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Design, synthesis, and application of novel triclosan prodrugs as potential antimalarial and antibacterial agents

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Abstract—A number of new triclosan-conjugated analogs bearing biodegradable ester linkage have been synthesized, characterized and evaluated for their antimalarial and antibacterial activities. Many of these compounds exhibit good inhibition against *Plasmodium falciparum* and *Escherichia coli*. Among them tertiary amine containing triclosan-conjugated prodrug (**5**) inhibited both *P. falciparum* (IC_{50}; 0.62 μM) and *E. coli* (IC_{50}; 0.26 μM) at lower concentrations as compared to triclosan. Owing to the presence of a cleavable ester moiety, these new prodrugs are hydrolyzed under physiological conditions and parent molecule, triclosan, is released. Further, introduction of tertiary/quaternary functionality increases their cellular uptake. These properties impart them with higher potency to their antimalarial as well as antibacterial activities. The best compound among them **5** shows close to four-fold enhanced activities against *P. falciparum* and *E. coli* cultures as compared to triclosan.
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1. Introduction

Malaria is a global health problem that threatens 300–500 million people and kills more than one million people annually.[1] Disease control is hampered by the occurrence of multi-drug-resistant strains of the malaria parasite *Plasmodium falciparum*.[2,3] *P. falciparum* infection is the most widespread form of malaria and is the predominant cause of severe disease and death. Traditional treatments with drugs, such as chloroquine and sulfadoxine-pyrimethamine, are now much less effective due to rampant resistance,[4] hence harnessing drug targets unique to the malaria parasite such as the type II fatty acid biosynthesis pathway[5] opens new avenues for drug development against malaria. Type II fatty acid synthesis is brought about by FAS-II(fatty acid synthase II), which is structurally different from FAS-I (fatty acid synthase I) found in the human host as well as other higher eukaryotes and yeast. While FAS-II has discrete enzymes catalyzing individual reactions of the pathway, FAS-I is a homodimer of a multifunctional polypeptide with various domains catalyzing all the reactions of the biosynthetic pathway.[6] In *P. falciparum*, type II fatty acid synthesis has been localized in the relict plastid called apicoplast which is evolutionarily related to cyanobacteria.[7] The striking difference in the organization of the enzymes catalyzing fatty acid synthesis in *P. falciparum* and *E. coli* from that operating in the human host makes it a potent drug target not only for treating malaria but also for many bacterial infections. The iterative cycle of fatty acid biosynthesis consists of four steps, decarboxylative condensation, NADPH-dependent reduction, dehydration, and NADH-dependent reduction.[8] The NADH-dependent reduction step is carried out by enoyl-acyl carrier protein (ACP) reductase (ENR) which reduces the trans-2 enoyl bond of enoyl-ACP substrates to saturated acyl-ACPs and plays a deterministic role in completing the fatty acid elongation cycles.[9] ENR has been validated as a potential antimalarial[5,10] and antibacterial[11] drug target by us and oth-

Abbreviations: DIPEA, *N*,*N*-diisopropylethylamine; DMAE, dimethylaminoethanol; CDI, 1,1′-carbonyldiimidazole; MDMAEG, mono-(dimethylaminoethanyl)glutaryl; *P. falciparum*, Plasmodium falciparum; *E. coli*, Escherichia Coli.

Keywords: Prodrug; Triclosan; Antimalarial; Antibacterial; *Plasmodium falciparum*; *Escherichia Coli*.

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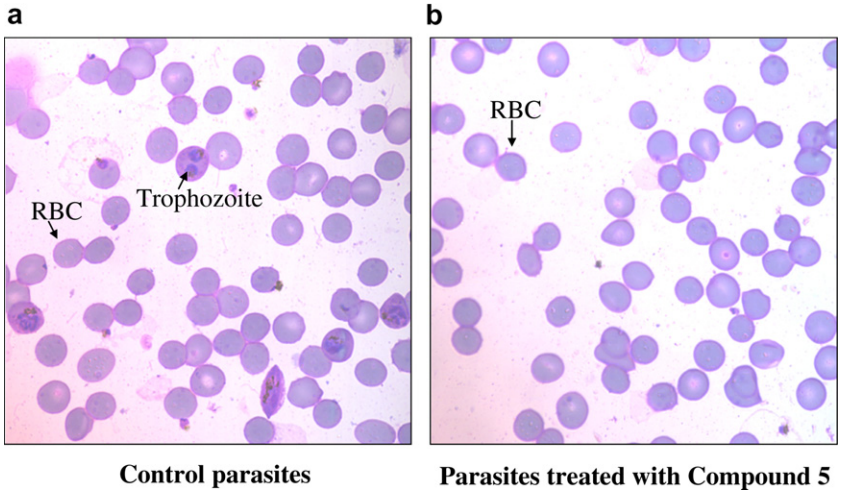


Figure 1. Inhibition of the growth of the parasites in red blood cell cultures. Synchronized parasites (5% parasitemia) were cultured in 96-well plates at 2–3% hematocrit and at an initial parasitemia of 1–2%, with different concentrations of the inhibitors in DMSO (final concentration, 0.05%). Parasite growth inhibition was assessed by microscopy of Giemsa-stained smears at 48 and 96 h. Inhibitions of the growth of parasites with compound **5** (Fig. 1b) have been shown as representative example.

ers. We have extensively studied *P. falciparum* ENR (*Pf*ENR) using biochemical as well as structural tools and also worked out the mechanism of its inhibition by triclosan.[12–16] Triclosan was found to be effective in killing *P. falciparum* in vitro and curing mice infected with the rodent malaria species *P. berghei* as well as systemic acute bacterial infection.[5,17] Structure–activity relationship (SAR)-based novel di-phenyl ethers have been evaluated for inhibition of *Pf*ENR and *Ec*ENR by us and others.[18–20] Triclosan has been traditionally used in consumer products such as toothpastes, mouthwashes, deodorants, soaps, and lotions and it was believed earlier to act by non-specific disruption of the bacterial cell wall. Now, with ENR having been identified as its target, triclosan provides us a promising scaffold, around which we can design newer compounds that can be tested for their possible therapeutic value against malaria and bacterial infections.

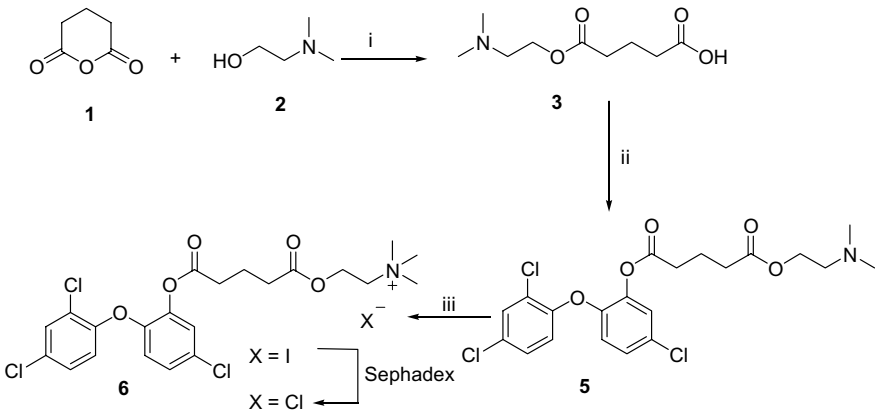
One approach towards improving the utility of such drugs, is to develop prodrug analogs capable of selective release of the parent triclosan. It is known that prodrug design comprises approaches that help in enhancing the efficacy and reducing the toxicity and unwanted effects of drugs by controlling their absorption, metabolism, and distribution. A prodrug is a derivative, which undergoes two independent reactions in order to regenerate the parent drug. In case of the prodrugs, the intermediate prodrug must be a chemically reactive entity, which rapidly undergoes a chemical conversion to release the parent drug under physiological conditions. However, this reactive molecule (viz. drug) is generated only, subsequent to an enzyme-catalyzed reaction on an otherwise chemically stable prodrug.[21] However, prodrug approach applied to triclosan (an antimalarial and antibacterial agent) indicates that a single chemical modification (as required in prodrugs) is sufficient to achieve the desired alteration in the biological properties to enhance the activity. In this paper, we report the characterization of a promising class of compounds designed to facilitate the transport of hydrophobic molecules across biological membranes. We focus on ester derivatives, because the ubiquitous presence of enzymes with esterase activity[22–24] ensures the rapid regeneration of the parent molecules 'triclosan'. Bioavailability and/or stability-improving ester prodrugs have been developed for many drugs with alcoholic or phenolic hydroxyls.[25,26] A releasable triclosan octaarginine derivatives (prodrug) were recently shown to be effective in inhibiting *T. gondii* tachyzoites with potency equivalent to that of triclosan while its non-hydrolyzable derivatives were devoid of activity.[27]

In the present paper, a series of prodrug esters were designed and synthesized in order to improve the cell penetration properties of the otherwise negatively charged agents. These prodrugs contain triclosan conjugated with appropriate acids, mono-(dimethylaminoethyl) glutarate, succinamic acid, levulinic acid, and glutaric anhydride via an enzyme-sensitive ester bond. Conjugated triclosan was expected to target the parasite where the labile bond is hydrolyzed so that the respective acids are released as antimalarial and antibacterial principles at the site of action. The literature survey suggests that triclosan ester-based conjugates have so far not been evaluated for their antimalarial and antimicrobial activities. Additionally, incorporation of a weak base in the prodrug of triclosan was expected to increase its uptake by the malaria parasite and bacteria improving its potency (Fig. 1).

2. Chemistry

2.1. Synthesis

Dimethylaminoethanol (DMAE) **2** reacts with an anhydride (glutaric **1**) to form a hemi-ester transferring its free carboxylic acid group to an imidazolide for reaction with the phenolic group of triclosan (Scheme 1). Mono-(dimethylaminoethanyl)glutaryl ester (MDMAGE; **3**) thus obtained is a hybrid of the DMAE and DMG (dimethyl glycine). Mono-(dimethylaminoethanyl)glutaryl

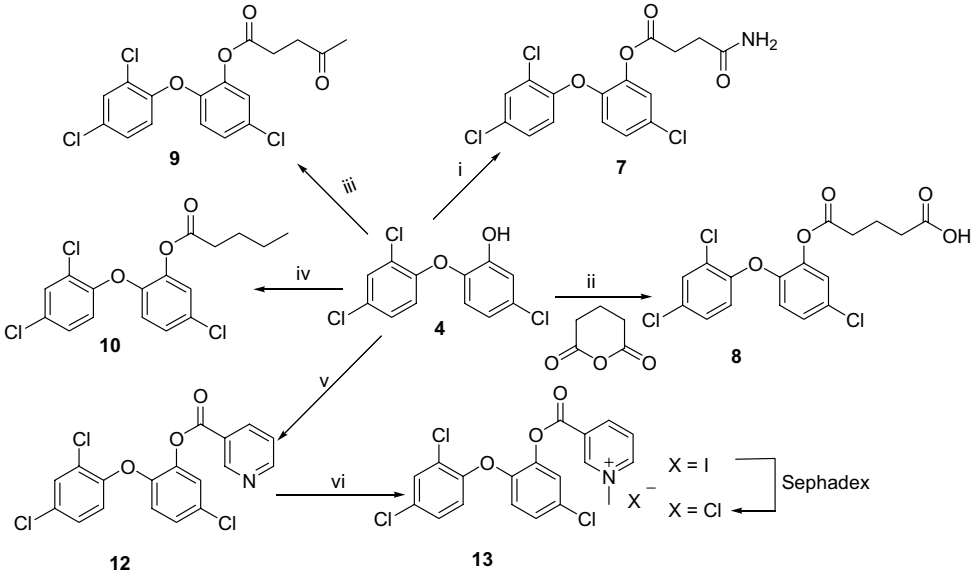


Scheme 1. Preparation of mono-(dimethylaminoethyl) glutarate (**3**), dimethylaminoethyl-glutaryl esters of triclosan (**5**) and trimethylaminoethyl-glutaryl esters of triclosan (**6**). Reagent and conditions: (i) CH_2Cl_2, DIPEA; (ii) a—CDI, DMF; b—triclosan (**4**), DCM, 4h; (iii) methyl iodide, THF, overnight.

imidazolide intermediate required for this purpose was prepared by the reaction of mono-(dimethylaminoethanol)glutaryl with CDI in dichloromethane. The corresponding quaternary ammonium salt of triclosan (**6**) was easily obtained by reaction of the tertiary amine (**5**) with methyl iodide at room temperature in dry THF. Subsequent ion exchange yielded the desired quaternary ammonium (**6**) as the more stable chloride salts.

Succinamic ester of triclosan (**7**) was synthesized by activating succinamic acid with CDI under N_2 and the activated imidazolide derivative of succinamic acid was coupled with triclosan. Triclosan glutaryl hemi ester (**8**) was synthesized by derivatization of a glutaric anhydride moiety to triclosan (**4**) in the presence of DIPEA. Esterification of triclosan with succinic anhydride was also attempted, but it was unsuccessful. Hence, a minimum length of five methylene units is apparently required for the esterification of triclosan. Another derivative was made with levulinic acid which was initially converted to the corresponding acid chloride using oxalyl chloride. This acid chloride was then coupled with triclosan in the presence of base DIPEA to give the levulinic acid ester of triclosan (**9**) (Scheme 2). In a similar way ester of valeric acid (pentanoic acid) with triclosan (**10**) was synthesized. Acid chlorides of respective acid, levulinic acid/valeric acid, were prepared by treatment of oxalyl chloride in DCM as solvent.

The nicotinoyl ester of triclosan (**12**) was synthesized by treatment of nicotinoyl chloride hydrochloride (**11**) with 1 equivalent of triclosan (**4**) and 2.1 equivalent of triethylamine as base, to yield the pyridine esters in moderate to good yields (Scheme 2). Quaternization of the pyridinium nitrogen atom with a five-fold excess of methyl iodide in acetone afforded the methylated product in quantitative yield. Resultant ion exchange yielded the desired quaternary ammonium (**13**) as the more stable chloride salts.



Scheme 2. Reagents and conditions: (i) succinamic acid, CDI, DMF, N_2; (ii) DCM, DIPEA; (iii) a—levulinic acid, $(COCl)_2$, cat. DMF, CH_2Cl_2, rt; b—triclosan; DIPEA, CH_2Cl_2; (iv) a—pentatonic acid, $(COCl)_2$, CH_2Cl_2, rt; (b) triclosan; DIPEA, CH_2Cl_2; (v) nicotinoyl chloride hydrochloride, Et_3N, toluene, reflux; (vi) CH_3I/acetone.

Due to transesterification of the ester products with the methanol eluent it was not possible to exchange the iodide for the chloride counter ion under the conditions routinely applied (Dowex 1 × 8, 200–400 mesh). By the use of the very mild ion exchange with Sephadex (DEAE A25, chloride form), however, transesterification was prevented despite the use of methanol as an eluent. Moreover, it also led to a quantitative ion exchange.

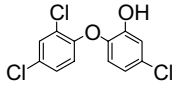
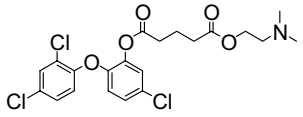
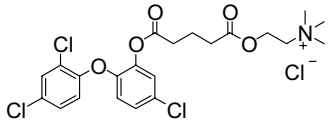
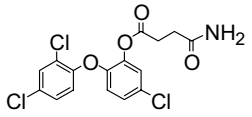
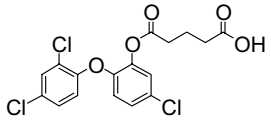
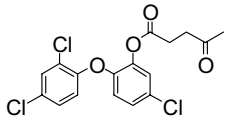
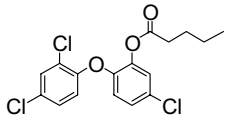
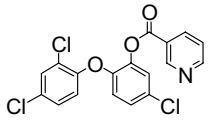
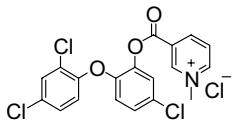
3. Results

3.1. Effect of various triclosan esters on *E. coli* culture

All the synthesized prodrugs were checked on *E. coli* cultures to determine the IC_{50} values of their inhibitory abilities. Each test was performed in triplicate and the IC_{50} reported represents the mean of at least three sets. Hence, while triclosan inhibited *E. coli* cultures with an IC_{50} of 0.75 μM, compounds **5–8** inhibited the cultures with IC_{50} values of 0.26, 0.42, 0.22, and 0.33 μM, respectively (Table 1). MIC (minimum inhibitory concentration) values of the compound (**5**– **8**) were found to be 0.55, 1.0, 0.6, and 0.75 μM, respectively. Nicotinoyl derivative of triclosan (compound **12**; pyridine derivative of triclosan), which is aromatic tertiary amine, inhibited growth of *E. coli* with IC_{50} of 3.1 μM (MIC = 9.0 μM) while pyridinium salt exhibits IC_{50} of 3.6 μM and MIC of 10.2 μM. From the above results it is clear that tertiary amine of aliphatic (**5**) is more active than an aromatic derivative (**13**).

The activity data (Table 1) suggest that compounds with tertiary amine ester of triclosan **5** (–$N(CH_3)_2$) group and

Table 1. IC_{50} and minimum inhibitory concentration (MIC) values of triclosan and its derivatives on cultures of *Escherichia coli* and *Plasmodium falciparum*

Compound ID	Structure	*E. coli**	*E. coli**	*P. falciparum**	*P. falciparum**
		IC_{50} (μM)	MIC (μM)	IC_{50} (μM)	MIC (μM)
4		0.75 ± 0.1	1.0 ± 0.1	2.29 ± 0.3	17.6 ± 2.1
5		0.26 ± 0.05	0.55 ± 0.07	0.62 ± 0.1	7.9 ± 0.7
6		0.42 ± 0.07	1.0 ± 0.1	3.02 ± 0.5	1 9.7 ± 1.6
7		0.22 ± 0.03	0.6 ± 0.12	6.70 ± 0.9	27.9 ± 3.4
8		0.33 ± 0.02	0.75 ± 0.2	0.52 ± 0.07	4.8 ± 0.3
9		77 ± 12.3	nd	0.68 ± 0.1	6.2 ± 0.6
10		140 ± 21	nd	8.90 ± 1.1	33.8 ± 4.7
12		3.1 ± 0.9	9.0 ± 2.3	1.15 ± 0.3	9.7 ± 1.1
13		3.6 ± 1.2	10.2 ± 3.5	2.07 ± 0.3	13.7 ± 1.3

* Data are expressed as means ± SD from at least three different experiments in duplicate. nd, not determine.

succinamic acid ester of triclosan **7** (–CO–NH_2) are more active than the compound with quaternary ammonium salt (**6**). This is because under assay conditions tertiary amine derivatives of triclosan (**5**) and succinamic ester of triclosan (**7**) do not undergo protonation and this protecting group is hydrophilic.

3.2. Effect of various ester of triclosan on *P. falciparum* cultures

With *P. falciparum* cultures, compound **5** (tertiary amine derivative of triclosan) showed IC_{50} of 0.62 μM and MIC of 7.9 μM (Fig. 2B), which are more than 3.9 times better than that of triclosan. Compound **6** (quaternary ammonium salt of triclosan) showed IC_{50} of 3.02 μM (Table 1). Similar pattern of outcome was obtained with aromatic tertiary amine (nicotinoyl ester of triclosan **12**) and quaternary ammonium salt (compound **13**). Compound **7**, succinamic acid derivative of triclosan, inhibits *P. falciparum* growth at higher concentrations (IC_{50} = 6.7 μM; MIC = 27.9 μM), whereas glutaryl ester of triclosan (**8**) and levulinic ester of triclosan (**9**) inhibited malaria parasite at lower concentrations as compared to compound **7** as well as the parent molecule (Table 1). Pentanoic acid-derived triclosan ester (prodrug) was active only at high micromolar concentrations (Table 1). Compounds **12** and **13** showed MIC values of 9.7 and 13.7 μM, respectively. Compound **12** is twice as potent as triclosan, perhaps due to its weak base properties.

3.3. Stability

Stabilities of the prodrugs were measured by FPLC (UV detection) in a Luria Broth at 37 °C (pH 7.2). All the prodrugs were found to be stable during 24 h of storage between −10 to 40 °C as no triclosan was liberated from them under these conditions.

3.4. FPLC assay of prodrug metabolism by *E. coli* and *P. falciparum* cultures

The conversion of the pro-drug to parent molecule triclosan (drug) was checked by the protocol developed in our laboratory,[17] in which solid-phase extraction procedure was coupled with a fast performance liquid chromatography (FPLC) method to determine the level of drug in the *E. coli* extract. The enzymatic hydrolyses of prodrugs, lead to the appearance of only two peaks characterized as triclosan and the given prodrug (Figs. 2A and 3A). The peak corresponding to the prodrug decreased until its complete disappearance while the parent molecule (triclosan) reached a plateau. The retention time of the prodrug (compound **5** shown as a representative example) was 31.9 min whereas for triclosan it was 37.9 min. With *P. falciparum* cultures

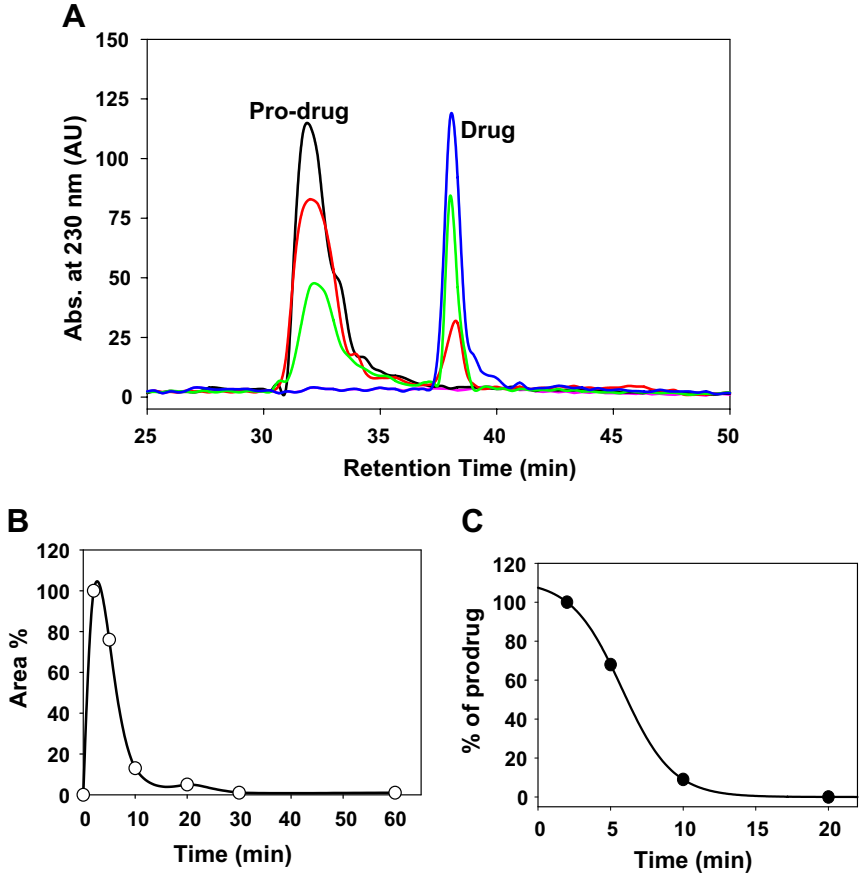


Figure 2. (A) FPLC detection of conversion of pro-drug (**5**) to drug in *E. coli* extract with a C_{18} reverse-phase column by monitoring the absorbance at 230 nm. Drug levels were detected at 0 (pink line), 2 min (black line), 5 min (red line), 10 min (green line), and 20 min (blue line) min after treatment. (B) Enzymatic cleavage of prodrug. (C) Disappearance of the pro-drug **5** in *E. coli* culture.

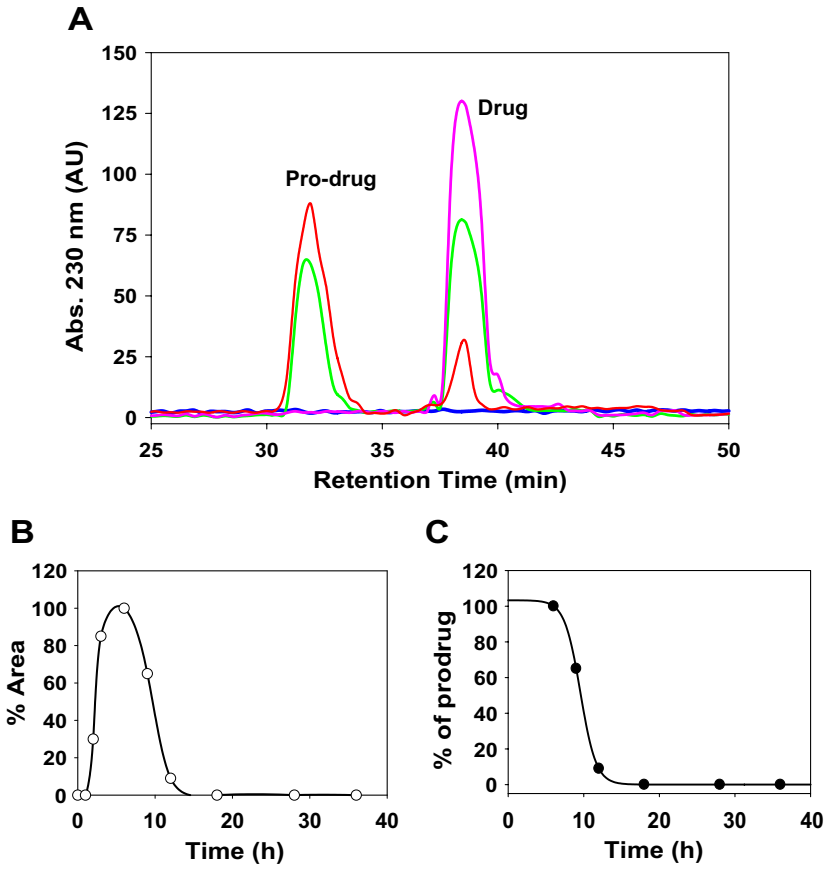


Figure 3. (A) FPLC detection of conversion of pro-drug (**5**) to drug in *P. falciparum* extract with a C_{18} reverse-phase column by monitoring the absorbance at 230 nm. Drug levels were shown at 3 h (blue line), 12 h (red line), 18 h (green line), and 24 h (pink line) after treatment. (B) Enzymatic cleavage of prodrug. (C) Disappearance of the pro-drug **5** in *P. falciparum*.

the half-life of prodrug disappearance for compound **5** was 9.45 h (Fig. 3C). For compounds **8** and **9** the half-lives were ~9.50 and 9.53 h, respectively (data not shown). However, the half-life of prodrug disappearance for compound **5** with *E. coli* cultures was approximately ~6 min (Fig. 2C). For compounds **6**, **7**, and **8** half-lives were approximately 8, 12, and 9 min, respectively. Thus a conversion of prodrug to parent molecule, triclosan, by the esterases present in *P. falciparum* and *E. coli* cultures is evident (Figs. 2A and 3A).

4. Discussion

The goal of the work is development of permeability-enhancing prodrug species for triclosan. Triclosan molecule has one active phenolic functional group, which is ideal for modification. In the present work, the phenolic group has been used to make prodrug candidates. The main objective for preparing this prodrug was

(i) to ease the transmembrane passage of triclosan, in order to build its significant intracellular concentration, (ii) to enhance the hydrophilicity of the molecule, and (iii) to have a biodegradable linkage in the inhibitors, which could be degraded by the cellular enzymes and triclosan released at the drug target site (pro-drug).

In order to achieve these objectives triclosan was covalently linked to various ligands viz. succinamic acid, levulinic acid, nicotinic acid etc.

Compound **5** (dimethylaminoethyl-glutaryl esters of triclosan), which is a tertiary amine ester of triclosan, exhibits the best growth inhibitory activity with an IC_{50} of 0.26 μM, whereas the quaternary ammonium salt derivative **6** (trimethylaminoethyl-glutaryl esters of triclosan) shows activity at 0.42 μM. Thus the tertiary amine (**5**) as well as the quaternary ammonium salt of triclosan (**6**) showed more activity as compared to triclosan against *E. coli*.

For the inhibition of the growth of *E. coli* cultures succinamic ester of triclosan (compound **7**) exhibited IC_{50} and MIC of 0.22 and 0.6 μM, respectively. It was noted that compound **7** is 3.5 times more active than triclosan. This may be due to the presence of a polar (–CO–NH_2) group at the end of succinamic ester of triclosan. Replacing NH_2, which is present at one of the termini of succinamic acid of triclosan **7**, with methyl group (–CH_3) or a non-polar polar group (levulinic acid, compound **9**) results in decrease in the activity. However, when NH_2 group of compound (**7**) is replaced by hydroxyl group (–OH; glutaryl ester of triclosan **8**), the potency exhibited is twice that of triclosan. This indicates

that the presence of polar groups at termini of the aliphatic esters of triclosan increases the activity of the prodrug in *E. coli* culture. This is further confirmed by the fact that when this terminus of ester is replaced by non-polar group (s) the activity of compounds decreases dramatically (Table 1). In case of *E. coli* compounds **5**, **6**, **7**, and **8** have better inhibition as compared to triclosan. Improved inhibition in **5**, **6**, **7**, and **8** was due to the presence of tertiary amine (–N–$(CH_3)_2$), quaternary amine (–N–$(CH_3)_3$), amido (–$CONH_2$), and carboxy (–COOH) groups, which are at the termini of respective ester.

With *P. falciparum* cultures, while triclosan inhibited growth of the cultures with an IC_{50} of 2.29 μM and MIC = 17.6 μM, compounds **5**, **8**, **9**, and **12** showed inhibition in the low micromolar range (Table 1). Improved inhibition in **5**, **8**, **9**, and **12** was due to the presence of tertiary amine (–N–$(CH_3)_2$), carboxy (–COOH), and keto (–$COCH_3$) group, which are at the termini of respective ester. Compounds **6**, **7**, **10**, and **13** inhibited cultures of the malaria parasite at high micromolar concentrations (Table 1).

The tertiary amine conjugate of triclosan (**5**) displays enhanced inhibition against both *E. coli* and *P. falciparum* cultures indicating that dimethylaminoethyl-glutaryl esters of triclosan (tertiary amine conjugate of triclosan) have the proclivity to diffuse rapidly across the biological membranes and are transformed to mono-protonated derivatives within *E. coli* and *P. falciparum*. Once they are protonated they are less permeable, and are perhaps trapped within the organisms increasing their accumulation. These findings are consistent with the earlier observations which show that accumulation of other anti-malarials within the parasite leads to improvement in their potencies.[28]

Previously in the literature it is reported that 5 (and 6)-carboxy-2,7,-dichloro di-acetate carries only one negative charge and is permeable to cells.[29] Similarly quinolone derivatives bearing free carboxylic acid have also been reported (viz. quinoline-3-carboxylic acid) that show more activity as compared to their uncharged parent compounds against bacterial and malarial strains.[30,31] In our case also carboxylic derivative (**8**) has shown good activity against both *E. coli* and *P. falciparum* cultures (Table 1). Although it is premature at this stage to speculate about any specific mechanism for the greater potency of compound (**8**), its observed potency is not surprising in view of the above reports.

Prodrugs were incubated with *E. coli* cultures for various time intervals. It was found that most of the inhibitor is taken up by the bacteria within minutes and hardly any amount of the ester used in these studies is observed in the culture supernatant as evident from the FPLC (Fig. 2B). On the contrary even after eight hours very little of prodrugs (**9** and **10**) is able to penetrate the cell explaining their poor activity. It is evident from the results that the better inhibition of these prodrugs is due to their increased permeability.

In *P. falciparum* pro-drug (**5**) entered the cell in 6 h (Fig. 3B) and the observed half-life of the pro-drug disappearance in *Plasmodium* is ~9.45 h (Fig. 3C). Similarly most of the other active compounds are permeable inside the cell within 6 h after that conversion of prodrugs to drug begins. The problem of delivery across the many membranes enveloping the malaria parasite could be a reason for the delay in their entry into the parasite.

We have tested these prodrugs for their inhibitory activity against ENRs and found that none of them possess the activity. Incubation of these prodrugs with *P. falciparum* and *E. coli* culture results in hydrolysis of ester linkage of these prodrugs releasing triclosan (Figs. 2A and 3A). Triclosan thus released apparently targets FabI inhibiting the growth of *E. coli* and the malaria parasite.

5. Conclusion

Several derivatives of triclosan, to enhance their uptake by malaria parasite and *E. coli*, have been synthesized. All the covalent bonds in the prodrugs thus synthesized are hydrolyzable by enzymes present in the target organisms. This makes these derivatives promising prodrugs, which can get accumulated inside the target organism and hydrolyzed therein releasing the parent biocide. Many of the compounds from the prodrug series (**5**–**13**) have shown promising in vitro biological activity against malaria (*P. falciparum*) and bacteria (*E. coli*) establishing that the triclosan could be converted to biologically effective esterase-sensitive prodrug analogs. This in turn opens up new avenues for exploring suitably designed triclosan-based prodrugs as potential antimalarial and antibacterial agents.

6. Experimental

All the starting materials were obtained from Aldrich or Fluka and used as supplied. Solvents used for the chemical synthesis acquired from commercial sources were of analytical grade of the highest purity, and were used without further purification unless otherwise stated. Column chromatography was performed using 100–200 mesh silica gel, whereas all TLC (silica gel) development was performed on silica gel-coated sheets (Merck Kiesel 60 F254, 0.2 mm thickness). Both ^{1}H NMR and ^{13}C NMR spectra were recorded on 300 and 400 MHz Bruker NMR spectrometer using tetramethylsilane as internal standard and the chemical shifts are reported in (δ) units. The sample concentration in each case was approximately 10 mg in chloroform-**d** (0.6 mL). Mass spectra were recorded on ESI-MS (Bruker Daltonis, Esquire 3000^{plus}) instrument.

6.1. Pentanedioic acid mono-(2-dimethylamino-ethyl) ester (3)

To an anhydrous dichloromethane solution (5 mL) of dimethylaminoethanol (0.5 mL; 5 mmol) was added dropwise at 0 °C an anhydrous dichloromethane solution (5 mL) of glutaric anhydride (0.684 g; 6 mmol)

and *N*,*N*-di-isopropylethylamine (DIPEA) (0.95 mL; 5.5 mmol) and stirred overnight at room temperature. The reaction mixture was diluted with DCM (150) and washed with water (3 × 50 mL). Organic layer was dried over Na_2SO_4 and concentrated under reduced pressure, giving compound (**3**), which was purified by column chromatography on silica gel (eluent; chloroform/methanol 9:1) to give desired product as a white syrupy mass. Yield 0.792 g (78%), ^{1}H NMR (400 MHz, $CDCl_3$)δ 1.94 (m, 2H), 2.3 (m, 4H), 2.26 (s, 6H), 3.15 (t, *J* = 7.6 Hz, 2H), 4.25 (t, *J* = 5.6 Hz, 2H), 10.46 (s, 1H, carboxy). ^{13}CNMR (400 MHz, $CDCl_3$) δ 175.1, 171.0, 64.4, 63.66, 43.06, 32.3, 30.2, 17.4. Anal. Calcd for $C_9H_{17}NO_4$: C, 53.19; H, 8.43; N, 6.89. Found C, 52.93; H, 8.65; N, 6.86. ESI-MS *m*/*z* $[M + H]^+$ 204.10.

6.2. Pentanedioic acid 5-chloro-2-(2,4-dichloro-phenoxy)-phenyl ester 2-dimethylamino-ethyl ester (5)

Mono-(dimethyl aminoethyl)glutatrate (2.03 g, 10 mmol) and CDI (2.03 g, 10 mmol) were suspended in dichloromethane (10 mL) with stirring at 25 °C under nitrogen for 1 h. Triclosan (2.895 g; 10 mmol) was added to this reaction mixture and stirred for another 3 h. Completion of the reaction was monitored on TLC and after completion the reaction mixture was poured in water and extracted with DCM. Organic layer was dried over Na_2SO_4, and concentrated in vacuum. The crude product was chromatographed on silica gel column, eluted with chloroform/methanol (9:1), Yield 2.554 g (54%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.9(m, 2H), 2.27 (s, 6H), 2.3–2.56 (m, 4H), 3.18 (t, J = 7.2 Hz, 2H), 4.16 (t, *J* = 5.6 Hz, 2H), 6.82 (m, 2H, *Ar-H*), 7.15 (m, 3H, *Ar-H*), 7.4 (d, *J* = 8.0 Hz, 1H, *Ar-H*). ^{13}CNMR (400 MHz, $CDCl_3$) δ 170.1, 169.8, 148.4, 144.1, 139.2, 127.9, 127.0, 126.7, 125.6, 124.4, 123.4, 121.8, 117.8, 117.5, 64.6, 63.7, 43.0, 32.5, 30.0, 17.4. Anal. Calcd for $C_{21}H_{22}Cl_3NO_5$: C, 53.13; H, 4.67; N, 2.95. Found C, 53.17; H, 4.69; N, 2.95. ESI-MS *m*/*z* $[M+H]^+$ 474.10.

6.3. (2-{4-[5-Chloro-2-(2,4-dichloro-phenoxy)-phenoxy-carbonyl]-butyryloxy}-ethyl)-trimethyl-ammonium chloride (6)

The dimethylaminoethyl-glutaryl esters of triclosan (compound **5**, 0.946 g; 2 mmol) dissolved in the minimum amount of dry THF was treated with methyl iodide (0.71 g; 0.32 mL; 10 mmol). Although a solid product separated in the very first few minutes, stirring at room temperature continued for another 3 h. The reaction mixture was left overnight at 4 °C, and then the precipitate was filtered off, washed with several portions of dry ether followed by drying in vacuum. Iodide salts were used in ion exchange without additional purification. Ion exchange chromatography on a Sephadex column (chloride form, DEAE, A25) with methanol as eluent afforded white (waxy) solid. The residue was further purified by flash chromatography over silica Merck-60, eluting with a gradient (from 1:9 to 4:6) of methanol in ethyl acetate to provide **6** (1.001 g, 82% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.9(m, 2 H), 2.4–2.6 (m, 4H), 3.2 (s, 9H), 3.27 (t, *J* = 7.8 Hz, 2H), 4.15 (t, *J* = 5.6 Hz, 2H), 6.9 (m, 2H, *Ar-H*), 7.2–7.3 (m, 3H, *Ar-H*), 7.5 (d, *J* = 8.6 Hz, 1H, *Ar-H*). ^{13}C NMR (400 MHz, $CDCl_3$) δ 171.4, 169.6, 152.4, 147.9, 143.5, 131.4, 130.5, 129.6, 129.2, 128.3, 125.6, 121.5, 120.1, 118.5, 64.2, 63.8, 49.03, 33.9, 33.6, 20.8. Anal. Calcd for $C_{22}H_{25}Cl_4NO_5$: C, 50.31; H, 4.80; N, 2.67. Found C, 50.42; H, 4.74; N, 2.65. ESI-MS *m*/*z* $[M-Cl]^+$ 488.11.

6.4. Succinamic acid 5-chloro-2-(2,4-dichloro-phenoxy)-phenyl ester (7)

To a solution of succinamic acid (**5**) (0.234 g, 2 mmol) in dry DMF (5 mL) was added CDI (0.324 g, 2 mmol) with stirring at room temperature under N_2. After evolution of CO_2 was complete (2 h), triclosan (**4**) (0.579 g, 2 mmol) was added and the reaction proceeded for an additional 24 h. The mixture was concentrated in vacuo to a white solid and washed with H_2O followed by CCl_4. The resulting solid was purified on a silica gel column; elution with EtOAc/methanol (9:1) gave **7**as a white crystalline solid (0.529 g, 68% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.55 (t, *J* = 6.8 Hz, 2H), 2.82–2.92 (m, 2H), 5.6 (d, *J* = 8.4 Hz, 2H), 6.67–6.90 (m, 2H, *Ar-H*), 7.15–7.26 (m, 4H, *Ar-H*). ^{13}C NMR (400 MHz, $CDCl_3$) δ 172.4, 169.9, 152.0, 148.1, 143.2, 131.0, 129.5, 128.5, 126.9, 125.5, 121.6, 120.9, 119.9, 118.4, 32.6, 29.9. Anal. Calcd for $C_{16}H_{12}C_{l3}NO_4$: C, 49.45; H, 3.11; N, 3.60. Found C, 49.45; H, 3.10; N, 3.56. ESI-MS *m*/*z* $[M+Na]^+$ 410, $[M + K]^+$ 427.9.

6.5. Pentanedioic acid mono-[5-chloro-2-(2,4-dichloro-phenoxy)-phenyl] ester (8)

Triclosan (1.158 g; 4 mmol), glutaric anhydride (0.684 g; 6 mmol) and 4-(dimethyl amino)pyridine (16 mg; 0.13 mmol) were dissolved in 20 mL of dry dichloromethane and left overnight at room temperature. The reaction mixture was diluted with dichloromethane (100 mL) and washed with water. The organic layer was dried over sodium sulfate and solvent removed by vacuum evaporation. The white solid was purified by silica gel chromatography (eluent: chloroform/methanol 10:1 to 5:1). Yield 0.900 g (56%), ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.98 (m, 2H), 2.40 (*J* = 4.8 Hz, 2H), 2.55 (t, *J* = 7.2 Hz), 6.8 (m, 2H, *Ar-H*), 7.1–7.4 (m, 4H, *Ar-H*), 10.6 (s, 1H, carboxy). ^{13}C NMR (400 MHz, $CDCl_3$) δ 176.5, 170.2, 150.8, 146.5, 141.6, 130.4, 129.4, 128, 126.9, 125.8, 124.3, 121.3, 120.5, 119.5, 32.8, 32.5, 19.2. Anal. Calcd for $C_{17}H_{13}C_{l3}O_5$: C, 50.59; H, 3.25. Found C, 50.45; H, 3.34. ESI-MS *m*/*z* $[M+K]^+$439.2.

6.6. 4-Oxo-pentanoic acid 5-chloro-2-(2,4-dichloro-phenoxy)-phenyl ester (9)

To a solution of levulinic (0.255 mL, 2.5 mmol), a few drops of DMF, and 20 mL CH_2Cl_2 were added followed by oxalyl chloride (0.435 mL, 5.0 mmol) dropwise at room temperature. After stirring at room temperature for 1 h, the mixture was concentrated to dryness. Then solution of triclosan (868 mg, 3 mmol), DIPEA (0.522 mL, 3.0 mmol), and 10 mL CH_2Cl_2 was added dropwise, and the reaction mixture was stirred at room

temperature overnight. After dilution with water and extraction with ethyl acetate, the organic extracts were dried over anhydrous Na_2SO_4 and evaporated to provide a light yellow syrupy mass. The residue was purified by silica gel chromatography, eluting with a gradient (from 1:1 to 6:4) of ethyl acetate in hexane to provide **9**(0.730 g, 63% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.1 (s, 3H), 2.7–2.8 (m, 4H), 6.8 (m, 2H, *Ar-H*), 7.1–7.2 (m, 3H, *Ar-H*), 7.4 (d, J = 2.4 Hz, 1H, *Ar-H*). ^{13}C NMR (300 MHz, $CDCl_3$) δ 205.9, 170.3, 150.9, 146.5, 141.7, 130.3, 129.4, 129.2, 128.1, 126.9, 125.7, 124.4, 120.2, 119.1, 36.7, 29.1, 27.6. Anal. Calcd for $C_{17}H_{13}Cl_3O_4$: C, 52.67; H, 3.38. Found C, 52.65; H, 3.35. ESI-MS *m/z* [M+Na] $^+$ 408.9.

6.7. Pentanoic acid 5-chloro-2-(2,4-dichloro-phenoxy)-phenyl ester (10)

To a solution of pentanoic acid (0.432 mL, 4 mmol) and 20 mL CH_2Cl_2 was added oxalyl chloride (0.87 mL, 10 mmol) dropwise at room temperature. After stirring at room temperature for 1 h, the mixture was concentrated to dryness. A solution of triclosan (1.158 g, 4 mmol), DIPEA (0.835 mL, 4.8 mmol), and 15 mL CH_2Cl_2 was added dropwise, and the reaction mixture was stirred at rt overnight. After dilution with water and extraction with ethyl acetate, the organic extracts were dried over anhydrous Na_2SO_4 . The solvent was evaporated in vacuo, yielding viscous syrupy mass that was purified on silica gel column eluted with ethyl acetate/hexane (15:85). Final yield was 1.116 g, (75%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.9 (t, J = 7.5 Hz, 3H), 1.2–1.4 (m, 2H), 1.5–1.6 (m, 2H), 2.4 (t, J = 7.5 Hz, 2H), 6.8 (m, 2H, *Ar-H*), 7.1–7.2 (m, 3H, *Ar-H*), 7.4 (d, J = 2.4 Hz, 1H, *Ar-H*). ^{13}C NMR (300 MHz, $CDCl_3$) δ 170.1, 154.5, 148.6, 144.0, 128.9, 127.8, 126.8, 125.5, 124.3, 123.2, 121.9, 119.8, 117.4, 32.4, 26.2, 20.5, 12.8. Anal. Calcd for $C_{17}H_{15}Cl_3O_3$: C, 54.64; H, 4.05.Found C, 54.46; H, 4.67. ESI-MS *m/z*$^+$ [M+H] 373.4.

6.8. 3-[5-Chloro-2-(2,4-dichloro-phenoxy)-phenoxy]-pyridine (12)

Nicotinoyl hydrochloride **11** (3 mmol) was added in small amounts to a refluxing solution of triclosan (**4**, 2.5 mmol)in toluene (20 mL). Triethylamine (0.7 mL; 7.0 mmol) was then added dropwise, and the mixture was refluxed overnight. After filtration, the solvents were removed and resulting yellow-white solid was purified on Al_2O_3 (act. II–III) with a dichloromethane/hexane (1:1) to pure dichloromethane gradient, followed by crystallization from acetonitrile to afford esters **12** as white solid. Yield 0.903 g (92%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.92 (m, 2H, *Ar-H*), 7.15–7.25 (m, 3H, *Ar-H*), 7.35 (d, J = 8.6 Hz, 1H, *Ar-H*), 7.47 (m, 1H, *py*), 8.35 (d, J = 5.9 Hz, 1H, *py*), 8.9 (d, J = 7.9 Hz, 1H, *py*), 9.27(s, 1H, *py*). ^{13}C NMR (400 MHz, $CDCl_3$) δ 164.7, 154.2, 151.3, 150.8, 146.7, 141.3, 137.5, 130.4, 129.7, 129.2, 128.1, 127.3, 126.0, 124.4, 123.3, 120.5, 120.1, 119.1. Anal. Calcd for $C_{18}H_{10}Cl_3NO_3$: C, 54.78; H, 2.55; N, 3.55. Found C, 54.74; H, 2.77; N, 3.54. ESI-MS *m/z* [M+H] $^+$ 394.12.

6.9. 3-[5-Chloro-2-(2,4-dichloro-phenoxy)-phenoxy]-1-methyl-pyridinium (13)

A solution of pyridine ester 11 (1 mmol) and methyl iodide (9 mmol) in dry acetone (20 mL) was refluxed for 5–6 h under nitrogen, with the progress being monitored by TLC. The mixture was taken to dryness and the crude iodide salts were used in ion exchange without additional purification. Ion exchange chromatography on a Sephadex column (chloride form, DEAE, A25) with methanol as eluent afforded light yellow (waxy) solids. That was purified on silica column chromatography (Merck-60), eluting with a gradient (from 1:9 to 3:7) of methanol in ethyl acetate to get desired compound. Yields (70%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.06 (s, 3H, –N *CH*$_3$), 6.76–6.85 (m, 3H, *Ar-H*), 7.03–7.18 (m, 2H, *Ar-H*), 7.42 (d, J = 8.0, 1H, *Ar-H*), 7.58 (m, 1H, *py*), 8.32 (d, J = 6.2 Hz, 1H, *py*), 9.15 (d, J = 8.08 Hz, 1H, *py*), 9.42 (m, 1H,*py*). ^{13}C NMR (400 MHz, $CDCl_3$) δ 164.4, 154.6, 151.2, 151.0, 146.2, 141.1, 136.8, 130.4, 129.5, 129.1, 128.4, 128.1, 126.2, 124.1, 129.9, 120.5, 120.2, 119.4, 49.2. Anal. Calcd for $C_{19}H_{13}Cl_4NO_3$: C, 51.27; H, 2.93; N, 3.15. Found C, 51.27; H, 2.93; N, 3.17. ESI-MS *m/z* [M+H] $^+$ 444.01.

6.10. Determination of antimicrobial activity

The whole-cell antimicrobial activity of the test compounds was determined by micro-dilution method. About 10 mM stock solutions of triclosan and its derivatives were made in DMSO. Wild-type *E. coli* K12 was grown in LB broth until the mid-log phase and then diluted hundred times in the same medium. One hundred microliters of this cell suspension was used to inoculate each tube containing 10 mL of the medium with the inhibitors. The tubes were incubated at 37 °C for 12 h. Turbidity was measured by the OD taken at a time gap of every 2 h at 600 nm. The absorbance values were normalized to the solvent-treated control.

6.11. Assessment of inhibition of growth of *P. falciparum* and determination of IC_{50} of the compounds

The experiments were performed using *P. falciparum* FCK2 strain (chloroquine-sensitive, IC_{50}, 18 nM), an isolate from Karnataka, India. *P. falciparum* was cultured using standard techniques[32] and synchronized using 5% sorbitol[33] at 4% hematocrit in RPMI 1640 medium (Invitrogen, CA, USA) supplemented with 10% human serum, 0.225% sodium bicarbonate, and 0.01 mg/mL of gentamicin. Growth inhibition was monitored using microscopic examination of the parasites by standard Giemsa staining. Typically uninfected or infected (1–2% parasitaemia, ring stage) red blood cells (2% hematocrit) were added to the culture medium in the wells of a 96-well plate (Nunc, Roskilde, Denmark), and different concentrations of inhibitor in Me_2SO did not exceed 0.05%. The experiment started with the synchronized parasite culture in the early trophozoite stage and inhibitors were added up to the fourth day. Solvent controls as well as triclosan as positive control were included. *P. falciparum* growth was compared with solvent control. The IC_{50} was calculated from a plot of relative

percent parasitemia versus the log concentration of the inhibitor by fitting it to non-linear regression analysis using Sigma Plot 2000 software (Systat Software Inc., CA, USA).

6.12. Assessment of inhibition of growth of *P. falciparum* and determination of IC_{50} of the compounds by [^{3}H]-hypoxanthine uptake assay

IC_{50} was also determined by the [^{3}H]-hypoxanthine uptake assay. The parasites were cultured using standard techniques as described above for the microscopic examination. The semi-automated microdilution technique of Desjardins et al., which is based on [^{3}H]-hypoxanthine uptake by parasites, was used to assess the sensitivity of the parasites to the various inhibitors.[34] Briefly, synchronized parasites were cultured in 96-well plates (Nunc, Copenhagen, Denmark) at 2–3% hematocrit and at an initial parasitemia of 1–2%, with varying concentrations of the inhibitors, and addition of the inhibitor in fresh medium every 24 h for 48 h. All additions were done in duplicate. Inhibitor stocks were made in sterile DMSO and dilutions made such that the final concentration of DMSO in the parasite culture did not exceed 0.1%. Parasites synchronized at the ring stage were cultured in the presence of varying concentrations of the inhibitors for the first 48 h and then incubated with [^{3}H]-hypoxanthine (1 μCi/well) for the next 36 h and harvested. They were then harvested using a Nunc cell harvester onto glass fiber filters, washed and subjected to liquid scintillation counting (Hewlett–Packard). IC_{50}s were calculated from plots of relative percent parasitemia versus the log concentration of the respective inhibitors, fitted to non-linear regression analysis using Sigma Plot 2000 software. The IC_{50} values using this method were similar to those obtained using microscopic examination.

6.13. Conversion of triclosan esters to parent molecule triclosan in vivo after the treatment with *E. coli* and *P. falciparum*

The conversion of pro-drug to drug was checked on *E. coli* and *P. falciparum* cultures. *E. coli* cells were treated with pro-drug for different time points (0, 2, 5, 10, and 20 min). Cells were harvested at 6000 rpm for 15 min at 4 °C, washed twice with Luria Broth, and the resultant pellet was resuspended in 50 mM Tris, pH 7.5. The cell suspension was sonicated (Vibra-Cells, Sonics and Materials). Cell debris was removed by centrifugation at 16,000 rpm for 30 min at 4 °C. Solid-phase extraction was done by using SEP-PAK columns (Millipore Waters). The columns were prepared by passing 3 ml of methanol and then 1 ml of water through them. After the sample was loaded, the column was rinsed with 1 ml of distilled water and dried under vacuum for 5 min. The samples were eluted with 700 μl of methanol. The eluate was diluted 1:1 in distilled water, loaded on a C_{18}reverse-phase column (4.6 mm by 25 cm), and eluted with an isocratic gradient of acetonitrile by using FPLC-AKTA Basic (Amersham Pharmacia). The absorbance was measured at 230 and 240. However, in case of *P. falciparum* culture synchronized parasites at young trophozoite stage were treated with pro-drug for different time points (0, 0.5, 3, 6, 12, 18, 24, and 36 h). Parasites were isolated and resuspended in PBS. Solid-phase extraction and FPLC assay have been done using the above protocol for *P. falciparum* cultures also.

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