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Pharmacomodulation on the 3-acetylursolic acid skeleton: Design, synthesis, and biological evaluation of novel *N*-{3-[4-(3-aminopropyl)piperazinyl]propyl}-3-*O*-acetylursolamide derivatives as antimalarial agents

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Abstract—A series of new piperazine derivatives of ursolic acid was synthesized and tested against *Plasmodium falciparum* strains. They were also tested on their cytotoxicity effects upon MRC-5 cells. Seven new piperazinyl analogues showed significant activity in the nanomolar range (IC_{50} = 78–167 nM) against *Plasmodium falciparum* CQ-resistant strain FcB1. A possible mechanism of interaction implicating binding of these compounds to β-hematin was supported by in vitro tests. Moreover, the importance of the hydrophilic framework attached at the terminal nitrogen atom of the bis-(3-aminopropyl)piperazine joined to the triterpene ring was also explored through molecular dynamic simulations.

1. Introduction

Malaria is one of the 10 most prevailing and fatal infectious diseases of the world and has been a public health problem in about 90 countries; whereas, approximately 40% of the world population is under risk of contamination. It causes between 1.2 and 2.7 million deaths each year.[1] Four species of *Plasmodium* cause malaria in human beings; however *P. falciparum* is the most dangerous of these infections.[2] Until now, several of the drugs used as antimalarial agents were developed from natural sources, like quinine, artemisinin, and their derivatives. Nevertheless, due to the emergence of resistant *Plasmodium* strains, the therapeutic impact of such compounds has been diminished creating a great urge to the development of new and effective antimalarial drugs.[3]

An additional group of natural products showing promising antimalarial activity can be found in saponins, naturally amphiphilic compounds composed by an aglycone (triterpenic or steroidal) and by carbohydrate chains attached by an ether or ester linkages. Saponins, as well as triterpenes, have been investigated for numerous biological activities such as anti-inflammatory,

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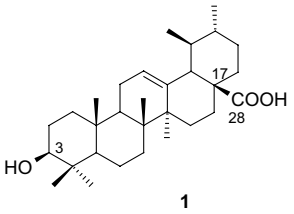

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Figure 1. Ursolic acid.

antimalarial, and anti-HIV.[4–8] Our group has been working for decades on saponins from South American *Ilex* species in which ursolic acid (**1**) (Fig. 1) is found free or as aglycone of these saponins.[9–11] In relation to antimalarial activity, ursolic acid (**1**) produced suppression of parasitemia against *P. berghei berghei* in mice.[12] Additionally, **1** was also reported to be capable to reduce parasite proliferation against *P. falciparum* 3D7, W2, and K1 strains in vitro.[8,13] Furthermore, the potential of triterpenoid scaffolds as pharmacophoric groups for the development of new antimalarial agents has been evidenced by other groups.[14,15]

Recently, the advancement of molecular biology techniques has improved the understanding of the biochemistry of malaria parasites concurring to the identification of potential targets for new drugs.[16] Considering the aminoalkyl side chain as an important requirement for a strong antimalarial activity,[17] the 1,4-bis(3-aminopropyl)piperazinyl function was identified as a promising pharmacophoric group associated with antimalarial activity against both chloroquine (CQ) sensitive and resistant strains of *P. falciparum*.[18–21] This was related to possible interactions of protonated piperazine nitrogen atoms with the carboxylate moiety of heme; thus, promoting the inhibition of hemozoin formation and preventing heme detoxification in the digestive parasite's vacuole.[21]

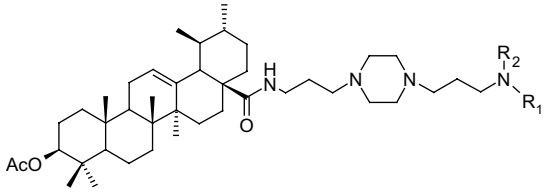
Hence, we describe the synthesis of a new series of derivatives showing promising antimalarial activities based on the condensation of ursolic acid (**1**) and 1,4-bis(3-aminopropyl)piperazine. While such approach explores the postulated interaction between polyamines and heme carboxylate groups, it evaluates the possible involvement of triterpenoid scaffold in inhibition of β-hematin formation. Additionally, since the major role of aromatic rings in compounds as chloroquine has been associated to π–π stacking with heme molecules, the replacement of π-interacting by non-π-interacting structural moieties on antimalarial agents has a potential to point new directions in the search of novel compounds, useful in the treatment of malaria. Moreover, we further explore the structure–activity relationship (SAR) on this series of compounds through molecular dynamic (MD) simulations of the packing between heme and antimalarial agents.

2. Results and discussion

2.1. Chemistry

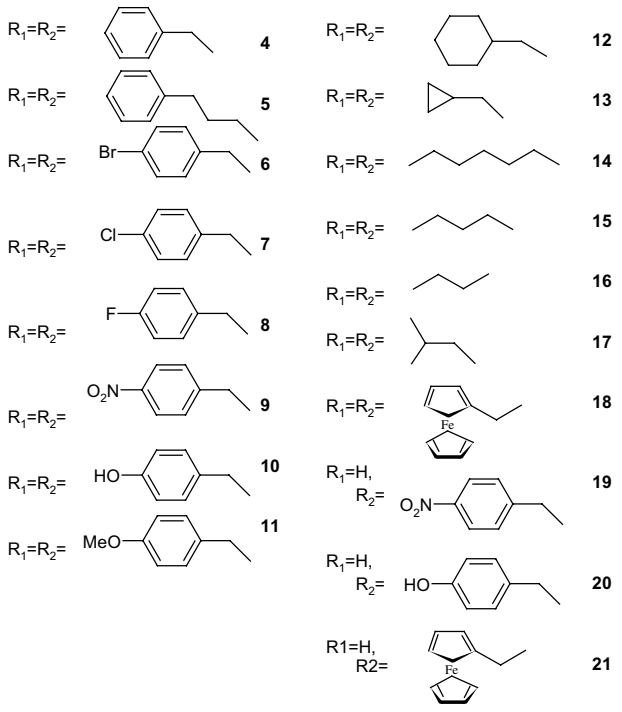
The source of ursolic acid (**1**) was the leaves of *Ilex paraguariensis*, a native plant in South America, that were submitted to maceration with EtOH 70% to provide crude saponin residue. This residue was submitted to acid hydrolysis to obtain the major aglycone (**1**) that was purified as previously mentioned.[10,22] Ursolic acid (**1**) was converted to its acetyl ester **2** in quantitative yield. The promising antimalarial activity obtained for this 3-acetylursolic acid (**2**) (24.93 μM for **2** versus 52.93 μM for **1**, Table 1) led us to use this skeleton as our bioactive pharmacophore for further pharmacomodulation. Thus, coupling reaction of **2** with *N*-Boc-bisaminopropylpiperazine and triethylamine (TEA) was performed after activation of the carboxylic acid at C-17 with oxalyl chloride. *N*-Boc-amino protecting group was, then, removed by a treatment with a trifluoroacetic acid/dichloromethane (TFA 10%/CH_2Cl_2) mixture to result into the deprotected analogue **3**. Reductive amination conditions using sodium triacetoxyborohydride ($NaHB(OAc)_3$)as the reducing agent and appropriate aldehydes were used to obtain compounds **4**–**21** (Scheme 1), according to the literature.[20,23] Ten derivatives were synthesized using aromatic (**4**–**11**, **19**, and **20**) and six with aliphatic (**12**–**17**) aldehydes, while two compounds were obtained by the reaction with ferrocenecarboxaldehyde (**18** and **21**).

Table 1. In vitro sensitivity of *P. Falciparum* FcB1 strain to compounds **1**–**21**



Compound	R_1	R_2	IC_{50} FcB1 (μM)
1			52.93 ± 5.71
2			24.93 ± 7.44
Chloroquine			0.13 ± 0.04
3	R_1=R_2=	H	0.17 ± 0.07
3a	R_1=R_2=	H	0.32 ± 0.18
4	R_1=R_2=	Benzyl	21.12 ± 2.90
5	R_1=R_2=	Benzylethyl	8.06 ± 0.65
6	R_1=R_2=	4-Bromobenzyl	>100
7	R_1=R_2=	4-Chlorobenzyl	46.04 ± 10.76
8	R_1=R_2=	4-Fluorobenzyl	20.27 ± 5.46
9	R_1=R_2=	4-Nitrobenzyl	3.57 ± 1.47
10	R_1=R_2=	4-Hydroxybenzyl	0.16 ± 0.02
11	R_1=R_2=	4-Methoxybenzyl	6.50 ± 1.74
12	R_1=R_2=	Methylcyclohexyl	>100
13	R_1=R_2=	Methylcyclopropyl	0.49 ± 0.21
14	R_1=R_2=	Heptyl	41.81 ± 6.72
15	R_1=R_2=	Butyl	1.00 ± 0.12
16	R_1=R_2=	Propyl	0.80 ± 0.13
17	R_1=R_2=	*tert*-Butyl	18.77 ± 1.14
18	R_1=R_2=	Methylferrocenyl	1.21 ± 0.46
19	R_1=H	R_2=4-nitrobenzyl	1.04 ± 0.33
20	R_1=H	R_2=4-hydroxybenzyl	0.08 ± 0.04
21	R_1=H	R_2=methylferrocenyl	0.77 ± 0.09

3a, compound **3** not acetylated.



In the aromatic series, substitution on the phenyl ring at *para* position was evaluated. The presence of electron-donating groups, such as OH (**10**) or OMe (**11**); or an electron-withdrawing group as NO_2 (**9**); and halogens as F (**8**), Cl (**7**) or Br (**6**), was studied. In the aliphatic series, two cyclic derivatives **12** and **13** were synthesized bearing a cyclohexyl and a cyclopropyl group, respectively. The effect of the elongation of the chain was studied in compounds **14**–**16** together with one compound **17** with branched chain. Finally, two ferrocenyl derivatives **18** and **21** were also synthesized.

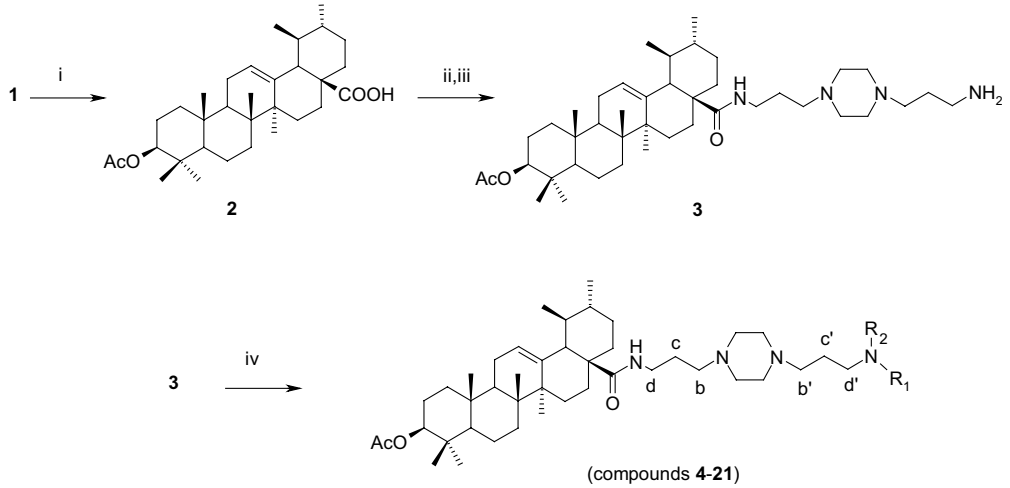
As far as we know, the syntheses of the new *N*-{3-[4-(3-aminopropyl)piperazinyl]propyl}-3-*O*-acetylursolamide derivatives, using a triterpene skeleton as pharmacophore, were accomplished for the first time.

2.2. Pharmacological

2.2.1. Antimalarial activity. All compounds were evaluated for their in vitro antimalarial activities against *P. falciparum* CQ-resistant strain FcB1 ($IC_{50}CQ$ = 130 nM). As shown in Table 1, piperazinyl derivatives (**3**–**21**) from ursolic acid (**1**) were all more active than their unsubstituted parent ($IC_{50(FcB1\ strain)}$ **1** = 52.93 μM), corroborating the relevance of the 1,4-bis(3-aminopropyl)piperazinyl group to the antimalarial activity. Additionally, **3**, **10**, and **20** (170, 160, and 78 nM, respectively) showed equivalent or superior activities when compared to chloroquine (130 nM), indicating that hydrogen bonding donor capabilities may be related to the SAR of our new compounds **1**–**20**. Moreover, the role of acetate at position 3 of steroids or triterpenoids is under investigation as already mentioned.[24]

For compounds lacking such interaction, the activity appeared to be mainly related to the lipophilicity of the substituent group, as can be observed for aromatic derivatives **6** (98 μM), **7** (46.04 μM), **4** (21.12 μM), **8** (20.27 μM), and **9** (3.57 μM). Moreover, not only lipophilicity but also the flexibility of the substituent group appeared to be related to activity, as shown with the 2.5-fold increase in activity from compound **4** to its superior homologue **5**. A similar profile could be observed between cyclic (less active) and acyclic (more active) substituents, indicating that the capability of the group attached to R_1 and R_2 for adopting specific conformations may be an important structural attribute for an antimalarial activity in this set of newly synthesized compounds.

The effect of an antimalarial activity related to the introduction of ferrocenyl moiety to chloroquine and artemisinin derivatives was previously studied, including a structure–activity relationship.[25–29] Unfortunately, in our series, the antimalarial activity of compounds bearing the ferrocene moiety (**18**, **21**) was not noticeable in comparison to the 4-hydroxybenzyl derivative **20**.



Scheme 1. Synthesis of ursolic acid derivatives (**2**–**21**). Reagents: (i) pyridine, acetic anhydride; (ii) oxalyl chloride, *N*-Boc-bisaminopropylpiperazine, TEA, CH_2Cl_2; (iii) TFA 10%/CH_2Cl_2; (iv) RCHO, $NaHB(OAc)_3$.

2.2.2. Assays using P. falciparum chloroquine-sensitive strain Thai and MRC-5 cells' cytotoxicity. Based on the results obtained for compounds tested in *P. falciparum* FcB1 strain (Table 1), the most active compounds were, then, assayed against the *P. falciparum* chloroquine-sensitive strain Thai (Table 2). In addition, they were also tested against a human diploid embryonic lung cell line (MRC-5 cell) in order to evaluate their cytotoxicity, allowing to obtain the resistance index FcB1/Thai and the selectivity index FcB1/MRC-5 (Table 2).

The most active ursolic acid derivatives against *P. falciparum* CQ-resistant strain FcB1 were also the most active against sensitive Thai strain, suggesting low levels of cross-resistance to CQ. The resistance index calculated from the ratio of the IC_{50} values of the sensitive and resistant strains of *P. falciparum* was in the range of 0.37–1.17, lower than that observed for chloroquine (RI = 11).

All tested compounds showed cytotoxicity upon MRC-5 cells in the μM range and, IC_{50} values varied from 1.57 to 18.28 μM. The selectivity index was defined as the ratio of the IC_{50} value on the MRC-5 cells to the IC_{50} value on the CQ resistant *P. falciparum* strain FcB1. The compound **20** presented the highest selectivity among the compounds tested with a selectivity index of 27 and could be further developed as a new lead for pharmacological investigations.

2.2.3. Inhibition of β-hematin formation test. Moreover, compounds **1**,**2**, **3**, **9**, and **10** were also submitted to an in vitro evaluation of their capabilities to inhibit β-hematin formation in order to further explore the mechanism of action of these newly synthesized ursolic acid derivatives. Then, the obtained results were compared to chloroquine. Considering the results obtained for the most active compounds, **3** and **10** (Table 3), it can be postulated that most of the antimalarial activity observed for compounds **1**–**21** is due to the inhibition of heme assembly in β-hematin. Such mechanism is in agreement with previous works suggesting the role of piperazine nitrogen atoms in complexation to heme carboxylate groups.[21]

2.3. Structure–activity of ursolic acid derivatives

The strategy employed in the design of derivatives **3**–**21** resulted in compounds with biological activities equivalent or superior to chloroquine. This strategy used hybridization of triterpenic skeleton of ursolic acid (**1**) and 1,4-bis(3-aminopropyl)piperazine- both potential antimalarial pharmacophores- followed by a reductive amination in order to obtain compounds with different groups at the terminal nitrogen atom of the second pharmacophore. After the biological evaluation, we proceeded to study the complexes formed by heme and the obtained ursolic acid derivatives through molecular modeling techniques in order to elucidate their structure–activity relationship (SAR) at the atomic level.

Table 2. In vitro sensitivity of *P. falciparum* Thai strain and MRC-5

Compound	IC_{50} (μM)		Resistance index FcB1	Selectivity index
	Thai	MRC-5		
3	0.46 ± 0.04	1.61 ± 0.14	0.37	9.6
9	3.05 ± 0.27	18.28 ± 0.10	1.17	5.1
10	0.35 ± 0.03	1.57 ± 0.67	0.46	9.7
13	0.95 ± 0.01	2.53 ± 0.51	0.51	5.1
16	1.00 ± 0.55	1.96 ± 0.78	0.79	2.5
18	1.48 ± 0	4.08 ± 0.09	0.81	3.5
19	0.98 ± 0.21	5.39 ± 0.10	1.06	5.2
20	0.18 ± 0.03	2.10 ± 0.20	0.43	27
21	1.13 ± 0.12	2.00 ± 0.20	0.68	2.6
Chloroquine	0.01 ± 0.01	NA	11	NA

NA, not available.

Table 3. Inhibition of β-hematin formation

Compound	Concentration (mM)	Inhibition[a] (%)	IC_{50} (mM)	CQ index[b]
Chloroquine phosphate	5	36.9 ± 8.6	5.9 ± 1.15	1
	10	97.4 ± 0.2		
1	5	17.9 ± 8.6	≫20	≫3.4
2	1–20	0		
3	5	12.9 ± 3.1	>20	>3.4
	10	24.6 ± 3.1		
9	5.7	3.6 ± 4.86	≫20	≫3.4
	11.4	18.6 ± 5.84		
10	4.2	39.6 ± 25.5	5.25 ± 2.82	0.89
	8.4	82.2 ± 14.2		

[a] Mean ± standard deviation from three independent experiments.
[b] IC_{50} compound/IC_{50} chloroquine.

So, considering the previous evidences for polar interactions between basic nitrogen atoms from piperazine containing derivatives,[21] as well as the influence of substituents R_1 and R_2 on the activity of compounds **3**–**21**, molecular dynamic simulations (MD) in explicit aqueous solutions were employed in order to study the complexation of compounds with heme. Although such methodology does not include π–π interactions, it is, indeed, capable to describe the effect of solvent over the solvated compound conformations, diffusion, and complexation at a high level of accuracy.

In fact, MD has been recently employed to study the interaction of two Fe(III)PPIX molecules in both vacuum and aqueous solutions.[30,31] So, in order to complement such observations we decided for the use of randomly placed heme molecules as the initial configuration for the simulated system. As doing so, a spontaneous complexation between two heme molecules in water was observed (Fig. 2a). As far as we know, such observation is one of the first descriptions, at the atomic level, of a non-enzymatic and spontaneous formation of an intermolecular precursor of the β-hematin dimer in solution. Additionally, the so obtained conformational ensemble confirmed previous descriptions of an ~180° orientation between carboxylate groups, strongly supporting the proposal that aqueous Fe(III)PPIX dimers consist of coplanar back-to-back complexes between two porphyrins.[31] Besides, the distance between two heme molecules is in agreement with previous data.[32]

Since the nanosecond time scale simulations of heme molecules were capable to, adequately, describe the formation of β-hematin dimer we used the same approach

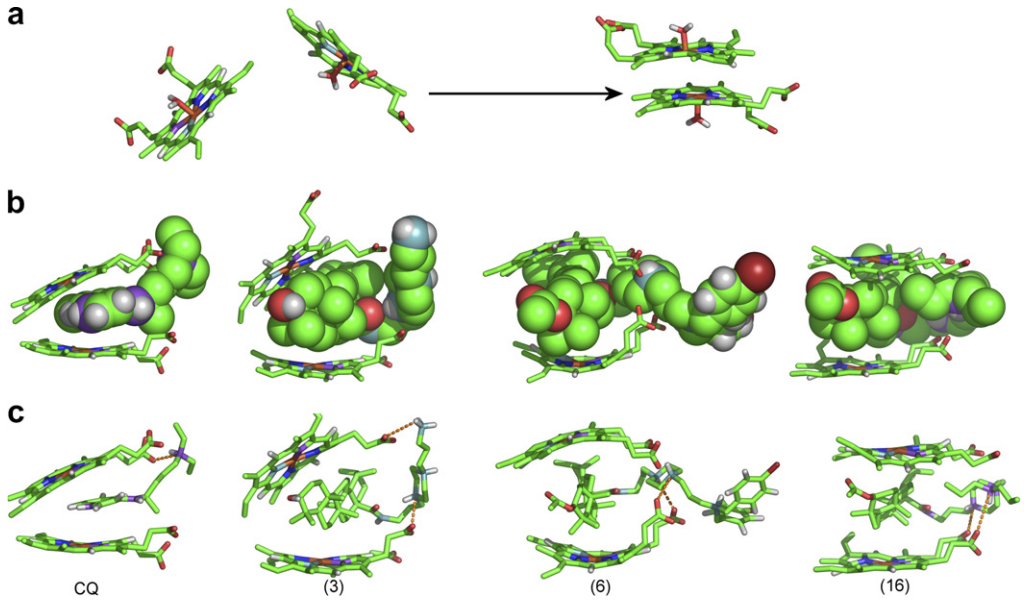


Figure 2. (a) Spontaneous association of heme molecules in aqueous solution during a MD simulation. (b and c) Complexes between heme and chloroquine (CQ), **3**, **6**, and **16**, obtained after spontaneous diffusion in solvent shown in space filing and tube representations, respectively.

to evaluate the interaction of heme with the synthesized compounds. Based on such approach, compounds **16**, **6**, **3** and chloroquine were observed to spontaneously interact with heme molecules by diffusion, forming stable complexes during the 10.0 ns trajectories (Fig. 2b and c). Furthermore, it is important to note that the geometry observed for the complex between chloroquine and heme is in agreement with the interatomic distances previously described to occur in such complex by NMR methods.[33]

Related to the ursolic acid derivative complexations to heme molecules, important details could be obtained from the performed simulations, confirming and expanding, at the atomic level, a SAR for this class of compounds, as follows (Chart 1):

1. The inverse relation between the hydrophobicity of the substituent attached to the terminal nitrogen atom of the *N*-[1,4-bis(3-aminopropyl)piperazinyl pharmacophoric group and the antimalarial activity could be explained on the basis of its capability to perform strong electrostatic interactions with carboxylate groups of heme molecules, that is, more hydrophobic and steric imposing groups would abolish or reduce such interaction and so decrease the activity of the compounds;
2. The molecular basis for the *N*-[1,4-bis(3-aminopropyl)piperazinyl pharmacophoric group can be rationalized in terms of its interaction with the carboxylate groups of heme: in systems composed only by heme molecules the carboxylate groups are in opposite orientations, when the heme molecules are packed with antimalarial agents, all the negatively charged groups show a re-orientation and point to the side where the positively charged amino groups are placed. As a result, several salt bridges can be performed simultaneously in solution between ligands and hematin. A similar scheme of interactions was recently proposed to occur with diethyl-amino-alkoxyxanthones,[34] supporting these observations;
3. One of the main interactions expected to take place in most of antimalarial agents evolves a π–π packing between the flat and aromatic rings from compounds

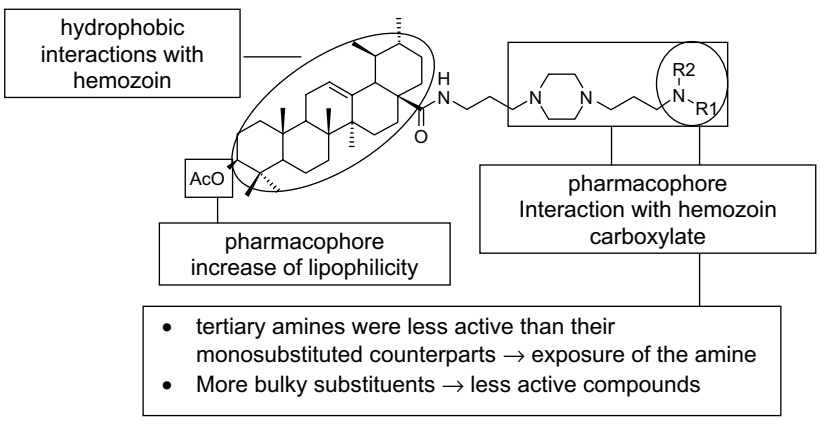


Chart 1. Structure–activity relationships of piperazinyl ursolic acid derivatives.

and heme. However, in molecules as artemisinin and the ursolic acid derivatives such interaction is not possible. At least for this last class of molecules, we observed that the substitution of an aromatic heterocyclic group by a triterpene skeleton is accompanied by the creation of a wide hydrophobic surface interacting with heme molecules (Fig. 2b). Besides, such group shows a perpendicular orientation related to the aromatic ring in compounds as chloroquine (Fig. 2c). That orientation can still be related to similar hydrophobic nucleus in complex with heme proteins.[35] If confirmed, such new orientation may represent a completely novel lead for future optimizations, exploring original interactions with heme and, mainly, the iron atom.

3. Conclusion

A series of new piperazine derivatives from ursolic acid were successfully synthesized through a rational design, based on the inhibition of β-hematin formation as one molecular mechanism of the malaria disease. These compounds were tested for their antimalarial in vitro activity upon the *P. falciparum* chloroquine-resistant strain FcB1 and, the most active compounds were tested against the *P. falciparum* chloroquine-sensitive strain Thai. Seven new piperazinyl analogues of ursolic acid (**1**) showed significant activity in the nanomolar range including compounds **20** (IC_{50} = 78 nM), **10** (IC_{50} = 161 nM), and **3** (IC_{50} = 167 nM), while chloroquine presented an IC_{50} of 130 nM. A comparison of the IC_{50} values related to inhibition of growth of the resistant and the sensitive strains of *P. falciparum* suggested low levels of cross-resistance to chloroquine. All tested compounds showed cytotoxicity upon the human diploid embryonic lung cell line (MRC-5 cells) in the micromolar range and, IC_{50} values varied from 1.57 to 18.28 μM. The most active compounds showed an in vitro inhibition of β-hematin formation, suggesting an effect on this target. These results indicate that they may share some similarities in their mechanism of action with chloroquine; but, this should be more deeply investigated.

Furthermore, the importance of the triterpene skeleton bearing an acetyl group at C-3 for antimalarial activity was demostrated and, the piperazine moiety was also identified as a required pharmacophore in this series. The importance of the hydrophilic framework attached at the terminal nitrogen atom of the bis-(3-aminopropyl)piperazine joined to the triterpene ring was also characterized through MD simulations, supporting a mechanism of action for these new ursolic acid derivatives on the binding of triterpene to heme. Based on the obtained results, the most active compounds could constitute suitable candidates for supplementary physicochemical and biological studies in order to use these molecules to improve our knowledge about the molecular mechanism involved in malaria. This means to understand their mechanism of action as antimalarial compounds and/or their capacity to interfere in the parasite mechanism of resistance. Finally, we believe that these results could provide suitable information for the development of new antimalarial compounds.

4. Experimental

4.1. Chemicals

All commercially available reagents were used without further purification unless otherwise stated. The solvents used were all of AR grade and were distilled under positive pressure of dry nitrogen atmosphere where necessary. All reactions were performed in pre-dried apparatus under an atmosphere of nitrogen unless otherwise stated. The progress of the reactions was monitored by analytical thin-layer chromatography (TLC) performed on Merck silica gel 60 F_{254} plates. Visualization was performed using phosphomolybdic acid in methanol. Column chromatography was carried out using matrix silica 60 (35–70 μm). The melting points were determined on a Kofler bench melting point apparatus and are not corrected. High-resolution spectra were obtained on a Micromass-Waters Q-TOF Ultima spectrometer. The IR spectra through the range from 4000 to 600 cm^{-1} were run on a Jasco FT/IR-4200 spectrometer. ^1H and ^{13}C NMR spectra were recorded on a Bruker AC 500 spectrometer operating at 500 and 125 MHz, respectively, with tetramethylsilane as internal standard, using deuterated chloroform. The chemical shifts are expressed as δ values in parts per million (ppm) and the coupling constants (J) are given in hertz (Hz). Yields were of purified compounds and were not optimized.

Dried-powdered leaves of *Ilex paraguariensis* A. St Hil. were submitted to maceration using 70% EtOH and, after evaporation of ethanol under vacuum, the saponins present in the resulting aqueous solution were submitted to acid hydrolysis to afford **1**.[10] After crystallization using AcOEt:MeOH (70:30), ursolic acid (**1**) was obtained with 10% yield and its purity (>95%) was determined by HPLC (R_t = 15 min).[22] This compound was identified by comparison of its NMR data with the literature.[36,37]

4.2. 3-O-acetylursolic acid (2)

This compound was prepared from **1** (91 mg, 0.2 mmol) as previously described[10] (100% yield): mp 170 °C; $[\alpha]_D^{20}$ +99.3° ($CHCl_3$, c 0.1); IR (ATR): 3562 (OH), 2947 (OH), 2866, 1732 (C=O acetyl), 1697 (C=O acid), 1460 (C–O), 1385, 1306 (C–O), 1248 (C–O–C), 1030 (C–OH). HRMS: $C_{32}H_{49}O_4$ 497.3635 $[M + H]^+$(100%).

4.3. N-{3-[4-(3-aminopropyl)piperazinyl]propyl}-3-O-acetylursolamide (3) and 3a

To a solution of **2** (1.48 g, 3 mmol) in dry CH_2Cl_2 (30 mL) at 0 °C was added dropwise oxalyl chloride (7.8 mL, 9 mmol) and stirred for 3 h at room temperature. The reaction mixture was allowed to cool at 0 °C, and TEA (25 mL, 18 mmol) and *N-tert*-butoxycarbonyl-1,4-bis(3-aminopropyl)piperazine (2.7 g, 9 mmol)

were added; then, it was allowed to warm at room temperature and stirred overnight. The reaction was quenched with H_2O (10 mL), and the organic phase was separated. The aqueous phase was extracted with CH_2Cl_2 (3 × 30 mL). The combined organic phases were dried over Na_2SO_4, filtered, and evaporated under vacuum. The crude residue was dissolved in CH_2Cl_2 (20 mL), acidified with TFA 10% (50 mL) for deprotection reaction, and stirred for 5 h. The reaction was quenched using saturated aqueous potassium carbonate solution (60 mL). The aqueous phase was extracted with CH_2Cl_2 (3 × 30 mL). The organic phases were washed with H_2O (3 × 30 mL), dried over Na_2SO_4, filtered, and evaporated under vacuum to afford **3** as a white solid (80%): mp 138 °C; IR (ATR): 3325 (NH_2), 2873, 2807, 1731 (C=O acetyl), 1683 (C=O amide), 1637 (C=C), 1462 (C–O), 1367, 1308 (C–O–C), 1245 (C–N). ^{1}H NMR ($CDCl_3$): 0.81 (d, 3 H, ^3J = 6.9, CH_3-30), 0.87 (m, 1H, CH-5), 0.91 (d, 3H, ^3J = 6.6, CH_3-29), 0.93 (m, 1H, $CH_{2(H\alpha)}$-1), 0.94 (s, 3H, CH_3-25), 0.95 (m, 1H, CH-11), 0.96 (m, 1H, CH_2-20), 0.99 (s, 3H, CH_3-24), 1.00 (s, 3H, CH_3-23), 1.01 (s, 3H, CH_3-26), 1.14 (s, 3H, CH_3-27), 1.21 (m, 1H, $CH_{2(H\alpha)}$-7), 1.33 (m; 1H, CH-19), 1.41 (m, 2H, CH_2-2), 1.55 (m, 4H, $CH_{2(H\beta)}$-6, $CH_{2(H\beta)}$-7, CH-9, $CH_{2(H\alpha)}$-21), 1.62 (m, 4H, $CH_{2(H\beta)}$-1, $CH_{2(H\alpha)}$-15, $CH_{2(H\beta)}$-16, $CH_{2(H\beta)}$-21), 1.70 (m, 5H, CH_2-c, CH_2-c′, $CH_{2(H\alpha)}$-6), 1.77 (td, 1H, ^3J = 14.0 and 4.0, $CH_{2(H\alpha)}$-16), 1.83 (m, 2H, CH_2-22), 2.00 (m, 1H, $CH_{2(H\beta)}$-15), 2.10 (s, 3H, CH_3COO), 2.51 (m, 13H, CH_2-piperazine, CH_2-b, CH_2-b′, CH-18), 3.03 (m, 2H, CH_2-d′), 3.49 (m, 2H, CH_2-d), 4.54 (dd, 1H, ^3J = 13.8 and 7.4, CH-3), 5.34 (tl, 1H, CH-12), 6.46 (sl, 1H, NH). ^{13}C NMR: 15.9 (C-25), 17.1 (C-26), 17.3 (C-24), 17.6 (C-29), 18.6 (C-6), 21.7 (C-30 and CH_3COO), 23.8 (C-11), 23.9 (C-27), 24.1 (C-2), 25.1 (C-16), 26.2 (C-c), 28.2 (C-23), 28.5 (C-15), 31.2 (C-21), 31.8 (C-c′), 33.1 (C-7), 37.2 (C-10), 37.8 (C-22), 38.1 (C-4), 38.5 (C-1), 39.2 (C-d), 39.4 (C-19), 39.9 (C-20), 40.0 (C-8), 40.2 (C-d′), 42.6 (C-14), 47.8 (C-9), 47.9 (C-17), 53.4 (C-18), 54.7 (piperazine), 55.6 (C-b and C-b′), 55.7 (C-5), 81.2 (C-3), 123.1 (C-12), 139.5 (C-13), 171.4 (CH_3COO), 179.5 (C-28). HRMS $[M+H]^+$ $C_{42}H_{73}N_4O_3$ requires 681.5683, found 681.5667.

Compound **3** was submitted to deacetylation reaction using potassium carbonate according to the literature[38] to obtain compound **3a**. Its ^{1}H and ^{13}C NMR spectral data were similar to those of **3** except the absence of the acetyl signals.

4.4. General procedure for the synthesis of 4–21 through reductive amination

To a solution of **3** (34 mg, 0.05 mmol) in dry THF (1 mL) were added the appropriate aldehyde (0.15 mmol, 3 eq) and $NaHB(OAc)_3$ (32.8 mg, 0.15 mmol, 3 eq) as the reducing agent to obtain compounds **4**–**18** according to the literature.[20,23] Similar reaction conditions using 1.5 equivalents of the appropriate aldehyde and reducing agent were used to obtain the compounds **19**–**21**. The mixture was stirred at room temperature for 5 h; then, it was quenched with an aqueous solution of 1 M NaOH (0.5 mL). The aqueous phase was extracted with CH_2Cl_2 (3 × 1 mL). The combined organic phases were washed with H_2O (2 × 1 mL); then, it was dried over Na_2SO_4, filtered, and evaporated under vacuum. The resultant crude products were purified by flash chromatography on silica gel using 0–10% MeOH in CH_2Cl_2 as eluent.

4.5. N-{3-[4-(3-(Bisbenzylamino)propyl)piperazinyl]propyl}-3-O-acetyl-ursolamide (4)

This compound was prepared from benzaldehyde (15.2 μL, 0.15 mmol, 3 eq) to afford **4** as yellow oil (55%): IR (ATR): 3351, 2926, 1732, 1643, 1519, 1452, 1367, 1244. ^{1}H NMR ($CDCl_3$): 0.81 (d, 3H, ^3J = 6.9, CH_3), 0.87 (1H, CH), 0.91 (d, 3H, ^3J = 5.2, CH_3), 0.93 (1H, CH_2), 0.96 (s, 3H, CH_3), 0.95 (1H, CH_2), 0.99 (sl, 4H, CH_3, CH-20), 1.00 (s, 3H, CH_3), 1.01 (s, 3H, CH_3), 1.14 (s, 3H, CH_3), 1.21 (m, 1H, CH_2), 1.33 (m, 3H, CH, CH_2-2), 1.48 (m, 4H, CH_2, CH), 1.60 (m, 4H, CH_2), 1.71 (m, 5H, CH_2), 1.77 (td, 1H, ^3J = 14.0 and 4.0, CH_2), 1.84 (2H, CH_2), 1.99 (m, 1H, CH_2), 2.10 (s, 3H, CH_3COO), 2.49 (m, 13H, CH_2-piperazine, 1H, CH), 3.09 (m, 2H, CH_2-d′), 3.47 (m, 2H, CH_2-d), 3.60 (s, 4H, –N(CH_2)$_2$), 4.54 (dd, 1H, ^3J = 9.5 and 5.9, CH), 5.35 (tl, 1H, CH), 6.48 (sl, 1H, NH), 7.27 (dd, 2× 1H, ^3J = 7.1, CH-*para*), 7.34 (dd, 2× 2H, ^3J = 7.3, CH-*meta*), 7.39 (d, 2× 2H, ^3J = 7.5, CH-*ortho*). ^{13}C NMR ($CDCl_3$): 15.8, 17.1, 17.4, 17.7, 18.6, 21.6 (C-30), 21.7 (CH_3COO), 23.8, 23.9, 24.1, 25.1, 26.2, 28.2, 28.5, 31.3, 32.8 (C-c′), 33.4, 37.3, 37.8, 38.1, 38.5, 38.7 (C-d), 39.5, 39.8, 39.9, 42.5, 47.9 (C-9 and C-17), 51.7 (C-b′), 52.9 (piperazine), 53.9 (C-d′), 54.0, 55.6, 56.6 (C-b), 58.9 (C-e), 81.3, 123.9, 127.3 (C-*para*), 128.6 (C-*meta*), 129.2 (C-*ortho*), 140.1, 145.3 (C-*ipso*), 171.4, 178.1 (C-28). HRMS $[M+H]^+$ $C_{56}H_{85}N_4O_3$ requires 861.6622, found 861.6649.

4.6. N-{3-[4-(3-(Bis(3-phenylpropyl)amino)propyl)piperazinyl]propyl}-3-O-acetylursolamide (5)

This compound was prepared from phenylpropionaldehyde (26.5 μL, 0.15 mmol, 3 eq) to afford **5** as yellow oil (30%): IR (ATR): 3318, 2922, 2854, 2811, 1731, 1648, 1455, 1370, 1245. ^{1}H NMR ($CDCl_3$): 0.81 (d, 3H, ^3J = 7.0), 0.87 (1H, CH), 0.91 (d, 3H, ^3J = 5.0, CH_3), 0.93 (1H, CH_2), 0.94 (1H, CH_2), 0.98 (s, 3H, CH_3), 0.99 (sl, 7H, CH_3, CH), 1.00 (s, 3H, CH_3), 1.14 (s, 3H, CH_3), 1.21 (1H, CH_2), 1.33 (m, 1H, CH), 1.42 (2H, CH_2), 1.53 (m, 4H, CH_2, CH), 1.63 (m, 4H, CH_2), 1.74 (m, 6H, CH_2), 1.97 (2H, CH_2-22), 2.02 (m, 5H, $CH_{2(H\beta)}$-15, 2× –$CH_2$$CH_2$Ar), 2.10 (s, 3H, CH_3), 2.54 (m, 21H, CH_2-piperazine, CH_2-b, CH_2-b′, CH-18, –N(CH_2)$_2$, 2× –CH_2Ar), 3.10 (m, 2H, CH_2), 3.48 (m, 2H, CH_2), 4.27 (dd, 1H, ^3J = 8.2 and 6.2, CH), 5.34 (tl, 1H, CH), 6.51 (sl, 1H, NH), 7.21 (d, 2× 2H, ^3J = 7.1, CH-*para*), 7.24 (d, 2× 2H, ^3J = 7.3, CH-*meta*), 7.32 (d, 2× 2H, ^3J = 6.2, CH-*ortho*). ^{13}C NMR ($CDCl_3$): 15.9; 17.1, 17.4, 17.7, 18.6, 21.6, 21.7, 23.8, 23.9, 24.2, 25.1, 26.1, 28.2, 28.5, 30.1 (2× –$CH_2$$CH_2$Ar), 31.3, 33.2, 33.9, 37.3, 37.8, 38.1, 38.7, 39.2, 39.5, 39.9, 40.1, 42.7, 47.9, 52.0, 53.3, 53.5, 54.1, 55.7, 56.6, 57.4, 68.6 (2× –CH_2Ar), 81.3, 125.8, 126.3 (C-*para*), 128.7 (C-*meta*), 129.2 (C-*ortho*), 134.3 (C-*ipso*), 140.1, 171.4,

178.2. HRMS $[M+H]^+$ $C_{60}H_{93}N_4O_3$ requires 917.7248, found 917.7242.

4.7. N-{3-[4-(3-(Bis(4-bromobenzyl)amino)propyl)piperazinyl]propyl}-3-O-acetylursolamide (6)

This compound was prepared from 4-bromobenzaldehyde (27.5 mg, 0.15 mmol, 3 eq) to afford **6** as yellow oil (56%): IR (ATR): 3325, 2927, 2872, 2810, 1730, 1644, 1519, 1368, 1245, 1069. ^{1}H NMR ($CDCl_3$): 0.79 (d, 3H, $^3J = 7.8$, CH_3), 0.86 (1H, CH), 0.90 (d, 3H, $^3J = 6.2$, CH_3), 0.93 (1H, CH_2), 0.94 (1H, CH_2), 0.98 (s, 7H, CH_3, CH), 0.99 (sl, 6H, CH_3), 1.13 (s, 3H, CH_3), 1.19 (1H, CH_2), 1.31 (m, 1H, CH), 1.41 (2H, CH_2), 1.51 (m, 4H, CH_2, CH), 1.59 (m, 4H, CH_2), 1.72 (m, 6H, CH_2), 1.84 (2H, CH_2-22), 2.11 (s, 3H, CH_3), 2.41 (m, 13H, CH_2, CH), 3.07 (m, 2H, CH_2), 3.48 (m, 2H, CH_2), 3.50 (s, 4H, CH_2), 4.53 (dd, 1H, $^3J = 9.7$ and 5.8, CH), 5.33 (tl, 1H, CH), 6.56 (sl, 1H, NH), 7.25 (d, 2× 2H, $^3J = 8.1$, CH-*ortho*), 7.47 (d, 2× 2H, $^3J = 8.2$, CH-*meta*). ^{13}C NMR ($CDCl_3$): 15.9; 17.1, 17.4, 17.7, 18.6, 21.6, 21.7, 23.8, 23.9, 24.1, 25.1, 26.2, 28.2, 28.5, 31.3, 33.1, 37.3, 37.8, 38.1, 38.7, 39.2, 39.5, 39.9, 40.1, 42.8, 47.9, 51.8, 53.4, 53.6, 54.1, 55.7, 56.6, 58.2, 81.3, 121.1 (C-*ortho*) 125.8, 130.8 (C-*meta*), 131.7 (C-*ipso*), 139.0 (C-*para*), 140.2, 171.4, 178.2. HRMS $[M+H]^+$ $C_{56}H_{83}N_4O_3Br_2$ requires 1017.4832, found 1017.4812.

4.8. N-{3-[4-(3-(Bis(4-chlorobenzyl)amino)propyl)piperazinyl]propyl}-3-O-acetylursolamide (7)

This compound was prepared from 4-chlorobenzaldehyde (21.9 mg, 0.15 mmol, 3 eq) to afford **7** as green oil (56%): IR (ATR): 3374, 2944, 2874, 1731, 1645, 1490, 1367, 1246. ^{1}H NMR ($CDCl_3$): 0.82 (d, 3H, $^3J = 6.9$, CH_3), 0.88 (1H, CH), 0.91 (d, 3H, $^3J = 5.6$, CH_3), 0.93 (1H, CH_2), 0.94 (1H, CH_2), 0.96 (s, 3H, CH_3), 0.99 (sl, 4H, CH_3, CH), 1.00 (s, 3H, CH_3), 1.01 (s, 3H, CH_3), 1.14 (s, 3H, CH_3), 1.21 (1H, CH_2), 1.34 (m, 1H, CH), 1.42 (2H, CH_2), 1.53 (m, 4H, CH_2, CH), 1.63 (m, 4H, CH_2), 1.72 (m, 6H, CH_2), 1.97 (2H, CH_2-22), 2.02 (m, 1H, CH_2), 2.10 (s, 3H, CH_3), 2.49 (m, 13H, CH_2, CH), 3.09 (m, 2H, CH_2), 3.47 (m, 2H, CH_2), 3.54 (s, 4H, CH_2), 4.55 (dd, 1H, $^3J = 9.3$ and 5.3, CH), 5.34 (tl, 1H, CH), 6.46 (sl, 1H, NH), 7.30 (d, 2× 2H, $^3J = 8.8$, CH-*ortho*), 7.32 (d, 2× 2H, $^3J = 8.5$, CH-*meta*). ^{13}C NMR ($CDCl_3$): 15.9; 17.1, 17.4, 17.7, 18.6, 21.6, 21.8, 23.8, 23.9, 24.1, 25.1, 26.2, 28.3, 28.5, 31.3, 33.2, 33.4, 37.3, 37.8, 38.1, 38.7, 39.1, 39.5, 39.8, 40.0, 42.5, 47.9, 51.8, 53.3, 53.5, 54.1, 55.7, 56.6, 58.1, 81.3, 122.9 128.8 (C-*meta*), 130.4 (C-*ipso*), 133.0 (C-*para*), 138.5(C-*ortho*), 140.2, 171.3, 178.2. HRMS $[M+H]^+$ $C_{56}H_{83}N_4O_3Cl_2$ requires 929.5842, found 929.5825.

4.9. N-{3-[4-(3-(Bis(4-fluorobenzyl)amino)propyl)piperazinyl]propyl}-3-O-acetylursolamide (8)

This compound was prepared from 4-fluorobenzaldehyde (16.2 μL, 0.15 mmol, 3 eq) to afford **8** as green oil (70%): IR (ATR): 3361, 2927, 2874, 1731, 1644, 1507, 1454, 1368, 1245. ^{1}H NMR ($CDCl_3$): 0.81 (d, 3H, $^3J = 7.0$, CH_3), 0.87 (1H, CH), 0.91 (d, 3H, $^3J = 5.8$, CH_3), 0.94 (1H, CH_2), 0.95 (s, 3H, CH_3), 0.94 (1H, CH_2), 0.98 (sl, 4H, CH_3,CH), 0.99 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 1.14 (s, 3H, CH_3), 1.20 (m, 1H, CH_2), 1.33 (m, 1H, CH), 1.41 (m, 2H, CH_2), 1.54 (m, 4H, CH_2, CH), 1.62 (m, 4H, CH_2), 1.70 (m, 5H, CH_2), 1.77 (td, 1H, $^3J = 13.6$ and 3.9, CH_2), 1.84 (2H, CH_2), 1.97 (m, 1H, CH_2), 2.10 (s, 3H, CH_3), 2.45 (m, 13H, CH_2, CH-18), 3.10 (m, 2H, CH_2), 3.47 (m, 2H, CH_2), 3.54 (s, 4H, CH_2), 4.54 (dd, 1H, $^3J = 9.3$ and 5.5, CH), 5.33 (tl, 1H, CH), 6.49 (sl, 1H, NH), 7.03 (dd, 2× 2H, $^3J_{HH} = 8.5$,$^3J_{HF} = 8.6$, CH-*meta*), 7.32 (dd, 2× 2H, $^3J_{HH} = 8.2$, $^3J_{HF} = 5.7$, CH-*ortho*).^{13}C NMR ($CDCl_3$): 15.9; 17.1, 17.4, 17.7, 18.6, 21.6, 21.7, 23.8, 23.9, 24.1, 25.1, 26.1, 28.2, 28.5, 31.3, 33.1, 33.4, 37.3, 37.8, 38.1, 38.7, 39.2, 39.5, 39.8, 40.1, 42.8, 47.9, 51.7, 53.5, 53.6, 53.8, 55.6, 56.8, 57.9, 81.3, 115.5 (C-*meta*), 122.9 125.6 (C-*ipso*), 130.6 (C-*ortho*), 140.2, 161.3 (C-*para*), 171.4, 178.2. HRMS $[M+H]^+$ $C_{56}H_{83}N_4O_3F_2$ requires 897.6433, found 897.6407.

4.10. N-{3-[4-(3-(Bis(4-nitrobenzyl)amino)propyl)piperazinyl]propyl}-3-O-acetylursolamide (9)

This compound was prepared from 4-nitrobenzaldehyde (22.6 mg, 0.15 mmol, 3 eq) to afford **9** as green oil (40%): IR (ATR): 3325, 2924, 2854, 1729, 1644, 1597, 1518, 1454, 1368, 1245. ^{1}H NMR ($CDCl_3$): 0.80 (d, 3H, $^3J = 6.9$, CH_3), 0.89 (1H, CH), 0.90 (d, 3H, $^3J = 5.7$, CH_3), 0.93 (1H, CH_2), 0.96 (s, 3H, CH_3), 0.94 (1H, CH_2), 0.97 (sl, 4H, CH_3, CH-20), 0.99 (s, 6H, CH_3), 1.12 (s, 3H, CH_3), 1.19 (1H, CH_2), 1.31 (m, 1H, CH), 1.40 (2H, CH_2), 1.57 (m, 8H, CH_2, CH), 1.72 (m, 6H, CH_2), 1.97 (2H, CH_2), 2.02 (m, 1H, CH_2), 2.11 (s, 3H, CH_3), 2.48 (m, 13H, CH_2, CH), 3.09 (m, 2H, CH_2), 3.51 (m, 2H, CH_2), 3.70 (s, 4H, CH_2), 4.53 (sl, 1H, CH), 5.34 (tl, 1H, CH), 6.46 (sl, 1H, NH), 7.58 (d, 2× 2H, $^3J = 8.2$, CH-*ortho*), 8.24 (d, 2× 2H, $^3J = 8.3$, CH-*meta*).^{13}C NMR ($CDCl_3$): 15.9, 17.1, 17.4, 17.7, 18.6, 21.6, 21.7, 23.8, 23.9, 24.1, 25.1, 26.1, 28.2, 28.5, 31.3, 33.1, 33.4, 37.2, 37.8, 38.1, 38.7, 39.2, 39.5, 39.9, 40.1, 42.8, 47.9, 51.8, 53.3, 53.5, 54.1, 55.4, 56.4, 58.4, 81.3, 124.1 (C-*ortho*), 125.8, 129.6 (C-*meta*), 139.1, 143.0 (C-*ipso*), 148.1 (C-*para*), 171.4, 178.1. HRMS $[M+H]^+$ $C_{56}H_{83}N_6O_7$ requires 951.6323, found 951.6326.

4.11. N-{3-[4-(3-(Bis(4-hydroxybenzyl)amino)propyl)piperazinyl]propyl}-3-O-acetylursolamide (10)

This compound was prepared from 4-hydroxybenzaldehyde (18.3 mg, 0.15 mmol, 3 eq) to afford **10** as green oil (30%): IR (ATR): 3309, 2925, 2872, 1728, 1629, 1514, 1453, 1367, 1246. ^{1}H NMR ($CDCl_3$): 0.79 (d, 3H, $^3J = 7.0$, CH_3), 0.87 (1H, CH), 0.91 (d, 3H, $^3J = 5.8$, CH_3), 0.93 (1H, CH_2), 0.96 (s, 3H, CH_3), 0.94 (1H, CH_2), 0.96 (sl, 4H, CH_3, CH), 0.97 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 1.13 (s, 3H, CH_3), 1.21 (1H, CH_2), 1.38 (m, 3H, CH, CH_2), 1.53 (m, 4H, CH_2, CH), 1.62 (m, 4H, CH_2), 1.69 (m, 6H, CH_2), 1.95 (2H, CH_2), 1.98 (m, 1H, CH_2), 2.11 (s, 3H, CH_3), 2.48 (m, 13H, CH_2, CH), 3.06 (m, 2H, CH_2), 3.45 (m, 2H, CH_2), 3.54 (s, 4H, CH_2), 4.54 (dd, 1H, $^3J = 9.3$ and 5.5, CH), 5.33 (tl, 1H, CH), 6.39 (sl, 1H, NH), 6.77 (d, 2× 2H,

^3J = 8.1, CH-*ortho*), 7.16 (d, 2× 2H, ^3J = 8.2, CH-*meta*). ^{13}C NMR ($CDCl_3$): 15.9, 17.1, 17.3, 17.7, 18.5, 21.6, 21.7, 23.8, 23.9, 24.1, 25.2, 26.2, 28.2, 28.5, 31.2, 33.0, 33.4, 37.2, 37.7, 38.1, 38.7, 39.1, 39.5, 39.9, 40.1, 42.6, 47.8, 48.1, 51.3, 52.7, 53.8, 53.8, 54.2, 55.6, 56.4, 58.3, 81.3, 115.8 (C-*meta*), 125.9, 130.6 (C-*ortho*), 130.9 (C-*ipso*), 140.2, 156.0 (C-*para*), 171.6, 179.6. HRMS $[M+H]^+$ $C_{56}H_{83}N_4O_5$ requires 893.6493, found 893.6498.

4.12. N-{3-[4-(3-(Bis(4-methoxybenzyl)amino)propyl)piperazinyl]propyl}-3-O-acetylursolamide (11)

This compound was prepared from 4-methoxybenzaldehyde (18.2 μL, 0.15 mmol, 3 eq) to afford **11** as yellow oil (30%): IR (ATR): 3363, 2927, 2873, 1731, 1645, 1510, 1456, 1367, 1244. ^{1}H NMR ($CDCl_3$): 0.81 (d, 3H, ^3J = 6.9, CH_3), 0.87 (1H, CH), 0.91 (d, 3H, ^3J = 5.3, CH_3), 0.93 (1H, CH_2), 0.96 (s, 3H, CH_3), 0.94 (1H, CH_2), 0.99 (sl, 4H, CH_3, CH), 1.00 (s, 3H, CH_3), 1.01 (s, 3H, CH_3), 1.14 (s, 3H, CH_3), 1.23 (m, 1H, CH_2), 1.33 (1H, CH), 1.41 (m, 2H, CH_2), 1.52 (m, 4H, CH_2, CH), 1.62 (m, 4H, CH_2), 1.73 (m, 6H, CH_2), 1.87 (2H, CH_2), 2.00 (m, 1H, CH_2), 2.10 (s, 3H, CH_3), 2.50 (m, 13H, CH_2, CH-18), 3.10 (m, 2H, CH_2), 3.47 (m, 2H, CH_2), 3.52 (s, 4H, CH_2), 3.85 (s, 6H, CH_3O-), 4.54 (dd, 1H, ^3J = 9.5 and 5.9, CH), 5.35 (tl, 1H, CH), 6.50 (sl, 1H, NH), 6.89 (d, 2× 2H, ^3J = 8.4, CH-*ortho*), 7.29 (d, 2× 2H, ^3J = 8.4, CH-*meta*).^{13}C NMR ($CDCl_3$): 15.9, 17.1, 17.4, 17.7, 18.6, 21.6, 21.7, 23.8, 23.9, 24.1, 25.1, 26.2, 28.2, 28.5, 31.3, 33.1, 37.2, 37.8, 38.1, 38.7, 39.1, 39.5, 39.8, 40.1, 42.5, 47.9, 51.7, 52.9, 53.1, 54.0, 55.6, 56.6 (CH_3O-), 57.9, 81.3, 113.9 (C-*meta*), 123.9, 125.8 (C-*ipso*), 130.3 (C-*ortho*), 140.1, 158.9 (C-*para*), 171.4, 178.1. HRMS $[M+H]^+$ $C_{58}H_{89}N_4O_5$ requires 921.6833, found 921.6792.

4.13. N-{3-[4-(3-(Bis(cyclohexylmethyl)amino)propyl)piperazinyl]propyl}-3-O-acetylursolamide (12)

This compound was prepared from cyclohexanecarboxaldehyde (18.14 μL, 0.15 mmol, 3 eq) to afford **12** as yellow oil (50%): IR (ATR): 3310, 2920, 2849, 1733, 1643, 1520, 1448, 1368, 1242. ^{1}H NMR ($CDCl_3$): 0.83 (d, 3H, ^3J = 6.7, CH_3), 0.87 (1H, CH), 0.92 (d, 3H, ^3J = 6.4, CH_3), 0.93 (1H, CH_2), 0.94 (1H, CH_2), 0.96 (s, 3H, CH_3), 0.97 (sl, 4H, CH_3, CH), 0.99 (s, 3H, CH_3), 1.01 (s, 3H, CH_3), 1.15 (s, 3H, CH_3), 1.20 (1H, CH_2), 1.25 (m, 12H, 6× CH_2 cyclohexyl), 1.33 (m, 13H, CH-19, CH_2-2, 4× CH_2 cyclohexyl), 1.54 (m, 6H, CH_2, 2× CH cyclohexyl), 1.74 (m, 10H, CH_2), 1.99 (m, 3H, CH_2), 2.10 (s, 3H, CH_3), 2.13 (d, 4H, ^3J = 6.3, –N(CH_2)$_2$), 2.48 (m, 13H, CH_2, CH), 3.10 (m, 2H, CH_2), 3.49 (m, 2H, CH_2), 4.55 (dd, 1H, ^3J = 9.3 and 5.5, CH), 5.35 (tl, 1H, CH), 6.53 (sl, 1H, NH).^{13}C NMR ($CDCl_3$): 15.9, 17.1, 17.4, 17.7, 18.6, 21.6, 23.8, 23.9, 24.1, 25.1, 26.1, 26.6 (6× CH_2 cyclohexyl), 27.4 (4× CH_2 cyclohexyl) 28.3, 28.5, 31.3, 32.3, 33.2, 36.7 (2× CH cyclohexyl), 37.3, 37.9, 38.1, 38.7, 39.3, 39.5, 40.0, 40.1, 42.9, 47.9, 53.0, 53.5, 53.6, 54.1, 55.7, 57.0, 57.7, 81.3, 125.8, 140.1, 171.3, 178.1. HRMS $[M+H]^+$ $C_{56}H_{97}N_4O_3$ requires 873.7561, found 873.7553.

4.14. N-{3-[4-(3-(Bis(cyclopropylmethyl)amino)propyl)piperazinyl]propyl}-3-O-acetylursolamide (13)

This compound was prepared from cyclopropanecarboxaldehyde (11.4 μL, 0.15 mmol, 3 eq) to afford **13** as yellow oil (30%): IR (ATR): 3322, 2922, 2852, 1733, 1646, 1524, 1457, 1369, 1244. ^{1}H NMR ($CDCl_3$): 0.82 (d, 3H, ^3J = 6.7, CH_3), 0.87 (1H, CH), 0.92 (d, 3H, ^3J = 5.5, CH_3), 0.94 (1H, CH_2), 0.95 (s, 3H, CH_3), 0.93 (1H, CH_2), 1.00 (sl, 4H, CH_3, CH), 1.01 (s, 6H, CH_3), 1.14 (s, 3H, CH_3), 1.21 (1H, CH_2), 1.32 (m, 11H, CH-19, CH_2-2, 4× CH_2 cyclopropyl), 1.55 (m, 6H, CH_2, 2× CH cyclopropyl), 1.72 (m, 10H, CH_2), 2.01 (m, 3H, CH_2), 2.09 (d, 4H, ^3J = 6.0, –N(CH_2)$_2$), 2.10 (s, 3H, CH_3), 2.55 (m, 13H, CH_2, CH), 3.10 (m, 2H, CH_2), 3.51 (m, 2H, CH_2), 4.55 (dd, 1H, ^3J = 9.2 and 5.5, CH), 5.35 (tl, 1H, CH), 6.55 (sl, 1H, NH).^{13}C NMR ($CDCl_3$): 15.9, 17.1, 17.4, 17.7, 18.6, 21.6, 23.8, 23.9, 24.1, 25.1, 26.1, 28.3, 28.5, 29.7 (4× CH_2 cyclopropyl), 30.1 (2× CH cyclopropyl), 31.9, 32.3, 33.2, 37.3, 37.8, 38.1, 38.7, 39.3, 39.5, 39.9, 40.1, 42.8, 47.9, 51.9, 53.6, 53.8, 54.1, 55.7, 57.7, 59.2, 81.3, 125.8, 140.1, 171.4, 178.1. HRMS $[M+H]^+$ $C_{50}H_{85}N_4O_3$ requires 789.6622, found 789.6609.

4.15. N-{3-[4-(3-(Bisheptylamino)propyl)piperazinyl]propyl}-3-O-acetyl-ursolamide (14)

This compound was prepared from heptaldehyde (22 μL, 0.15 mmol, 3 eq) to afford **14** as yellow oil (30%): IR (ATR): 3318, 2926, 2854, 1735, 1640, 1519, 1456, 1366, 1244. ^{1}H NMR ($CDCl_3$): 0.82 (d, 3H, ^3J = 6.8, CH_3), 0.87 (1H, CH), 0.91 (d, 3H, ^3J = 6.7, CH_3), 0.93 (1H, CH_2), 0.94 (8H, CH_2, 2× CH_3 heptyl), 0.95 (s, 3H, CH_3), 0.97 (sl, 4H, CH_3, CH), 0,.99 (s, 3H, CH_3), 1.01 (s, 3H, CH_3), 1.14 (s, 3H, CH_3), 1.21 (1H, CH_2), 1.32 (m, 23H, CH-19, CH_2-2, 10× CH_2 heptyl), 1.53 (m, 4H, CH_2, CH), 1.74 (m, 10H, CH_2), 1.99 (m, 3H, CH_2), 2.10 (s, 3H, CH_3), 2.46 (m, 17H, CH_2, CH, –N(CH_2)$_2$), 3.09 (m, 2H, CH_2), 3.48 (m, 2H, CH_2), 4.56 (dd, 1H, ^3J = 9.3 and 5.5, CH), 5.35 (tl, 1H, CH), 6.53 (sl, 1H, NH).^{13}C NMR ($CDCl_3$): 14.4 (2× CH_3 heptyl), 15.9, 17.1, 17.4, 17.7, 18.6, 21.6, 23.0 (2× –$CH_2$$CH_3$ heptyl), 23.8, 23.9, 24.1, 25.1, 26.1, 27.9 (2× –CH_2(CH_2)$_3$$CH_3$ heptyl), 28.3, 28.5, 28.7 (2× –CH_2(CH_2)$_2$$CH_3$ heptyl), 29.7 (2× –CH_2(CH_2)$_4$$CH_3$ heptyl), 30.1 (2× –$CH_2$$CH_2$$CH_3$ heptyl), 31.3, 32.3, 33.2, 37.3, 37.8, 38.1, 38.7, 39.3, 39.5, 39.9, 40.1, 42.8, 47.9, 48.0, 52.3, 53.6, 54.1, 54.4, 55.7, 57.1, 57.8, 81.3, 125.8, 140.1, 171.4, 178.1. HRMS $[M+H]^+$ $C_{56}H_{101}N_4O_3$ requires 877.7874, found 877.7901.

4.16. N-{3-[4-(3-(Bisbutylamino)propyl)piperazinyl]propyl}-3-O-acetyl-ursolamide (15)

This compound was prepared from butyraldehyde (13.2 μL, 0.15 mmol, 3 eq) to afford **15** as yellow oil (30%): IR (ATR): 3320, 2926, 2870, 1733, 1640, 1520, 1461, 1368, 1242. ^{1}H NMR ($CDCl_3$): 0.82 (d, 3H, ^3J = 6.9, CH_3), 0.88 (1H, CH), 0.91 (d, 3H, ^3J = 6.7, CH_3), 0.94 (2H, CH_2), 0.93 (m, 6H, 2× CH_3 butyl), 0.95 (s, 3H, CH_3), 0.97 (sl, 4H, CH_3, CH), 0.98 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 1.14 (s, 3H, CH_3), 1.21 (1H,

CH_2), 1.35 (m, 11H, CH-19, CH_2-2, 4× CH_2butyl), 1.53 (m, 4H, CH_2, CH), 1.73 (m, 10H, CH_2), 1.99 (m, 3H, CH_2), 2.10 (s, 3H, CH_3), 2.51 (m, 17H, CH_2, CH, –N(CH_2)$_2$), 3.10 (m, 2H, CH_2), 3.47 (m, 2H, CH_2), 4.54 (dd, 1H, ^3J = 9.3 and 5.5, CH), 5.35 (tl, 1H, CH), 6.51 (sl, 1H, NH).^{13}C NMR ($CDCl_3$): 14.0 (2× CH_3 butyl), 15.8, 17.1, 17.4, 17.7, 18.6, 20.6 (2× –$CH_2$$CH_3$ butyl), 21.6, 21.7, 23.7, 23.9, 24.1, 25.1, 25.4 (2× –$CH_2$$CH_2$$CH_3$ butyl), 26.2, 28.3, 28.5, 30.1, 31.3, 33.1, 37.3, 37.9, 38.1, 38.5, 39.3, 39.5, 39.9, 40.0, 42.7, 47.9, 48.1, 51.2, 52.8, 53.4, 53.7, 54.7, 54.8, 55.6, 81.3, 125.8, 140.2, 171.4, 178.1. HRMS [M+H]$^+$ $C_{50}H_{89}N_4O_3$ requires 793.6935, found 793.6981.

4.17. N-{3-[4-(3-(Bispropylamino)propyl)piperazinyl]propyl}-3-O-acetyl-ursolamide (16)

This compound was prepared from propionaldehyde (11.2 μL, 0.15 mmol, 3 eq) to afford **16** as yellow oil (45%): IR (ATR): 3325, 2923, 2870, 1734, 1639, 1523, 1457, 1369, 1243. ^{1}H NMR ($CDCl_3$): 0.80 (d, 3H, ^3J = 7.0, CH_3), 0.88 (1H, CH), 0.91 (d, 3H, ^3J = 6.5, CH_3), 0.93 (1H, CH_2), 0.93 (m, 6H, 2× CH_3 propyl), 0.94 (1H, CH_2), 0.96 (s, 3H, CH_3), 0.99 (s, 3H, CH_3, 1H, CH), 1.00 (s, 3H, CH_3), 1.01 (s, 3H, CH_3), 1.14 (s, 3H, CH_3), 1.21 (m, 1H, CH_2), 1.32 (m, 7H, CH, 2× CH_2 propyl), 1.53 (m, 4H, CH_2, CH), 1.62 (m, 4H, CH_2), 1.74 (m, 6H, CH_2), 1.99 (m, 3H, CH_2), 2.10 (s, 3H, CH_3), 2.56 (m, 17H, CH_2, CH, –N(CH_2)$_2$), 3.08 (m, 2H, CH_2), 3.49 (m, 2H, CH_2), 4.54 (dd, 1H, ^3J = 9.3 and 5.5, CH), 5.35 (tl, 1H, CH), 6.43 (sl, 1H, NH).^{13}C NMR ($CDCl_3$): 12.3 (2× CH_3 propyl), 15.9, 17.1, 17.4, 17.7, 18.6, 20.5 (CH_2 propyl), 21.6, 23.8, 23.9, 24.1, 25.1, 26.1, 28.2, 28.5, 31.3, 32.3, 33.2, 37.3, 37.8, 38.1, 38.7, 39.3, 39.5, 39.9, 40.1, 42.8, 47.9, 48.1, 52.5, 53.7, 53.9, 54.1, 55.7, 56.6, 57.8, 81.3, 125.8, 140.2, 171.6, 179.0. HRMS [M+H]$^+$ $C_{48}H_{85}N_4O_3$ requires 765.6622, found 765.6600.

4.18. N-{3-[4-(3-(Bisisobutylamino)propyl)piperazinyl]propyl}-3-O-acetyl-ursolamide (17)

This compound was prepared from isobutyraldehyde (26.4 μL, 0.15 mmol, 3 eq) to afford **17** as yellow oil (39%): IR (ATR): 3335, 2926, 2868, 1735, 1637, 1521, 1458, 1368, 1244. ^{1}H NMR ($CDCl_3$): 0.82 (3H, d, ^3J = 6.5, CH_3), 0.87 (1H, CH), 0.91 (m, 15H, CH_3-29, 2× CH_3 isobutyl), 0.93 (sl, 6H, CH_3, CH_2), 0.94 (sl, 4H, CH_3, CH), 0.97 (s, 6H, CH_3), 1.12 (s, 3H, CH_3), 1.20 (1H, CH_2), 1.31 (m, 5H, CH, CH_2, 2× CH isobutyl), 1.54 (m, 4H, CH_2, CH), 1.73 (m, 10H, CH_2), 1.99 (m, 3H, CH_2), 2.10 (s, 3H, CH_3), 2.41 (m, 17H, CH_2, CH, –N(CH_2)$_2$), 3.09 (m, 2H, CH_2), 3.50 (m, 2H, CH_2), 4.54 (dd, 1H, ^3J = 9.0 and 5.7, CH), 5.35 (tl, 1H, CH), 6.62 (sl, 1H, NH).^{13}C NMR ($CDCl_3$): 15.9, 17.1, 17.4, 17.7, 18.6, 21.3 (4× CH_3 isobutyl), 21.6, 21.7, 23.8, 23.9, 24.1, 25.1, 26.0, 27.0 (2× CH isobutyl), 28.3, 28.5, 30.1, 31.3, 33.2, 37.3, 37.8, 38.1, 38.7, 39.3, 39.5, 39.9, 40.1, 42.8, 47.8, 47.9, 53.0, 53.6, 54.1, 54.5, 55.7, 57.2, 57.7, 81.3, 125.8, 140.1, 171.4, 178.2. HRMS [M+H]$^+$ $C_{50}H_{89}N_4O_3$ requires 792.6935, found 793.6942.

4.19. N-{3-[4-(3-(Bisferrocenylamino)propyl)piperazinyl]propyl}-3-O-acetyl-ursolamide (18)

This compound was prepared from ferrocenecarboxaldehyde (65.5 mg, 0.15 mmol, 3 eq) to afford **18** as yellow oil (60%): IR (ATR): 3328, 2924, 2854, 1733, 1646, 1518, 1455, 1369, 1244. ^{1}H NMR ($CDCl_3$): 0.81 (d, 3H, ^3J = 6.0, CH_3), 0.86 (1H, CH), 0.90 (d, 3H, ^3J = 6.3, CH_3), 0.91 (1H, CH_2), 0.94 (1H, CH_2), 0.98 (s, 3H, CH_3), 0.99 (sl, 4H, CH_3, CH), 1.00 (s, 6H, CH_3), 1.13 (s, 3H, CH_3), 1.20 (1H, CH_2), 1.32 (m, 3H, CH, CH_2), 1.53 (m, 4H, CH_2,CH), 1.62 (m, 4H, CH_2), 1.72 (m, 6H, CH_2), 1.84 (2H, CH_2), 2.02 (m, 1H, CH_2), 2.11 (s, 3H, CH_3), 2.34 (m, 13H, CH_2, CH), 3.08 (m, 2H, CH_2), 3.48 (m, 2H, CH_2), 3.53 (s, 4H, –N(CH_2)$_2$), 4.17 (s, 10H, ferrocene), 4.26 (m, 8H, ferrocene), 4.54 (dd, 1H, ^3J = 9.0 and 5.3, CH), 5.33 (tl, 1H, CH), 6.54 (sl, 1H, NH).^{13}C NMR ($CDCl_3$): 15.9, 17.1, 17.4, 17.7, 18.6, 21.6, 21.7, 23.8, 23.9, 24.1, 25.1, 26.1, 28.3, 28.5, 29.7, 31.3, 33.1, 37.3, 37.8, 38.1, 38.7, 39.0, 39.5, 39.9, 40.1, 42.8, 47.9, 53.1, 53.4, 54.0, 54.1, 55.7, 69.1 (ferrocene), 69.7 (ferrocene), 70.0 (ferrocene), 70.9, 81.3, 125.8, 140.1, 171.4, 179.2. HRMS [M+H]$^+$ $C_{64}H_{93}N_4O_3Fe_2$ requires 1077.5946, found 1077.5945.

4.20. N-{3-[(4-(3-(4-Nitrobenzyl)amino)propyl)piperazinyl]propyl}-3-O-acetyl-ursolamide (19)

This compound was prepared from 4-nitrobenzaldehyde (13.7 mg, 0.075 mmol) to afford **19** as green oil (20%): $[\alpha]_D^{20}$ +29.1° ($CHCl_3$, c 0.1); IR (ATR): 3332 (NH); 2854; 1729 (C=O acetyl); 1644 (C=O amide); 1597 (C=C); 1518 (aromatic-NO_2); 1454 (C–O); 1368; 1245 (C–N); 854 (aromatic *p*-substituted). ^{1}H NMR ($CDCl_3$): 0.81 (d, 3H, ^3J = 7.0, CH_3-30), 0.89 (1H, CH-5), 0.90 (d, 3H, ^3J = 5.8, CH_3-29), 0.96 (s, 3H, CH_3-25), 0.95 (2H, CH_2-1, CH_2-11), 0.97 (sl, 4H, CH_3-24, CH-20), 1.00 (s, 6H, CH_3-23, CH_3-26), 1.13 (s, 3H, CH_3-27), 1.19 (1H, $CH_{2(H\alpha)}$-7), 1.36 (m, 3H, CH_2-2, CH-19), 1.57 (m, 8H, $CH_{2(H\beta)}$-1, $CH_{2(H\beta)}$-6, $CH_{2(H\beta)}$-7, CH-9, $CH_{2(H\alpha)}$-15, $CH_{2(H\beta)}$-16, CH_2-21), 1.74 (m, 8H, CH_2-c, CH_2H-c′, $CH_{2(H\alpha)}$-6, $CH_{2(H\alpha)}$-16, CH_2-22), 2.04 (m, 1H, $CH_{2(H\beta)}$-15), 2.11 (s, 3H, H_3CCOO), 2.47 (m, 13H, CH_2-a1, CH_2-a1′, CH_2-a2, CH_2-a2′, CH_2-b, CH_2-b′, CH-18), 3.04 (m, 2H, CH_2-d′), 3.50 (m, 2H, CH_2-d), 3.75 (s, 2H, –NCH_2), 4.53 (dd, 1H, ^3J = 11.7 and 4.6, CH-3), 5.34 (tl, 1H, CH-12), 6.57 (sl, 1H, NH), 7.58 (d, 2H, ^3J = 8.2, H-*ortho*), 8.22 (d, 2H, ^3J = 8.3, H-*meta*). ^{13}C NMR ($CDCl_3$): 15.9 (C-25), 17.1 (C-26), 17.4 (C-24), 17.7 (C-29), 18.6 (C-6), 21.6 (C-30), 21.7 (H_3CCOO), 23.8 (C-11), 23.9 (C-27), 24.1 (C-2), 25.1 (C-16), 26.0 (C-c), 28.2 (C-23), 28.5 (C-15), 30.2 (C-c′), 31.3 (C-21), 33.1 (C-7), 37.2 (C-10), 37.8 (C-22), 38.1 (C-4), 38.7 (C-1), 39.2 (C-d), 39.5 (C-19), 39.9 (C-20), 40.1 (C-8), 42.8 (C-14), 47.9 (C-17), 48.0 (C-9), 48.8 (C-b), 53.5 (C-a1, C-a1′, C-a2, C-a2′), 53.5 (C-d′), 54.1 (C-18), 55.6 (C-5), 57.1 (C-b′), 57.5 (C-e), 81.3 (C-3), 124.1 (C-*ortho*), 125.8 (C-12); 129.6 (C-*meta*); 139.2 (C-13); 143.0 (C-*ipso*); 148.0 (C-*para*); 171.5 (H_3C*C*O*O*); 178.1 (C-28). HRMS [M+H]$^+$ $C_{49}H_{78}N_5O_5$ requires 816.6003, found 816.5983.

4.21. N-{3-[(4-(3-(4-Hydroxybenzyl)amino)propyl)piperazinyl]propyl}-3-O-acetylursolamide (20)

This compound was prepared from 4-hydroxybenzaldehyde (9.15 mg, 0.075 mmol) to afford **20** as green oil (62%): $[\alpha]_D^{20}$ +58.3 ($CHCl_3$, c 0.15). IR (ATR): 3309; 2853; 1732; 1637; 1518; 1457; 1369; 1246. ^{1}H NMR ($CDCl_3$): 0.81 (d, 3H, $^3J = 6.9$, CH_3), 0.87 (1H, CH), 0.91 (d, 3H, $^3J = 5.9$, CH_3), 0.96 (s, 3H, CH_3), 0.95 (2H, CH_2), 0.96 (sl, 4H, CH_3 and CH), 0.99 (s, 3H, CH_3), 1.01 (s, 3H, CH_3), 1.14 (s, 3H, CH_3), 1.21 (1H, CH_2), 1.37 (m, 3H, CH and CH_2), 1.53 (m, 4H, CH_2, CH), 1.62 (m, 4H, CH_2), 1.75 (m, 8H, CH_2), 2.01 (m, 1H, CH_2), 2.11 (s, 3H, CH_3), 2.44 (m, 13H, CH_2, CH), 3.04 (m, 2H, CH_2), 3.45 (m, 2H, CH_2), 3.75 (s, 2H), –N(CH_2), 4.55 (dd, 1H, $^3J = 11.3$ and 5.5, CH), 5.34 (tl, 1H, CH), 6.47 (sl, 1H, NH), 6.78 (d, 2H, $^3J = 8.1$, CH-*ortho*), 7.20 (d, 2H, $^3J = 8.2$, CH-*meta*). ^{13}C NMR ($CDCl_3$): 15.9, 17.1, 17.3, 17.7, 18.6, 21.6, 21.7, 23.8, 23.9, 24.1, 25.1, 26.0, 28.2, 28.5, 30.1, 31.2, 33.1, 37.2, 37.8, 38.1, 38.7, 39.2, 39.5, 39.9, 40.1, 42.8, 47.9, 48.0, 48.9, 53.5, 54.1, 54.2, 55.6, 57.3, 57.7, 81.3, 115.7 (C-*meta*), 125.9 (C-12), 130.3 (C-*ortho*et C-*ipso*), 140.1, 156.0 (C-*para*), 171.5 (H_3CCOO), 178.6.

4.22. N-{3-[(4-(3-Ferrocenylamino)propyl)piperazinyl]propyl}-3-O-acetyl-ursolamide (21)

This compound was prepared from ferrocenecarboxaldehyde (16.5 mg, 0.075 mmol) to afford **21** as yellow oil (52%): IR (ATR): 3328, 2813, 1731, 1639, 1524, 1457, 1370, 1246, 1105, 1031. ^{1}H NMR ($CDCl_3$): 0.81 (3H, d, $^3J = 6.0$, CH_3), 0.87 (1H, CH), 0.91 (d, $^3J = 6.3$, 3H, CH_3), 0.93 (2H, CH_2), 0.96 (m, 7H, CH_3, CH), 1.00 (s, 6H, CH_3), 1.14 (s, 3H, CH_3), 1.21 (1H, CH_2), 1.34 (m, 3H, CH, CH_2), 1.52 (m, 4H, CH_2, CH), 1.61 (m, 4H, CH_2), 1.73 (m, 6H, CH_2), 1.86 (2H, CH_2), 2.01 (m, 1H, CH_2), 2.10 (s, 3H, CH_3), 2.36 (m, 13H, CH_2, CH), 3.06 (m, 2H, CH_2), 3.47 (m, 2H, CH_2), 3.78 (s, 2H, –NCH_2), 4.21 (s, 5H, CH), 4.28 (m, 4H, CH), 4.54 (dd, $^3J = 11.3$, 1H, 5.5, CH ferrocene), 5.35 (tl, 1H, CH ferrocene), 6.41 (sl, 1H, NH). ^{13}C NMR ($CDCl_3$): 15.9, 17.1, 17.3, 17.7, 18.6, 21.6, 23.8, 23.9, 25.1, 26.2, 28.2, 28.5, 30.1, 31.3, 33.1, 37.3, 37.8, 38.1, 38.7, 39.0, 39.5, 39.9, 40.1, 42.8, 47.9, 48.2, 48.6, 53.4, 53.6, 54.1, 55.6, 57.3, 58.1, 68.5 (ferrocene), 69.2 (ferrocene), 69.6 (ferrocene), 81.3, 125.8, 140.2, 171.4, 178.5.

4.23. Molecular modeling

The calculations employed methodology previously described by the group.[39–41] Briefly, the compounds were constructed and geometry optimized at the HF/3-21G level using GAMESS.[42] The so-obtained geometries were submitted to a single-point calculation at the HF/6-31G** level in order to obtain atomic charges suitable for MD simulations. These charges were included in PRODRG derived topologies[43] for each compound, allowing the description of the molecules through MD. All simulations were performed under GROMACS simulation suite[44] and GROMOS96 force field for 10.0 ns using explicit water molecules and counter-ions to neutralize the system.

4.24. In vitro P. falciparum culture and drug assays

Plasmodium falciparum strains were maintained continuously in culture on human erythrocytes as described by Trager and Jensen.[45] In vitro antiplasmodial activity was determined using a modification of the semi-automated microdilution technique of Desjardins et al.[46] *P. falciparum* CQ-sensitive (Thai) and CQ-resistant (FcB1) strains were used in sensitivity testing. Stock solutions of chloroquine diphosphate and test compounds were prepared in sterile distilled water and DMSO, respectively. Drug solutions were serially diluted with culture medium and added to asynchronous parasite cultures (1% parasitemia and 1% final hematocrite) in 96-well plates for 24 h, at 37 °C, prior to the addition of 0.5 μCi of [^{3}H]hypoxanthine (1–5 Ci/mmol; Amersham, Les Ulis, France) *per* well, for 24 h. The growth inhibition for each drug concentration was determined comparing the radioactivity incorporated into the treated culture with that in the controlled culture (without drug) maintained on the same plate. The concentration causing 50% inhibition (IC_{50}) was obtained from the drug concentration-response curve and the results were expressed as means ± standard deviations determined from several independent experiments. The DMSO concentration never exceeded 0.1% and did not inhibit the parasite growth.

4.25. Inhibition of β-hematin formation test

The methodology was the same as Baelmans and co-workers.[47] Deferoxamine mesylate presented 0% inhibition from 1 to 20 mM.

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