new anti-parasitic drugs for the developing world.

As with all single molecule targeted drugs, there are significant risks of resistance, a problem seen in the growing number of plant species resistant to ACCase inhibitor based herbicides [65]. Given the extensive use of these herbicides, the rise of resistance does not seem unusual and suggests that ACCase inhibitors would seem no more prone to resistance than many other anti-parasitic drugs being developed.

Continuing discoveries about the structure-function relationships between the ACCases and their inhibitors is providing the molecular basis for effective drug development. The ongoing search for new forms of ACCase inhibiting herbicides and, more recently, the focus on ACCases as a target in combating human disease [66] provides a significant source of novel compounds on which to piggyback apicomplexan-specific drug development. Considering the low cost of synthesis, ACCase inhibitors represent a promising, and surprisingly untapped, resource for novel anti-apicomplexan drugs.

ACYL CARRIER PROTEIN (ACP) AND ACP SYNTHASE

The acyl carrier protein is the heart of fatty acid synthesis, binding the growing fatty acyl chain as it undergoes multiple rounds of elongation. In Type II FAS, ACP is a small (9 kDa) protein that is essential for apicoplast fatty acid synthesis and for parasite survival in *Toxoplasma gondii* [67]. ACP is synthesized in an inactive state with the gene product, apo-ACP, being modified through the addition of a 4'-phosphopantetheine prosthetic group from Coenzyme A to form the active holo-ACP. In most organisms with Type II FAS, the conversion of apo-ACP to holo-ACP is catalyzed by ACP synthase [68], a nuclear-encoded, apicoplast-targeted gene in *P. falciparum* [5]. The conversion process is very efficient, with the apo-form of the enzyme being undetectable except under situations of extreme CoA depletion [69-71].

ACP synthases are members of the phosphopantetheinyl transferase (PPTase) protein superfamily; proteins involved in the activation of carrier proteins in fatty acid, polyketide and non-ribosomal peptide synthesis [72]. In *E. coli*, functional ACP synthases are essential for bacterial survival [73, 74] as blocking the conversion of apo-ACP to holo-ACP blocks fatty acid synthesis. Crystal structures of ACP synthase from two bacterial species show that, despite sequence divergence, these enzymes share a common secondary structure [75, 76]. Gene predictions suggest that the apicomplexan ACP synthases are divergent from their bacterial counterparts. They also appear to be unique in having a hydrolase domain fused to the ACP synthase domain [77]. The hydrolase activity has not been confirmed by experimentation and the purpose of this extra functional domain is unclear.

Another member of the phosphopantetheinyl transferase enzyme family, first characterized as the PPTase for surfactin non-ribosomal peptide synthesis in *Bacillus subtilis* (SFP), is found in eukaryotes with Type I FAS and some bacteria. SFP PPTases are more promiscuous than ACP synthases, being capable of catalyzing the addition of 4' phosphopantetheine to multiple targets, including ACP [78]. The human genome has a single SFP that catalyzes the

[1] Zollinger, NDSU extension, www.ag.ndsu.nodak.edu/weeds/w253/w253-5a.htm.

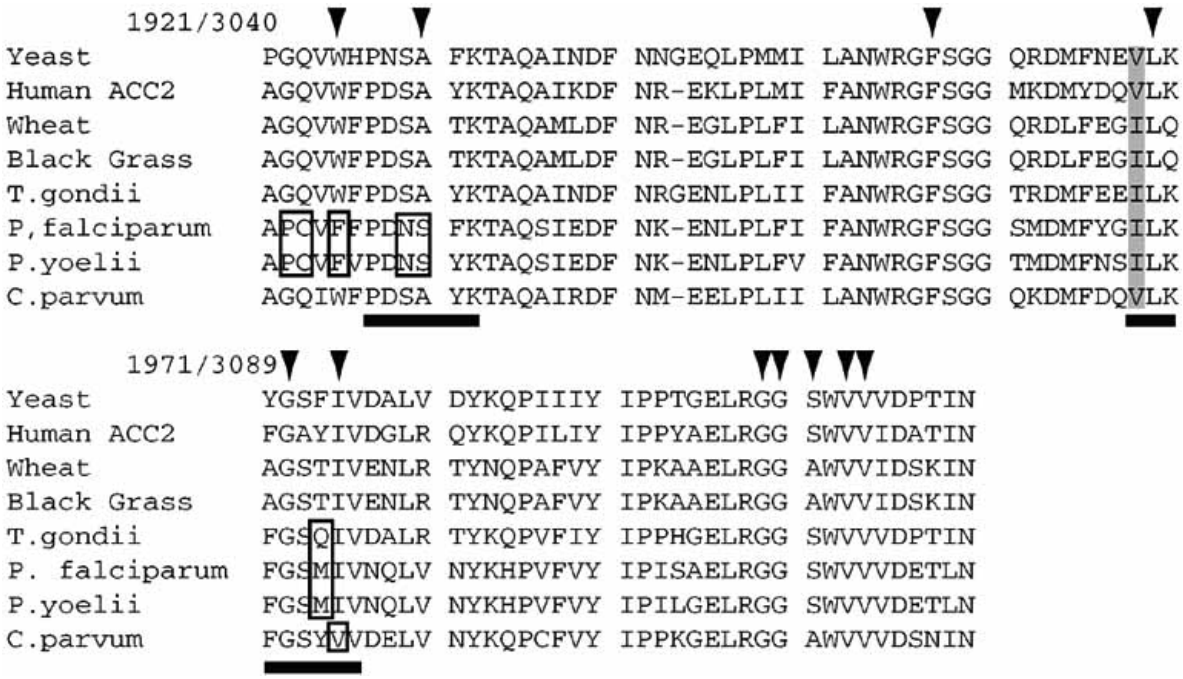


Fig. (4). Important amino acids in the CT domain of apicomplexan ACCases.

Clustal W alignment of a portion of the CT domain encompassing several sites of dimer interaction. Boxes represent amino acids that differ from both the eukaryotic cytosolic and grass plastidic ACCases. Arrows indicate amino acids involved in binding of the ACCase inhibitor haloxyfop. Gray shaded box indicates residues known to confer herbicide (fop) resistance. Black bars indicate residues forming helices situated on the dimer interface in yeast. Numbering is for yeast / *P. falciparum* sequences. Adapted from [64].

conversion of apo-ACP to holo-ACP for both the cytosolic Type I and mitochondrial Type II FAS, as well as the phosphopantetheinylation of an enzyme involved in lysine degradation [79]. Searches of the *Plasmodium* genome reveal only a single, ACP synthase-like phosphopantetheinyl transferase. *Cryptosporidium parvum* contains an SFP-like member of this family and *T. gondii* has both [77]. The broad substrate specificity of SFP may confer redundancy on ACP-synthase activity in those apicomplexans where it is found, precluding the use ACP synthase inhibitors in parasites that also encode an SFP.

Problems with redundancy notwithstanding, inhibiting ACP synthase may yet represent a valuable avenue for anti-parasitic drug development. The requirement for functional ACP synthases in bacterial survival [73, 74] demonstrates the key role this enzyme plays in fatty acid biosynthesis. The first reports of targeted drug studies suggest that small molecule inhibitors of ACP synthase are produced in natural systems [80] and can also be synthesized [81]. Despite the sequence divergence that is evident between enzymes of this class, ACP synthase inhibitors are active against a broad range of bacteria [81] suggesting that they may also be useful against apicomplexans. The absence of SFP in *Plasmodium* indicates that inhibiting ACP synthase is a viable strategy for developing anti-malarials. A better understanding of the functional redundancy of ACP synthase and SFP in other apicomplexans will be required before the usefulness of ACP synthases inhibitors in treatment of these parasites can be properly assessed.

MALONYL-CoA:ACP TRANSACYLASE (MCAT/FabD)

MCAT, a serine hydrolase, catalyzes the reversible reaction that attaches malonyl-CoA to a serine residue in the signature peptide of ACP [82]. Given its role in the initiation of Type II FAS, it is somewhat surprising that no MCAT inhibitors have been reported. The gene is required for functional fatty acid biosynthesis and its deletion is lethal in many bacteria [83-85]. MCAT is responsible for maintaining the equilibrium between malonyl-CoA and malonyl-ACP rather than as a regulator of flux through Type II FAS [24]. Therefore, inhibitors need to be extraordinarily effective to significantly effect fatty acid synthesis. Obviously, these enzymatic characteristics raise questions about the efficacy of MCAT inhibitors, but the unusual properties of this enzyme leave open the possibility of developing effective inhibitors.

Analysis of the crystal structures of MCAT from *Streptomyces coelicolor* and *E. coli* indicate that MCAT has a different reaction mechanism to other hydrolases in that the acyl-enzyme intermediate is unusually stable. The stability of this intermediate is essential for the subsequent reaction with the 4' phosphopantetheine moeity on ACP [86, 87]. A stable intermediate creates the opportunity for the development of inhibitors that interact with this enzyme conformation. Such inhibitors would be very specific, an important factor given that MCAT is a member of a large protein family. If such inhibitors were effectively irreversible, the activity of this enzyme could be reduced to the point where it would impact the entire fatty acid synthesis pathway.

Obviously the design of such highly targeted inhibitors requires a thorough understanding of the structure of the target enzyme. While crystal structures of apicomplexan MCATs have not been solved, heterologously expressed *P. falciparum* MCAT can be recovered in its active conformation and its activity has been assayed [71]. With the purified enzyme as a resource for both structure/function studies and large-scale screening of chemical libraries, MCAT could become an important target for novel anti-apicomplexan drugs in the future, particularly if it garners further interest as a target for anti-bacterial drugs.

β-KETOACYL-ACP SYNTHASE III (KASIII/FabH)

KASIII represents one of the control points in Type II FAS, both in terms of how many fatty acids are produced and the branching qualities of those fatty acids. The presence of functional KASIII is essential for survival in bacteria [88] and this enzyme has been the subject of significant investigation as a drug target [89-93].

KASIII is a multifunctional enzyme whose primary function is condensing acetyl-CoA and malonyl-ACP to form ß-ketobutyryl-ACP, the starting point for straight chain fatty acid chain synthesis [94]. It can also catalyze the related condensation reaction that joins acyl-CoA derivatives to malonyl-ACP to form branched chain fatty acids [95]. KASIII enzymes have also been shown to have some acetyl-CoA transacylase (ACAT) activity, catalyzing the transfer of acetyl from acetyl-CoA to ACP [94]; a reaction that can represent up to 10% of KASIII enzymatic activity in some organisms [95].

P. falciparum KASIII (PfKASIII) was among the first apicoplast drug targets identified [7]. The PfKASIII protein is most similar to plastid and cyanobacterial enzymes [7] and mutation analysis showed that key conserved residues of the cysteine-asparagine-histidine catalytic triad identified in bacterial KASIII [96] are required for PfKASIII function [97]. When assayed with malonyl-ACP and either acetyl-CoA or butyryl-CoA as substrates, PfKASIII shows similar affinities and reaction rates as bacterial KASIII [71], confirming its ability to initiate fatty acid synthesis. Isobutyryl-CoA is a poor substrate, indicating that PfKASIII is not involved in the synthesis of branched chain fatty acids [71]. The ACAT activity of PfKASIII is negligible when compared to the reaction rate of the condensation reaction [71] confirming that the primary function of *Pf*KasIII is the initiation of straight chain fatty acid synthesis.

KASIII shares little primary sequence identity but significant structural homology with KASI/II, the condensation enzymes involved in fatty acid elongation. The most significant structural differences are the presence in KASIII of an asparagine rather than a second histidine in the catalytic triad and a smaller substrate binding pocket that is unable to accommodate a growing acyl chain [24]. These unique features combine to make most bacterial KASIII enzymes resistant to the two known KASI/II inhibitors - thiolactomycin and cerulenin [21]. However, testing of compounds structurally related to thiolactomycin provided some of the first leads for KASIII inhibitors. Three such compounds inhibited both PfKASIII and *P. falciparum* growth at low µM concentrations [71]. Subsequent work showed that these compounds were also potent inhibitors of bacterial KASIII enzymes and of bacterial growth [90]. The similarity in efficacy between PfKASIII and bacterial KASIII inhibitors points out the similarity between these enzymes and suggests that the indole, benzoylaminobenzoic acid and 1,2-dithiole-3-one classes of inhibitors being developed as KASIII specific antibacterials [89-91] could provide an important source of lead compounds for new anti-apicomplexan drugs.

APICOPLAST FATTY ACID SYNTHESIS (III) – ELONGATION

The enzymes of the fatty acid chain elongation in *P. falciparum* are well-studied and have received much attention as drug targets, in part owing to the existence of well-established antibacterials targeting several of these enzymes. In bacteria, the relative activity of different isoforms of these elongation enzymes is important for regulating the characteristics of the fatty acid produced, particularly the ratio of saturated to unsaturated fatty acid chains produced [24]. Apicomplexans appear have a much less complex pathway of fatty acid synthesis with only a single isoform of each enzyme present and saturated fatty acids as the only product [5].

β-KETOACYL-ACP REDUCTASE (KAR/FabG)

The ß-ketobutyryl-ACP product of the KASIII reaction is reduced to ß-hydroxyacyl-ACP by ß-ketoacyl-ACP reductase (KAR) in the first of two steps catalyzed by enzymes of the short-chain dehydrogenase/reductase (SDR) superfamily. KAR is recognized as a promising target for broad-spectrum anti-bacterials because it is essential for fatty acid synthesis and exists as a single isoform in most bacteria [98]. KAR appears to be highly conserved in structure in bacteria and plants [24]. Both the *P. falciparum* and *T. gondii* genomes appear to contain single copies of KAR [99]. The mature forms of these proteins are highly conserved with 52.9% identical and 70.6% similar amino acids[2]. *P. falciparum* KAR (*Pf*KAR) has been characterized and is structurally and functionally similar to other published KARs [99, 100]. This suggests that inhibitors directed against KAR will either be effective against apicomplexans or provide solid platforms on which to develop such drugs. Indeed, the antihelminthic compound hexachlorophene inhibits *Pf*KAR and has antimalarial activity *in vitro* [100] and may, therefore, represent a compound on which to base novel KAR inhibiting antimalarials.

A survey of the inhibitory effect of plant polyphenols showed that KAR, the related fatty acid reductase ENR and the fatty acid dehydratase HAD (see below), are inhibited by several plant flavonoids in the low to sub µM range. These compounds also have significant antimalarial activity *in vitro* [101] but it is unclear whether the antimalarial activity is due to the inhibition of fatty acid synthesis, with evidence from bacteria suggesting that similar compounds inhibit bacterial KAR and ENR *in vitro* but do not target fatty acid synthesis [102]. MabA, the *Mycobacterium tuberculosis* homologue of KAR is inhibited by the frontline anti-tuberculosis drug isoniazid [103], although inhibition of MabA does not appear to play an important role in the inhibition of *M. tuberculosis* growth *in vivo* [104]. Therefore, it seems likely that flavonoids exert their impact by inhibiting other fatty acid enzymes, or *via* a process unrelated to fatty acid synthesis.

The relative lack of progress in developing KAR inhibitors into effective drugs may reflect the activity of this enzyme and how it is integrated into the FAS pathway. Reconstruction of the complete elongation cycle of *E. coli* Type II FAS *in vitro* demonstrates that KAR activity is not limiting in the pathway [105] nor does it appear that KAR is a focus for the regulation of fatty acid synthesis [106]. This strongly suggests that inhibitors will have to almost eliminate KAR activity before they will effectively inhibit fatty acid synthesis. While this is true for *E. coli*, in *Pseudomonas aeruginosa* the KAR homologue is a control point in the production of acyl-homoserine lactones and as such may be a more effective drug target [107]. The role, if any, that KAR plays in apicomplexan metabolism beyond Type II FAS is unknown and until there is a clearer picture of the function and regulation of apicomplexan KAR its usefulness as a drug target will remain limited.

β-HYDROXYACYL-ACP DEHYDRATASE (HAD/FabZ)

The ß-hydroxyacyl-ACP produced by KAR is dehydrated to enoyl-ACP by the ß-hydroxyacyl-ACP dehydratase (HAD). FabZ is the most common of the HAD isoforms, being found in apicomplexans, most bacteria and plants [24]. *E. coli* and other Gram negative bacteria that produce unsaturated fatty acids also contain FabA, a HAD isoform that functions as both a dehydratase and as an isomerase that converts trans-2 decenoyl-ACP to cis-3 decenoyl-ACP [108]. Isomerization is an essential step in unsaturated fatty acids biosynthesis and FabA is paired with the FabB isoform of KAS to elongate the unsaturated fatty acyl chain [109]. An alternative mechanism of unsaturated fatty acid synthesis is found in *Streptococcus* spp lacking FabA. In these species, FabM catalyses the isomerization of trans-2 decenoyl-ACP to cis-3 decenoyl-ACP [110, 111]. Even in organisms with multiple HAD isoforms, FabZ is the most active and is necessary for the elongation of unsaturated fatty acid chains [109]. Therefore, FabZ is considered to be the primary HAD isoform in Type II fatty acid synthesis.

FabZ is the only HAD isoform present in the genomes of *T. gondii* and *P. falciparum* [7, 112]. *P. falciparum* HAD (*Pf*HAD) has been characterized and has 40.5% identity with the *T. gondii*

[2] Based on Clustal W comparisons of *Pf*KAR, Genbank accession AA032669 and Tg KAR, www.toxodb.org accession Tg Twinscan_1472.

FabZ[3] and 21% amino acid identity with *E. coli* FabA [112]. *Pf*HAD exists as a dimer and its enzymology is similar to other HAD proteins in that it is more efficient at catalyzing the reverse (hydration) reaction than the forward (dehydration) reaction [105, 112]. HAD is only an effective dehydratase when the pool of ß-hydroxyacyl-ACP is reduced by the activity of the downstream enzyme ENR [105]. Despite its enzymatic characteristics, HAD is indispensable for Type II FAS and is emerging as a valid drug target, particularly for *Plasmodium*.

The first HAD inhibitor described was 3 decynoyl-NAC, an analogue of cis-3 deconyl-ACP that was the first mechanism-based, or suicide, inhibitor reported [113]. This compound binds irreversibly to the active site of the FabA isoform and blocks both isomerase and dehydratase activity [113]. 3 decynoyl-NAC has antibacterial activity and is not toxic to mammals [114] but has not been used as a lead compound for drug development [21]. This may reflect the narrow range of efficacy. As an analogue of the product of the isomerase reaction, 3 decynoyl-NAC is only effective against bacteria using FabA to catalyze the HAD reaction. Other organisms, such as the apicomplexans, that rely on exclusively on the FabZ isoform for HAD activity should be resistant to this inhibitor. However, recent structural work indicated that 3 decynoyl-NAC is covalently bound to *Pf*FabZ and inhibits enzyme activity *in vitro* [115] and suggests the need to assess this compound for antimalarial activity.

Recently several specific inhibitors of the *P. falciparum* HAD have been reported. Homology modeling of *Pf*HAD was used for the identification and design of several inhibitors. Two of these, NAS-21 and NAS-91, were found to be effective inhibitors of *Pf*HAD *in vitro* and NAS-91 inhibits the *in vitro* growth of *P. falciparum* with an IC_{50} of less than 10μM [112]. Also, several plant-derived flavonoids show anti-HAD and antimalarial activity at low μM concentrations [101]. These are clearly valuable lead compounds for the development of anti-malarial, and possibly anti-bacterial, drugs and further studies on the structure and function of this recently crystallized enzyme [115, 116] should greatly improve the prospects of targeting HAD in drug development.

ENOYL-ACP REDUCTASE (ENR/FabI)

Enoyl-ACP reductase (ENR/FabI), the second member of the SDR superfamily involved in fatty acid biosynthesis, reduces enoyl-ACP to acyl-ACP in a reaction that requires NADH. FabI, one of three ENR isoforms in bacteria, is the target for two of the most important Type II FAS inhibitors, triclosan and isoniazid [117, 118] and is being actively pursued as a target for new anti-bacterials. The focus on ENR as a drug target reflects its importance as a regulator of Type II FAS. As stated above, ENR activity drives the HAD reaction and flux through the pathway [105], making ENR a rate-limiting step in Type II FAS. ENR is also the target of regulation by the cell, with *E. coli* ENR being subject to negative feedback inhibition by long chain acyl-ACP [106]. There two other ENR isoforms in bacteria – FabK and FabL – and the presence of either of these enzymes confers resistance to ENR inhibitors directed against FabI. Both the *T. gondii* and *P. falciparum* genomes contain a single ENR. This protein is most similar to the FabI isoform from bacteria [119, 120] indicating that anti-bacterials targeting FabI will be useful as drugs against apicomplexans.

The enzymology and molecular structure of *P. falciparum* ENR (*Pf*ENR) have been thoroughly characterized. Sequence comparisons of *Pf*ENR to other ENR proteins reveals a highly conserved enzyme, with more similarity to the plant plastid ENR than bacterial forms of ENR [121]. Enzymatically, the preference shown by *Pf*ENR for NADH over NADPH as a cofactor and its sensitivity to triclosan [6, 121, 122] support the inclusion of *Pf*ENR in the FabI group of ENRs. The structural basis for this similarity in action is supported by the crystal structure of *Pf*ENR in complex with triclosan and NAD^+ [121]. This shows that the important residues of the active site and for subunit interaction are conserved between *Pf*ENR and the FabI ENR from *Brassica napus, M. tubercuolsis* and *E. coli*. Somewhat surprisingly, there is no *in vivo* evidence for apicoplast localization of this extensively characterized enzyme, although targeting prediction algorithms place this enzyme in the apicoplast [25].

Of the bacterial ENR inhibitors, triclosan is the most fully characterized in terms of its activity against PfENR. The most effective

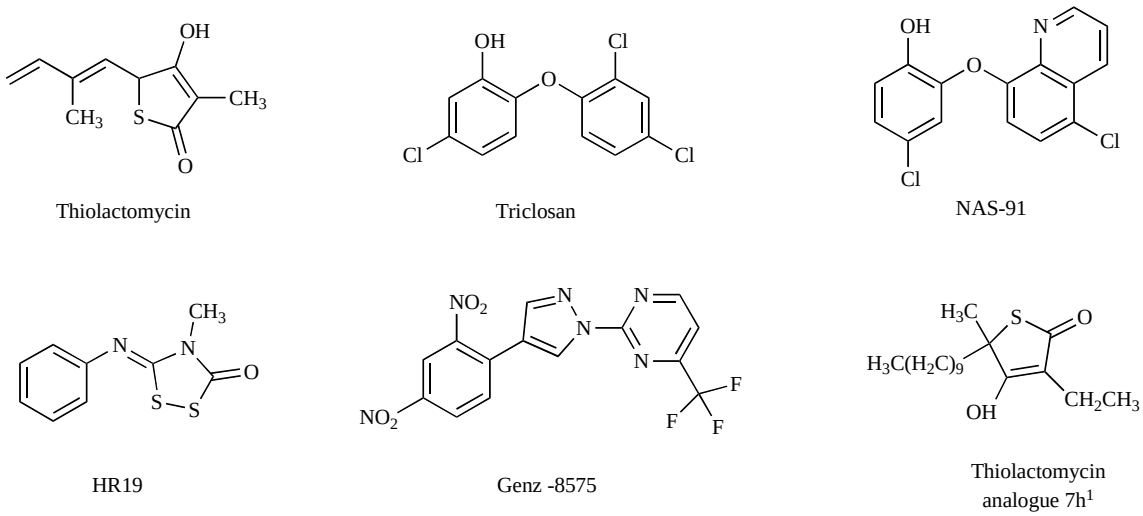


Fig. (5). Chemical structures of Apicomplexan Type II FAS inhibitors.

[1] Thiolactomycin analogue reported in [93].

ENR inhibitor of the 2-hydroxydiphenyl ether family, triclosan has been used a broad spectrum antibacterial in a wide variety of household products such as soaps and toothpaste for many years, but it was only recently shown that it inhibited lipid synthesis *via* its activity against ENR [118, 123]. Triclosan shows potent activity

[3] Clustal W comparisons of sequences identified in [7] 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) *Proc Natl Acad Sci USA*, **95**(21), 12352-7.

against *T. gondii* and *P. falciparum*, inhibiting fatty acid biosynthesis and showing IC_{50}s in the low micromolar range for *in vitro parasite* cultures. It also significantly inhibits the growth of *P. berghei in vivo* [6, 122]. Triclosan acts by forming a ternary complex with PfENR and NAD^+ that is reversible, but its tight binding and very slow dissociation rate make this inhibitor very potent [124, 125]. The pharmacokinetic properties of triclosan render it ineffective as an *in vivo* treatment, but it has been used in conjunction with structural information as a platform for the design of other ENR inhibitors [121, 126]. Unfortunately, none of those reported to date have proven to be clinically viable. There are also concerns over bacterial resistance to triclosan [127], although no resistant strains have been isolated from natural bacterial populations. Resistance may be of concern in the development of triclosan analogues for the treatment of apicomplexan diseases, as several single amino acid mutations in PfENR confer triclosan resistance in enzymatic assays [128].

The frontline anti-tuberculosis drug isoniazid targets InhA, the ENR homologue *M. tuberculosis* [104] but with a different mechanism than triclosan. When activated to it acyl radical form, isoniazid forms a covalent bond with NAD^+ and this adduct is a slow, tight binding inhibitor of InhA [129, 130]. Isoniazid was included in an early combination therapy for malaria treatment and did appear to improve the efficacy of the treatment [131, 132]. However, this appears to be a secondary effect, as isoniazid is a prodrug that requires activation by the *Mycobacterium* enzyme KatG to become active against InhA [133] and isoniazid alone does not show significant inhibition of *P. falciparum* in *in vitro* culture [134]. There are no published reports of work towards the creation of analogues of isoniazid, possibly because of its unique activating mechanism and the high level of resistance that has already developed to this drug. Reports of InhA mutants with cross-resistance to triclosan [135] also raise concerns about the development of cross-resistance to enoyl reductase inhibitors in cases of dual infection with malaria and tuberculosis.

Several novel classes of ENR inhibitor have been recently reported. Genz-8575 and Genz-10850, which were originally developed as InhA inhibitors, have been tested for their anti-plasmodial activity. While markedly less effective than triclosan, these compounds inhibited PfENR and blocked *in vitro* parasite growth at concentrations ranging from 10-32 μM [136]. Several plant products have also been identified which inhibit both *Pf*ENR and parasite growth [101, 137].

Although no other ENR inhibitors with anti-plasmodial activity have been reported, there are several families of inhibitors that are being explored as anti-bacterials. High throughput screening has identified the 1,4-disubstituted imidazoles, aminopyridines, benzodiazepines, indole naphthyridones and thiopyridines [138-142] as inhibitors of both the enoyl reductase of various bacteria and the growth of those bacteria *in vivo*. Testing of these classes of compounds against PfENR and for *in vitro* anti-plasmodial activity should provide a wealth of leads for the development of novel anti-apicomplexan drugs.

β-KETOACYL-ACP SYNTHASES I AND II (KASI/FabB AND KASII/FabF)

In bacteria, two closely related isoforms of ß-ketoacyl-ACP synthase, KASI and KASII, are responsible for the addition of subsequent malonyl-ACP units to the elongating fatty acid chain; with KASI being the primary enzyme [143] and KASII controlling fatty acid composition in a temperature sensitive manner [144, 145]. While there is limited sequence similarity between the KASI/II and KASIII proteins, they share a common structure and catalytic mechanism. As outlined above, the most important differences between KASI/II and KASIII are the catalytic triad of residues – a cysteine-histidine-histidine triad in KASI/II and a cysteine-asparagine-histidine triad in KASIII – and the presence of a binding pocket in KASI/II to accommodate the growing acyl chain [21, 146]. These features make KASI/II enzymes sensitive to two natural products – thiolactomycin and cerulenin [147, 148] - that have provided the basis for the development of most of the inhibitors of KASI/II.

Little is known about the KAS I/II enzyme in apicomplexans. The genomes of *P. falciparum* [43], *P. yoelii* [149], *P. berghei* [150] and *T. gondii* [4] each encode a single copy of this enzyme and prediction programs suggest that *the P. falciparum* enzyme is targeted to the apicoplast [5]. Also, thiolactomycin and several of its analogues inhibit the growth of *in vitro* cultures of *P. falciparum* [7, 19] as does cerulenin when applied in concert with the ENR inhibitor triclosan [6]. Since neither thiolactomycin nor cerulenin inhibit KASIII [71, 94], the *in vitro* effect of these drugs supports the conclusion that KASI/II homologue is active in apicoplast localized fatty acid synthesis in *Plasmodium* and *Toxoplasma*. There remains, however, no experimental evidence to support either the cellular localization or the relevant enzymatic activity of these enzymes in the apicomplexa.

As drug targets, KASI/II have, until recently, taken something of a backseat to other enzymes in the elongation cycle. Despite the significant understanding of the structural and functional basis for its activity [21], little progress has been reported in developing cerulenin as an antibacterial. This may reflect the inhibitory effect that this compound has on Type I FAS [151] and the issues this raises for selectivity.

There are no reports of thiolactomycin analogues being the focus of commercial anti-bacterial development programs but some progress has been made using thiolactomycin as an anti-parasitic compound. Two recent studies reported thiolactomycin analogues with IC_{50}s as low as 1μM against *in vitro P. falciparum* cultures [152, 153]. Thiolactomycin analogues have also been the subject of recent investigations as inhibitors of fatty acid synthesis in humans [154] and as drugs to combat tuberculosis [155]. Some of these analogues target Type I FAS so KAS I/II inhibitors may also impact drug development for apicomplexans such as *Cryptosporidium* and *Toxoplasma* that utilize the Type I pathway.

Recently, a screen of natural product extracts resulted in the discovery of a novel class of KAS I/II inhibitors termed platensimycins [156]. These inhibitors are both highly selective for KAS I/II activity and extremely potent inhibitors of the enzyme and of bacterial growth. While the efficacy of platensimycin against apicomplexan parasites remains to be explored, it clearly presents a novel source of compounds on which to piggyback novel anti-parasitic drugs.

FATTY ACID SYNTHESIS PATHWAYS IN OTHER APICOMPLEXAN PARASITES

The discovery and characterization of Type II FAS in *Plasmodium* and *Toxoplasma*, combined with the apparent lack of other fatty acid synthesis pathways in these two parasites, led to an initial assumption that apicoplast Type II FAS was the sole mechanism of fatty acid synthesis in the apicomplexans. As further genome sequencing and FAS characterization has taken place, however, the story has become more complicated. The first unusual finding was that *Cryptosporidium parvum*, which lacks both the apicoplast and Type II FAS [157], contains a eukaryotic Type I FAS (*Cp*FAS) with unusual structural characteristics [158]. *Cp*FAS is more closely related to polyketide synthases (PKS) from bacteria than mammalian Type I FAS "Fig. (**2**)" [119, 158]. *C. parvum* encodes a polyketide synthase (*Cp*PKS) of its own [159]; the only protist known to carry this class of protein. The presence of *Cp*PKS raises

[4] Genbank Accession #: Pb CAH94281, Pf NP_703921, Py XP_675741. Toxoplasma sequence: Tg Twinscan_0386 from www.toxodb.org.

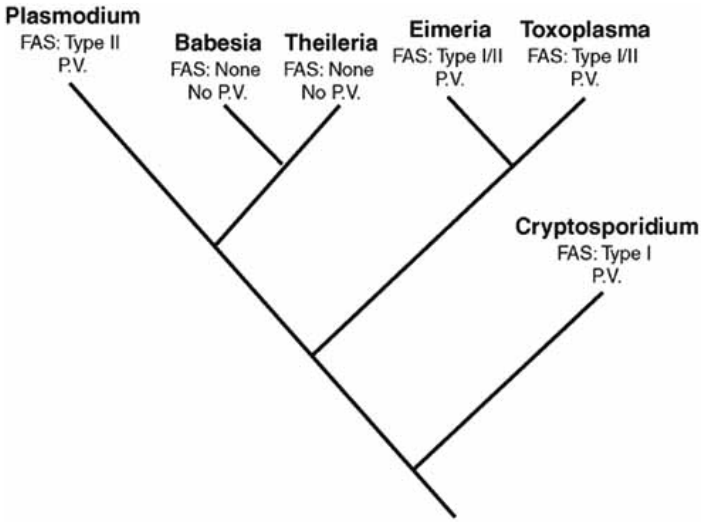


Fig. (6). Distribution of fatty acid synthesis pathways in the Apicomplexa.

Simplified evolutionary tree adapted from [177], showing the types of fatty acid synthesis pathways present and indicating the presence or absence of the parasitophorous vacuole throughout the intracellular life cycle.

interesting questions about the origin of the Type I FAS in *Cryptosporidium*. *T. gondii* and *Eimeria tenella* encode similar Type I FAS enzymes in addition to their apicoplast localized Type II pathways [26]. More recently it has emerged that the *Theileria annulata*, *Theileria parva* and *Babesia bigemina*[5] genomes do not encode either fatty acid synthesis pathway [38, 39]; suggesting a complete lack of *de novo* fatty acid biosynthesis in these parasites of cattle.

Superimposing the distribution of fatty acid pathways onto a simplified phylogenic tree of the apicomplexa suggests that the presence of both pathways was the ancestral state for the members of this phylum "Fig. (**4**)". Several independent losses of each pathway appear to have occurred. It is likely that *Cryptosporidium* lost the Type II pathway when it dispensed with its apicoplast, while it appears that the haematozoa branch of apicomplexans, which includes *Plasmodium*, *Theileria* and *Babesia*, lost the Type I pathway early on, with *Theileria* and *Babesia* subsequently dispensing with the Type II pathway "Fig. (**4**)".

Correlating the types of fatty acid synthesis pathways with the metabolism and cell biology of the apicomplexan parasites provides novel insight into the unresolved question of what role *de novo* fatty acid synthesis plays in the growth and development of these organisms. All of the characterized intracellular apicomplexans form and occupy a parasitophorous vacuole upon invasion. This vacuole is formed by both host cell lipids and lipids released from the apical organelles of the invading parasite [160, 161]. *Plasmodium*, *Eimeria*, and *Toxoplasma* maintain the parasitophorous vacuole throughout the intracellular life cycle and all of these parasites possess both types of fatty acid biosynthesis pathways. Conversely, while *Theileria* and *Babesia* create a parasitophorous vacuole upon invasion, they dispense with it early on in the life cycle. This correlation strongly suggests the involvement of *de novo* fatty acid synthesis with the parasitophorous vacuole.

Cryptosporidium is the only characterized apicomplexan that encodes for Type I FAS but lacks the Type II pathway [162]. As such, it sheds more light on the role played by each of the two types of fatty acid synthesis, particularly as it pertains to the creation and maintenance of the parasitophorous vacuole. Studies of the morphology of the parastiphorous vacuole in *C. parvum* indicate that the vacuole formed during the initial infection is replaced by an outgrowth of the host cell membrane, which encloses the parasite. [163]. Apparently, *Cryptosporidium* cannot maintain its own parasitophorous vacuole and this suggests that the Type II fatty acid synthesis pathway is required for the persistence of this structure throughout the entire parasite life cycle.

Type II FAS in *Toxoplasma gondii* plays an important role in lipoic acid synthesis. In parasites carrying an inducible knockout of type II FAS, repression of apicoplast fatty acid synthesis completely inhibits the lipoylation of apicoplast PDH, the only apicoplast protein known to be lipoylated [67]. The mitochondria contains several lipoylated proteins which are unaffected by type II FAS repression [67] reflecting the fact that lipoic acid in the *Toxoplasma* mitochondria is scavenged from the host cell [164]. These studies demonstrate that the apicoplast localized Type II FAS has functions beyond the generation or maintenance of membranes and these function may be more fundamental to parasite survival [34].

TYPE I FAS

The *C. parvum* Type I FAS *(Cp*FAS*)* is the only apicomplexan Type I FAS to be cloned and characterized. It is an unusual enzyme in that it has three complete sets of FAS domains "Fig. (**2**)". While this structure has more in common with the bacterial polyketide synthases, the sequence of the functional domains is identical to that of the mammalian Type I FAS [23, 158]. Enzymatic characterization of the individual domains of *Cp*FAS indicate that it uses long chain fatty acids (12:0-24:0) as its initial substrates, with a strong preference for palmitic acid (16:0), and its function is to elongate these substrates by six carbons to form very long chain fatty acids [162]. It does not appear that *Cp*FAS is able to generate fatty acids *de novo* but is a "fatty acid elongase" responsible for modifying fatty acids scavenged from the host [162]

The origin of Type I FAS in the apicomplexa presents an intriguing question. The *Cryptosporidium* was the first member of a distinct class of Type I FAS to be identified. At this time, the similarity between *Cp*FAS and *Cp*PKS and the apparent lack of Type I FAS in other apicomplexans led to the hypothesis that *Cp*FAS was derived from *Cp*PKS. More recently, the analysis of the *T. gondii* and *E. tenella* genomes indicated that these organisms encoded Type I FAS of the same class as *Cp*FAS and *Cp*PKS. [26, 159,

[5] *Babesia bigemina* data from complete shotgun sequence deposited at www.Sanger.ac.uk.

162]. As *C. parvum* is the only apicomplexan known to encode a PKS [159], it seems more likely that *Cp*PKS was derived from a duplication of the Type I FAS. This conclusion receives further support from the existence of two versions of the ACP-activating PPTases in the apicomplexa. The characteristically bacterial ACP-synthase is found in organisms with apicoplast Type II FAS pathways and the typically eukaryotic SFP in those with Type I FAS [77]. However, the similarity that Type I FAS from apicomplexans has to bacterial PKS suggests an interesting evolutionary history; one that will have to wait for more data on the members of this unusual class of Type I FAS to be thoroughly understood.

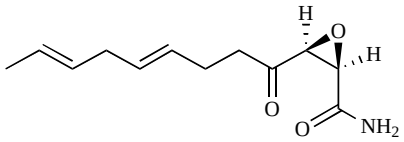
Another oddity in the fatty acid synthesis pathways of the apicomplexan is seen in the Type I FAS from *T. gondii*. Preliminary reports indicate that in immunofluorescence assays, antibodies the *C. parvum* Type I FAS cross-reacted with a mitochondrially localized protein in *Toxoplasma*.[6] This is a very intriguing finding in that a eukaryotic, cytosolic enzyme is targeted to an organelle of bacterial origin, the mitochondrion. Presumably, the Type I FAS was originally cytosolic but was re-targeted to replace the Type II pathway originally present in the mitochondrion; a more direct solution than re-targeting all of the enzymes of the Type II pathway.

The *T. gondii* SFP required for activation of the ACP subunit of this enzyme has been identified [77] and the *T. gondii* genome contains two distinct ACCases [57]. It is interesting to note, however, that neither the SFP nor the non-plastidic ACCase is localized to the mitochondria [57, 77]. Restriction of SFP to the cytosol is not incompatible with it activating the ACP of a mitochondrially localized FAS, as this is the situation for human mitochondrial FAS [79] but the absence of ACCase from the mitochondria raises questions about availability of malonyl-CoA for fatty acid synthesis in this organelle. Further studies on the localization of the *T. gondii* Type I FAS and its associated enzymes are needed to better understand this unusual enzyme and its role in *T. gondii*.

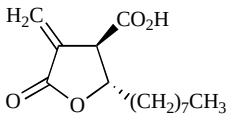
TYPE I FAS AS A DRUG TARGET

The obvious complication in developing inhibitors to target the Type I FAS in *Cryptosporidium* and *Toxoplasma* is the presence of homologous enzymes in the host. The question of selectivity for the parasitic enzyme has been key to strategies for developing novel drugs. *Cp*FAS is significantly different from human FAS in both protein sequence and molecular structure. The catalytic activity of several *Cp*FAS subunits also differs markedly from the human homologues [162], suggesting functional differences that might be exploited to achieve such selectivity. The characteristic fusion of several complete FAS enzymes into a single apicomplexan Type I FAS has implications for the development of resistance to drugs targeting this enzyme. If each subunit must be functional for the enzyme to be active, then multiple mutations, one for each repeated subunit, would be needed for resistance to develop [162]. This would present a distinct advantage for the treatment of *Cryptosporidium*, which currently has few viable treatment alternatives that could be included in a combined drug therapy strategy. Further insight into the activity and structure-function relationships of this unusual Type I FAS will be needed to point the way in identifying and developing effective and specific inhibitors.

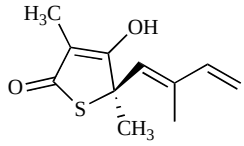
Given the economic realities of these diseases, it is valid to wonder where the lead compounds for the development of such inhibitors are going to come from. The compounds being developed to attack the Type II pathway of bacteria do not, by design, inhibit eukaryotic fatty acid synthesis. One exception is cerulenin, a broad-spectrum inhibitor of both Type I and Type II FAS. Cerulenin blocks the condensation reaction catalyzed by the KASI/II enzymes in the Type II pathway and by the corresponding KS subunit of Type I FAS [147]. Cerulenin can also block *C. parvum* growth *in vitro* at concentrations below those that are toxic to the host cells [158]. Due to its efficacy as a mammalian fatty acid synthesis inhibitor, cerulenin and its derivatives have not been the subject of significant interest as anti-bacterials [21]. It is, however, widely used as an *in vitro* inhibitor of fatty acid synthesis for functional and structural studies, so there is a significant body of knowledge regarding the molecular mechanisms of cerulenin inhibition [24]. With a more complete characterization of *Cp*FAS, it may be possible to use new structural information to identify and develop cerulenin-based compounds with greater specificity for the apicomplexan enzyme.



Cerulenin



C75



(5R)-thiolactomycin

Fig. (7). Chemical structures of Type I FAS inhibitors.

[1] Thiolactomycin analogues reported in [154]. Both are effective human FAS inhibitors but analogue 13b causes weight loss in rats without being toxic to cancer cells while analogue 16b has anti-cancer activity without causing weight loss.

A recently expanding source of leads for Type I FAS drugs comes from the interest in mammalian fatty acid synthesis as a target for both anti-cancer and anti-obesity drugs. In humans, most fatty acids are obtained from the diet. Consequently, the activity of cellular FAS in normal tissue is extremely low [165]. By contrast FAS is dramatically up regulated in cancerous tumors, with the endogenous FAS providing the entire supply of fatty acids in these tissues [166]. Elevated fatty acid synthesis plays a defining role in the development and proliferation of many cancers and the fatty acid synthesis pathway has become an important target for cancer therapy [167-169]. Cerulenin and the malonyl-CoA mimetic C75 inhibit mammalian Type I FAS and are effective in limiting the tumor growth *in vitro* and in animal models with low toxicity [151, 170]. The main side effect of these drugs is a loss of appetite and reversible weight loss in animals [171]. It is unclear how directly this weight loss is related to FAS inhibition, as these inhibitors impact multiple targets involved in the regulation of fatty acid metabolism [172, 173]. Structure-based design efforts have led to the development of a large number of thiolactomycin derivatives that are effective Type I FAS inhibitors [154]. Comparing the *in vivo* effects of these inhibitors demonstrated that the anti-cancer and weight loss effects of Type I FAS inhibitors can be targeted independently [154], suggesting that *Cp*FAS inhibitors can be devel-

[6] Crawford, M.J., G. Zhu, and D.S. Roos, Both Type I and Type II Fatty Acid Synthase in Toxoplasma gondii, in Molecular Parasitology Meeting XIV, Abstract No. 14C. 2003.

oped that have very limited impact on the normal metabolism of the host.

Another component of the eukaryotic fatty acid synthesis pathway that is being actively researched as a drug target for human disease is Acetyl-CoA carboxylase. The rising incidence of metabolic syndrome, a combination of symptoms including Type 2 diabetes, cardiovascular disease and atherosclerosis that are thought to stem from obesity [174], has led to the development of several different inhibitors of the two isoforms of human ACCases as a therapeutic approach to weight reduction [56]. One of these compounds, CP640186, inhibits human ACCases in the nanomolar range and also reduces malonyl-CoA production and fatty acid synthesis *in vivo*, with resulting weight loss and increased sensitivity to insulin [175]. Soraphen A, a polyketide from a soil bacterium, also inhibits eukaryotic ACCases at nanomolar concentrations [176] and is being developed as an agricultural fungicide. As with the anti-bacterial inhibitors and Type II FAS, these eukaryotic ACCase inhibitors could be fertile sources for the development of *Cp*FAS (and possibly *Cp*PKS) inhibiting drugs.

In terms of drug development for *C. parvum*, and possibly other apicomplexans, the most significant finding may be how well mammals tolerate compounds inhibiting their own Type I FAS and ACCase. Studies using such inhibitors *in vivo* indicate that the effective doses show no toxic side effects, at least in the short term [56, 154, 175]. Therefore, the concerns over selectivity that have been a major factor in the development strategy of both Type I and Type II fatty acid synthesis inhibitors assume diminished importance. It need no longer be assumed that if a compound inhibits components of the human fatty acid synthesis pathway it will not be a useful drug. This change in focus should broaden the range of candidates for drugs targeting both pathways, allowing compounds such as cerulenin, which have a broad spectrum of targets, to be re-examined as treatments for *Cryptosporidium* and other apicomplexan diseases.

CONCLUSION

The fatty acid biosynthesis pathways in apicomplexans remains one of the most important targets for developing novel drugs to combat these parasitic diseases. Although the burgeoning number of FAS combinations presents challenges for determining the specific roles played by newly synthesized fatty acids, it is clear that functional fatty acid synthesis pathways are absolutely required for parasite viability. The interest in novel inhibitors of fatty acid synthesis enzymes from bacteria, plants and humans will continue to provide a wealth of lead compounds for use in the development of apicomplexan specific drugs. Also, the renewed interest in the biology of human fatty acid synthesis and the relatively mild effects of inhibiting these pathways in humans makes the development of FAS inhibiting drugs for the treatment of *Cryptosporidium* and *Toxoplasma* a more promising proposition.

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