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Antiparasitic hybrids of *Cinchona* alkaloids and bile acids

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ABSTRACT

A series of 16 hybrids of *Cinchona* alkaloids and bile acids (**4a**–**h**, **5a**–**h**) was prepared by means of a Barton–Zard decarboxylation reaction. Quinine, quinidine, cinchonine and cinchonidine were functionalized at position C-2 of the quinoline nucleus by radical attack of a *nor*cholane substituent. The newly synthesized hybrids were evaluated *in vitro* for their antitrypanosomal, antileishmanial and antiplasmodial activities, along with their cytotoxicity against WI38, a normal human fibroblast cell line. Seven compounds (**4d**, **4f**, **4h**, **5b**, **5d**, **5f**, **5h**) showed promising trypanocidal activity with IC_{50} values in the same range as the commercial drug suramine. Moreover all the 16 hybrids showed antiplasmodial activity ($IC_{50} \leq 6$ μg/ml), particularly those containing a *nor*-chenodeoxycholane moiety (**4b**, **4d**, **4f**, **4h**, **5b**, **5d**, **5f**, **5h**) with IC_{50} values comparable to those of the natural alkaloids, and selectivity indices in the range of 5.6–15.7.

1. Introduction

Infectious diseases caused by protozoan parasites, still remain as major health problems, affecting particularly developing countries in regions such as Latin America and Africa. Among them, leishmaniasis, malaria and trypanosomiasis, caused by the parasites *Leishmania* sp., *Plasmodium* sp. and *Trypanosoma* sp. respectively, are of great concern. According to the World Health Organisation (WHO), 12 million people are currently affected by leishmaniasis, 10 million patients suffer Chagas' disease and 30,000 patients are affected by the African sleeping sickness (the last two both caused by *Trypanosoma* species). In the case of malaria, as of 2011, 258 million clinical cases have been reported [1]. Some forms of these parasites are lethal for humans and, as an example, the WHO estimated that 660,000 people died of malaria in 2010 [1]. Moreover, the medical treatment of these diseases suffers growing difficulties due of the increase of resistant strains to classical medication, and the toxic effects and relative efficacy of some of the drugs currently in use. These observations show the necessity and the growing interest for the discovery of new safer and more efficient antiprotozoal compounds to treat these infectious diseases.

Among the drugs currently in use in the treatment of malaria, quinine (**1a**) and its derivatives still remain the drugs of choice to treat severe cases. Generally speaking, compounds possessing a quinoline nucleus display a variety of biological activities [2], and particularly potent antiparasitic properties, such as antimalarial [3], antileishmanial [4,5] and trypanocidal activities [6]. For this reason, several examples of syntheses of derivatives possessing a quinoline nucleus have been reported, and quinoline itself as a scaffold, still remains a good starting material in the preparation of new antiparasitic drugs. On the other hand, there is a growing interest in the synthesis of hybrids of natural compounds to find new active substances. Nature itself is an example of the biosynthesis of hybrid molecules, with fragments belonging to the same or different biosynthetic pathways. Probably the best known examples of active natural hybrids are the antitumoral *Vinca* alkaloids, which are asymmetric dimers composed of two inactive fragments, i.e. a vindoline ring connected to a catharantine ring. Bioconjugation has emerged as a fast growing methodology in medicinal chemistry and aims at the binding of two or more active molecules to form new complex substances with the combined properties of their individual components [7]. As an example, Walsh et al. reported the synthesis and biological evaluation of a covalently linked artemisinin–quinine hybrid, which showed better efficacy against drug-sensitive and drug-resistant malaria than the individual drugs *per se* or a 1:1 mixture of both individual components [8]. Recently,

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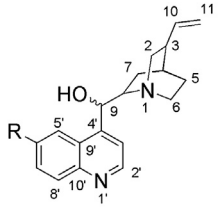
some examples of aminoquinoline–steroid conjugates were reported to have good *in vitro* activities against *Leishmania major* and *Mycobacterium tuberculosis* [9]. In this context, several bioactive hybrids based on a steroidal framework have been reported [10]. Some of these hybrids showed antiparasitic activity, for example 6-thiopurine derivatives containing 1,2,3-triazole and steroidal moieties were reported to exhibit promising antimalarial and antileishmanial activities [11].

We have recently started a project on synthetic modifications of abundant and easily accessible natural starting materials such as bile acids and plant terpenoids. In this context, we decided to synthesise hybrids of *Cinchona* alkaloids and bile acids as possible antiparasitic compounds. The choice of bile acids as ligands was based on their wide occurrence and accessibility, their role as good drug transporters [12,13], and, moreover, the various examples of steroids that display antiparasitic activity [14]. We recently reported the synthesis of cytotoxic steroidal quinones and hydroquinones derived from bile acids using a Barton–Zard radical decarboxylation reaction [15], and we decided to apply this methodology to the *Cinchona* alkaloids. The Barton–Zard decarboxylation reaction of *O*-acyl esters formed from carboxylic acids and *N*-hydroxy-2-thiopyridone is a versatile tool that can be used for functional group conversions and the formation of carbon–carbon bonds [16]. The N–O bond of Barton esters can be cleaved either in thermal or photolytic conditions to generate a carboxyl radical and a pyridine-2-thyil radical. The carboxyl radical then undergoes CO_2 loss to generate an alkyl radical which can react with the electron-deficient position of an electron-rich substrate to form a new carbon–carbon bond. Some examples were reported with heteroaromatic compounds such as pyridine and quinoline, leading to the radical addition at positions 2 and 4 of the heterocycle [17]. This work is the first example of the use of a Barton–Zard reaction on natural *Cinchona* alkaloids (**1a**–**d**) (Fig. 1), in which position 4′ is occupied by the bicyclic quinuclidine moiety, leaving position 2′ as the only electron-deficient position of the quinoline nucleus available for radical addition. The synthesis of new bioactive compounds by functionalization of quinine at position 2′ was previously achieved using organo-magnesium and organo-lithium chemistry [18]. However, this strategy faces the usual problems of functional group incompatibility in the presence of such strongly basic and/or nucleophilic reagents, which limits its use in the case of highly complex and multiply-functionalized natural products. On the other hand, the Barton–Zard reaction takes place in mild conditions, and represents an original and simple approach to obtain C-2′-substituted *Cinchona* alkaloids. The only requirement for the substituent group is the presence of a carboxylic acid, a frequent functional group in natural products by itself, or easily accessible by oxidation of primary alcohols or aldehydes. Furthermore, this strategy can also be applied in the preparation of new *Cinchona* alkaloids modified at position 2′, which may be useful as organocatalysts in asymmetric reactions [19]. Herein we report the application of the radical Barton–Zard decarboxylation reaction strategy for the preparation of 16 new *Cinchona* alkaloid-bile acid hybrids (**4a**–**h**, **5a**–**h**) (Fig. 2). All the synthesized compounds were completely characterized by spectroscopic techniques, and were evaluated *in vitro* for their antiparasitic activities against *Trypanosoma brucei brucei*, *Leishmania mexicana mexicana* and *Plasmodium falciparum*, along with their cytotoxicity against a human normal fibroblast cell line, WI-38.

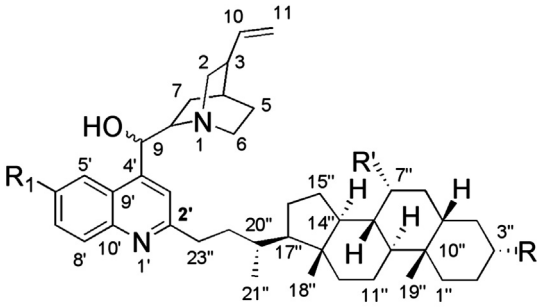


Quinine (**1a**) R = OCH_3, 9*R*
Quinidine (**1b**) R = OCH_3, 9*S*
Cinchonine (**1c**) R = H, 9*S*
Cinchonidine (**1d**) R = H, 9*R*

Fig. 1. Structures of *Cinchona* alkaloids **1a**–**d**.

2. Chemistry

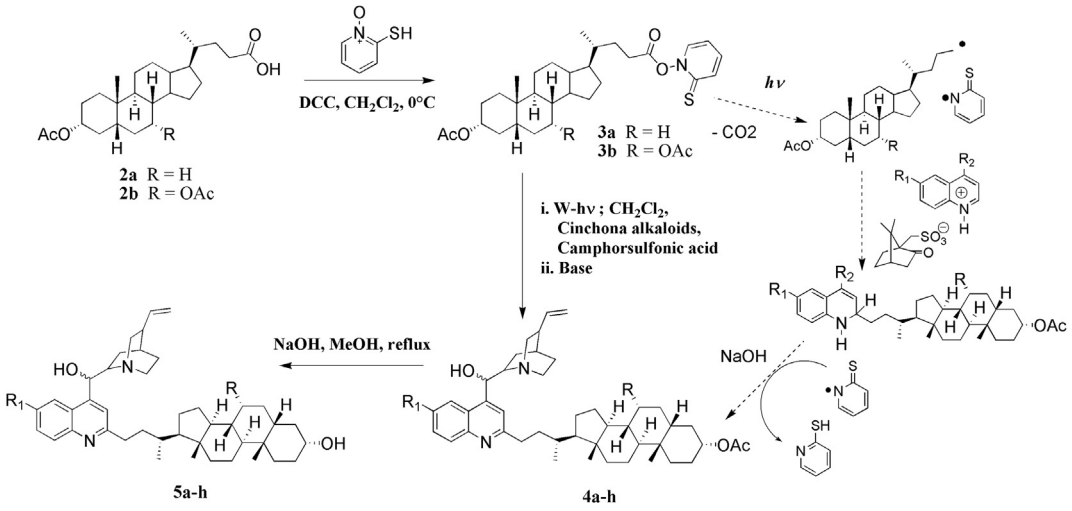
In the present work, the Barton–Zard decarboxylation was used for the preparation of compounds **4a**–**h**, following the synthetic pathway outlined in Scheme 1. The Barton esters **3a**–**b** were prepared from peracatylated chenodeoxycholic and lithocolic acids respectively, by reaction with 2-mercaptopyridine N-oxide and DCC (*N*,*N*′-dicyclohexylcarbodiimide) in dry CH_2Cl_2 at 0 °C, as previously reported [15]. These esters were used without purification, after removal of dicyclohexylurea by simple filtration trough a cotton plug. They were then subjected to photolytic decarboxylation in the presence of a large excess (10 eq.) of a protonated *Cinchona* alkaloid (**1a**–**b**) as radical trap, by irradiation with a 300 W tungsten lamp at 0 °C in CH_2Cl_2 under N_2 atmosphere to yield the corresponding hybrids **4a**–**h**. Quinine (**1a**), quinidine (**1b**), cinchonine (**1c**) and cinchonidine (**1d**) were used as substrates. Contrary to other examples of Barton–Zard reactions with simpler protonated heterocycles [20,21], two equivalents of camphorsulfonic acid *per* equivalent of *Cinchona* alkaloid had to be used, taking into account the presence of two basic nitrogen atoms. The nitrogen atom of the bicyclic quinuclidine has a pKb ~4.5, which is far lower than that of the aromatic quinoline ring (pKb ~ 9) of the alkaloids. For the same reason, the reaction was quenched with aqueous NaOH (2N) to obtain the free basic form of the hybrids. When the reaction was quenched with $NaHCO_3$ the compounds were obtained in their protonated forms, as evidenced in the ^{1}H NMR spectra by the deshielding of some protons of the bicyclic quinuclidine moiety. For example H-9 was shifted from 5.76 to 6.27 ppm and H-6a from 3.69 to 4.42 by protonation of compound **4a** (Fig. 3). Considering the



	R_1	OH-9	R	R'
4a	OCH_3	*R*	OAc	H
4b	OCH_3	*R*	OAc	OAc
4c	OCH_3	*S*	OAc	H
4d	OCH_3	*S*	OAc	OAc
4e	H	*S*	OAc	H
4f	H	*S*	OAc	OAc
4g	H	*R*	OAc	H
4h	H	*R*	OAc	OAc

	R_1	OH-9	R	R'
5a	OCH_3	*R*	OH	H
5b	OCH_3	*R*	OH	OH
5c	OCH_3	*S*	OH	H
5d	OCH_3	*S*	OH	OH
5e	H	*S*	OH	H
5f	H	*S*	OH	OH
5g	H	*R*	OH	H
5h	H	*R*	OH	OH

Fig. 2. Structures of hybrids **4a**–**h**, **5a**–**h**.



Scheme 1. General procedure for the preparation of compounds **4a**–**h** and **5a**–**h**.

large excess of *Cinchona* alkaloids used in the reaction and their similar polarity compared with the hybrids, the purification of the desired products **4a**–**h** had to be done in two steps. In the first step, the less polar side-products were removed by normal phase chromatography. Then, a final purification by reversed-phase VLC was performed to purify the product from the excess of starting alkaloids. The reaction yields after purification are expressed in Table 1.

Cleavage of the acetate groups was achieved by treatment of compounds **4a**–**h** with 20% NaOH in methanol at reflux, yielding quantitatively the deacetylated hybrids **5a**–**h** (Scheme 1).

The use of 10 equivalents of alkaloid, despite the difficulties in the removal of the excess after reaction completion, was maintained in order to favour the formation of the desired hybrids instead of the addition product of the pyridine-2-thyil radical to the bile acid radical, which occurs in the absence of a radical trap, and is one of the main by-products of this reaction [22].

3. Pharmacology

The antiparasitic activity of the 16 *Cinchona* alkaloid − bile acid hybrids (**4a**–**h**, **5a**–**h**) was evaluated *in vitro* against *Trypanosoma brucei brucei* bloodstream forms, *L. mexicana mexicana* promastigotes, and a chloroquine-sensitive strain of *Plasmodium falciparum* (3D7). The cytotoxicity of the hybrids against a human normal fibroblast cell line, WI-38, was also tested. Suramine, amphotericin B, artemisinin and campthotecin were used respectively as positive controls. The compounds were evaluated in a concentration range from 0.009 μg/ml to 20 μg/ml, from which their IC_{50}s (drug concentration resulting in 50% inhibition of parasite or cell growth) were calculated. Selectivity indices were calculated for each parasite with the formula IC_{50}(WI38)/IC_{50}(parasite). The results of the biological evaluation are listed in Table 2.

4. Results and discussion

A series of new hybrids (**4a**–**h**) obtained by radical decarboxylative addition of a peracetylated bile acid (**2a**–**b**) to position 2′ of *Cinchona* alkaloids (**1a**–**d**) were prepared as illustrated in Scheme 1, with overall yields of 20–30% considering the two reaction steps and final purification (Table 1). The structure of compounds **4a**–**h** and particularly the new C–C bond between C-2′ of the quinoline nucleus and C-23 of the *nor*cholane group could be ascertained by NMR analysis. In the ^{1}H NMR spectra of the products, the absence of a signal around 8.70 ppm (H-2′) of the *Cinchona* alkaloids was evident (Fig. 3). The ^{13}C NMR spectra revealed that the CH-2′ signal (typically at δ 147–150 ppm) of the *Cinchona* alkaloids was replaced by a quaternary carbon signal at 160 ppm (see spectra in Supplementary material). Finally, HMBC correlations between H-3′, H-23″ and H-22″ and C-2′ and between H-3′ and C-23″ confirmed undoubtedly the new C–C bond and the structure of the compounds (Fig. 4).

The initial set of hybrids **4a**–**h** was first obtained as the peracetylated forms, since previous observations dictated that the preparation of the Barton esters gave better yields using the peracetylated derivatives instead of the free bile acids [15]. In order to obtain the free hybrids **5a**–**h**, deacetylation of **4a**–**h** was quantitatively achieved by treatment with a solution of NaOH in MeOH (20%) at reflux. The structure of the deacetylated hybrids was confirmed by the disappearance of the acetyl singlets in the ^{1}H NMR spectra (typically at 2.02 ppm for OAc-3β and 2.04 for OAc-7β) (Fig. 3), and of the C=O intense band in the IR spectra (1730 cm^{-1}), along with the appearance of a wide band at 3300 cm^{-1} corresponding to the hydroxyl group.

The assignment of all ^{1}H and ^{13}C resonances was performed by analysis of a complete set of 1D and 2D NMR experiments. These assignments are provided in the Supplementary material.

Compounds **4a**–**h** and **5a**–**h** were evaluated for their antiparasitic activities along with the four starting *Cinchona* alkaloids (**1a**–**d**). Their cytotoxicity against normal human fibroblast WI38 cells was also evaluated. According to R. Pink et al. an antiparasitic hit should be active *in vitro* against protozoa with $IC_{50} \leq 1$ μg/ml and should be selective (at least tenfold more active against the parasite than against a mammalian cell line) [23].

Concerning the antitrypanosomal assay, all the hybrids showed interesting activities, with IC_{50}s comprised between 0.37 and 3.53 μg/ml and selectivity index larger than 1.3. The trypanocidal activity of the *Cinchona* alkaloids is increased when linked to a bile acid. However, the activity of the hybrids **4a**–**h** and **5a**–**h** seems less dependent on the nature of the alkaloid than on the nature of the bile acid. An IC_{50} ratio: IC_{50}(litho)/IC_{50}(cheno) was calculated for each pair of hybrids with a different alkaloid for comparative purposes. For a given steroidal substituent, the activity is indeed generally similar when linked to any of the alkaloids but increases with the hydroxylation degree of the steroid nucleus (Table 3), up to 9.5 fold of IC_{50} ratio in the case of the quinine/chenodeoxycholic acid hybrid (**5a**) compared to the quinine/lithocholic acid hybrid

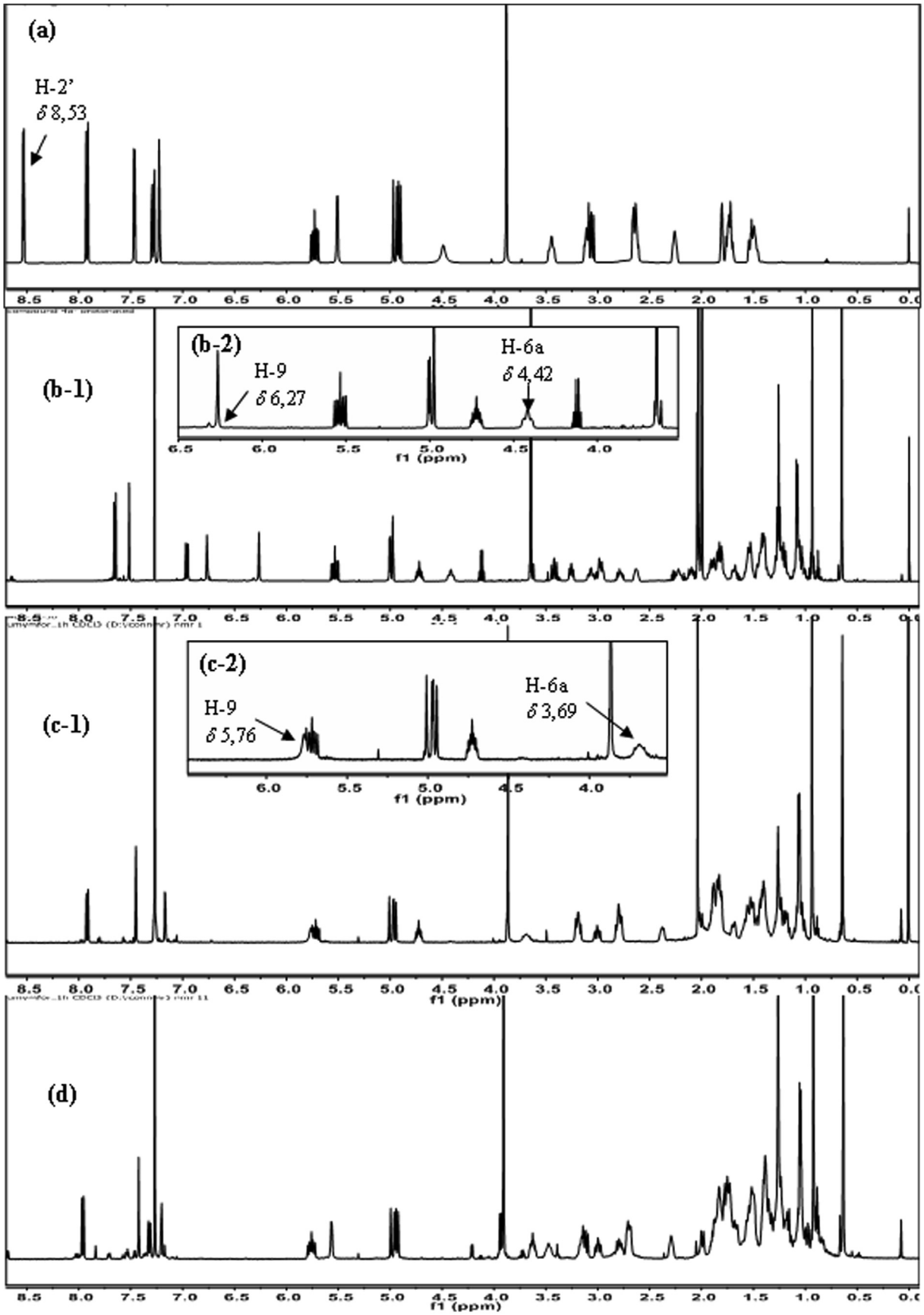


Fig. 3. ^{1}H NMR spectra of quinine (a), protonated form of **4a** (b-1), non-protonated **4a** (c-1) and **5a** (d) and zoom of spectra between 3,5 and 7 ppm of protonated **4a** (b-2) and non-protonated **4a** (c-2), at 500 MHz in $CDCl_3$.

Table 1
Summary of the obtained *Cinchona* alkaloid – bile acid hybrids.

Compound	Alkaloid	Bile acid (peracetated)	Yield (%)[a]	Deprotected compound[b]
4a	Quinine	Lithocholic	21	**5a**
4b	Quinine	Chenodeoxycholic	23	**5b**
4c	Quinidine	Lithocholic	13	**5c**
4d	Quinidine	Chenodeoxycholic	24	**5d**
4e	Cinchonine	Lithocholic	23	**5e**
4f	Cinchonine	Chenodeoxycholic	34	**5f**
4g	Cinchonidine	Lithocholic	19	**5g**
4h	Cinchonidine	Chenodeoxycholic	35	**5h**

[a] The yields were calculated after the two steps of the reaction and 2 steps of purification, and are based on the amount of starting bile acid peracetate.
[b] Compounds obtained quantitatively after treatment of the corresponding peracetylated hybrids with NaOH in methanol at reflux.

(**5b**). Acetylation of the hydroxyl groups of the bile acids does not seem to have a real impact on the *in vitro* assay but should be considered in case of future studies, due to its different solubility. In this series of compounds, the most promising hybrids are the ones having a chenodeoxycholic-derived *nor*cholane substituent (IC_{50} around 0.40 μg/ml), and particularly hybrids **4d** and **5b** which have a selectivity indices of 7.1 and 11.1 respectively, faced to their cytotoxicity against the normal human fibroblast cell line WI38.

The antileishmanial assays revealed that, except **5a**, all the hybrids inhibit 50% of parasite growth at concentrations lower than 20 μg/ml (Table 2). Once again, the activity is increased when a chenodeoxycholic-derived *nor*cholane substituent is linked to the alkaloid instead of a lithocholic-derived substituent. This trend is particularly evident in the case of the peracetylated derivatives of the hybrids (three-fold more active). However, regarding the selectivity index (most often < 1), the semisynthetic compounds were more toxic against normal cells than against the parasite, making them unattractive for further evaluation.

Finally, the antiplasmodial evaluation of the compounds revealed interesting results. Although none of the hybrids exhibits a better *in vitro* activity than the corresponding natural alkaloids, they are all active with IC_{50} < 6 μg/ml and 12 of them with $IC_{50} \leq 1$ μg/ml with good selectivity indices (Table 2). As in the case of the trypanocidal activity, an IC_{50} ratio: IC_{50}(litho)/IC_{50}(cheno) was calculated (Table 4). The difference in antiplasmodial activity in the series can also be attributed to the bile acid linked to the alkaloid. The presence of two hydroxyl groups in the steroidal

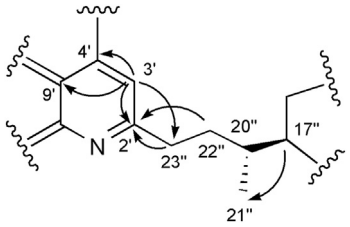


Fig. 4. Typical NMR HMBC correlations observed for compounds **4a**–**h** and **5a**–**h**.

Table 3
Comparison of the *in vitro* antitrypanosomal activity of the compounds.

Alkaloid	Lithocholic acid	Ratio	Chenodeoxycholic acid
	IC_{50} (μg/ml) (selectivity)	IC_{50} (litho)/ IC_{50}(cheno)	IC_{50} (μg/ml) (selectivity)
	Peracetylated acid		*Peracetylated acid*
Quinine	**4a** 2.03 (S = 8.0)	1.7	**4b** 1.22 (S = 3.2)
Quinidine	**4c** 2.97 (S = 2.6)	6.8	**4d** 0.44 (S = 7.1)
Cinchonine	**4e** 1.26 (S = 5.6)	2.9	**4f** 0.43 (S = 4.5)
Cinchonidine	**4g** 1.27 (S = 4.4)	3.1	**4h** 0.41 (S = 4.3)
	Free acid		*Free acid*
Quinine	**5a** 3.53 (S = n.d.)	9.5	**5b** 0.41 (S = 11.1)
Quinidine	**5c** 0.79 (S = 3.3)	2.1	**5d** 0.37 (S = 5.3)
Cinchonine	**5e** 3.01 (S = 1.3)	7.7	**5f** 0.39 (S = 3.6)
Cinchonidine	**5g** 2.16 (S = 4.3)	5.4	**5g** 0.40 (S = 4.3)

Selectivity: S = IC_{50}(WI38)/IC_{50}(*Tbb*); n.d. = not determined.

Table 2
In vitro antiparasitic and cytotoxic activities, and selectivity indices of compounds **4a**–**h** and **5a**–**h**.

Compound	Cytotoxicity	*T. brucei brucei*		*L. mexicana mexicana*		*P. falciparum*	
	IC_{50} (μg/ml) ± SD	IC_{50} (μg/ml) ± SD	Selectivity	IC_{50} (μg/ml) ± SD	Selectivity	IC_{50} (μg/ml) ± SD	Selectivity
	WI38	*Tbb*	WI38/*Tbb*	*Lmm*	WI38/*Lmm*	3D7	WI38/3D7
4a	16.16 ± 1.24	2.03 ± 1.12	8.0	13.97 ± 1.83	1.2	6.07 ± 3.29	2.7
4b	3.92 ± 1.33	1.22 ± 0.03	3.2	3.49 ± 0.46	1.1	0.69 ± 0.19	5.6
4c	7.79 ± 1.96	2.97 ± 0.26	2.6	13.67 ± 0.27	0.57	0.81 ± 0.13	9.7
4d	3.10 ± 1.11	0.44 ± 0.01	7.1	4.15 ± 0.13	0.75	0.38 ± 0.08	8.1
4e	7.02 ± 1.31	1.26 ± 0.01	5.6	12.06 ± 2.71	0.58	2.26 ± 0.46	3.1
4f	1.93 ± 0.06	0.43 ± 0.03	4.5	4.11 ± 0.45	0.47	0.19 ± 0.06	10.2
4g	5.58 ± 1.74	1.27 ± 0.07	4.4	12.19 ± 1.29	0.46	1.21 ± 0.48	4.6
4h	1.77 ± 0.46	0.41 ± 0.01	4.3	3.91 ± 0.04	0.45	0.21 ± 0.04	8.4
5a	>20	3.53 ± 0.19	>5.7	>20	n.d.	4.24 ± 0.45	>4.7
5b	4.57 ± 1.43	0.41 ± 0.04	11.1	4.67 ± 1.10	0.98	0.30 ± 0.06	15.5
5c	2.59 ± 1.72	0.79 ± 0.19	3.3	4.05 ± 0.07	0.64	0.68 ± 0.03	3.8
5d	1.95 ± 0.84	0.37 ± 0.03	5.3	3.86 ± 0.09	0.51	0.14 ± 0.01	14.0
5e	3.97 ± 1.81	3.01 ± 0.03	1.3	4.08 ± 0.14	0.97	0.86 ± 0.28	4.6
5f	1.42 ± 0.48	0.39 ± 0.01	3.6	3.39 ± 0.72	0.42	0.09 ± 0.01	15.7
5g	9.28 ± 0.76	2.16 ± 0.32	4.3	12.81 ± 0.82	0.72	1.07 ± 0.28	8.7
5h	1.71 ± 0.54	0.40 ± 0.02	4.3	3.45 ± 0.54	0.50	0.23 ± 0.02	7.5
Quinine **1a**	6.56 ± 0.01	6.87 ± 2.60	1.0	>20	n.d.	0.20 ± 0.11	32.0
Quinidine **1b**	11.28 ± 2.29	8.42 ± 0.42	1.3	>20	n.d.	0.06 ± 0.05	179.1
Cinchonine **1c**	>20	>20	n.d.	>20	n.d.	0.09 ± 0.05	>222
Cinchonidine **1d**	>20	18.28 ± 1.23	n.d.	5.74 ± 0.28	0.96	0.28 ± 0.27	>71
Campthotecin	0.4 ± 0.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Suramine	n.d.	0.11 ± 0.02	n.d.	n.d.	n.d.	n.d.	n.d.
Amphotericin B	n.d.	n.d.	n.d.	0.10 ± 0.01	n.d.	n.d.	n.d.
Artemisinin	n.d.	n.d.	n.d.	n.d.	n.d.	0.013 ± 0.003	n.d.

SD = Standard deviation; Selectivity = IC_{50}(WI38)/IC_{50}(*Tbb* or *Lmm* or 3D7); n.d. = not determined.

Table 4
Comparison of the *in vitro* antiplasmodial activity of the compounds.

Alkaloid		IC_{50} 3D7 (μg/ml) (selectivity index)		Ratio
		Lithocholic	Chenodeoxycholic	IC_{50}(litho)/ IC_{50}(cheno)
Quinine	Peracetylated (Ac) acid	**4a** 6.07 ($S = 2.7$)	**4b** 0.69 ($S = 5.6$)	**8.8**
	Free (OH) acid	**5a** 4.25 (S = n.d.)	**5b** 0.30 ($S = 15.5$)	**14**
	Ratio IC_{50}(Ac)/ IC_{50}(OH)	**1.4**	**2.3**	
Quinidine	Peracetylated (Ac) acid	**4c** 0.81 ($S = 9.7$)	**4d** 0.38 ($S = 8.1$)	**2.1**
	Free (OH) acid	**5c** 0.68 ($S = 3.8$)	**5d** 0.14 ($S = 14.0$)	**4.9**
	Ratio IC_{50}(Ac)/ IC_{50}(OH)	**1.2**	**2.7**	
Cinchonine	Peracetylated (Ac) acid	**4e** 2.26 ($S = 3.1$)	**4f** 0.19 ($S = 10.2$)	**11.9**
	Free (OH) acid	**5e** 0.86 ($S = 4.6$)	**5f** 0.09 ($S = 15.7$)	**9.6**
	Ratio IC_{50}(Ac)/ IC_{50}(OH)	**2.6**	**2.0**	
Cinchonidine	Peracetylated (Ac) acid	**4g** 1.21 ($S = 4.6$)	**4h** 0.21 ($S = 8.4$)	**5.8**
	Free (OH) acid	**4g** 1.07 ($S = 8.7$)	**5h** 0.23 ($S = 7.5$)	**4.7**
	Ratio IC_{50}(Ac)/ IC_{50}(OH)	**1.1**	**0.9**	

Selectivity index: S = IC_{50}(WI38)/IC_{50}(3D7); n.d. = not determined.

substituent increases strongly the activity of the hybrids, up to 14-fold in the IC_{50} ratio in the case of the quinine/chenodeoxycholic acid hybrid (**5b**) compared to the quinine/lithocholic acid hybrid (**5a**). However, contrary to the antitrypanosomal activity results, a trend can be observed with respect to the activity of deacetylated compounds: they are nearly twice as active as the acetylated hybrids (Table 4). An IC_{50} ratio between the acetylated and deacetylated hybrids was calculated for comparative purposes and included in Table 4. After careful analysis of these results, the most promising antiplasmodial compounds in this series of hybrids, are those with a chenoxydeoxycholic-derived norcholane substituent, particularly compounds **5b**, **5d** and **5f** with IC_{50}s of 0.30, 0.14 and 0.09 μg/ml, respectively, which are comparable to the respective values of the natural alkaloids, and selectivity indices > 14.

5. Conclusion

In conclusion, a novel series of hybrids (**4a**–**h**, **5a**–**h**) has been synthesised by attack of a bile acid-derived *nor*cholane radical at position 2′ of *Cinchona* alkaloids. These hybrids have an original structural framework due to the formation of a new C–C bond between C-2′ of the quinoline nucleus and C-23 of the *nor*cholane group. This work is the first example of the use of a Barton–Zard decarboxylation on this family of alkaloids, and represents a new strategy for the functionalization of *Cinchona* alkaloids at position 2′. Although the yields of the hybrids are not very high, the great structural complexity achieved in the final products after only two reaction steps should be considered. Besides, the starting bile acids are cheap and readily accessible materials, and the large excess of *Cinchona* alkaloids can eventually be recovered from the reaction mixture. All the 16 hybrids exhibited antiparasitic activity against the growth of *T. brucei brucei*, *L. mexicana mexicana* and/or *P. falciparum*. The leishmanicidal activity could be attributed to the general toxicity of the compounds observed also against normal human fibroblast WI38 cells. This was not the case for the antitrypanosomal and antiplasmodial activities, and some of the hybrids may be considered good antiparasitic hits, according to the criteria of R. Pink et al. [23], i.e. $IC_{50} \leq 1$ μg/ml and selectivity index > 10. In both cases an increase of the antiparasitic activity could be observed when increasing the hydroxylation degree of the bile acid. This may be attributed to a better solubility in physiological conditions and to an increase of cell penetration. The *in vitro* trypanocidal activity of the natural *Cinchona* alkaloids was strongly increased when linked to a bile acid and seven hybrids containing a chenodeoxycholic moiety had an IC_{50} around 0.40 μg/ml, in the same range as the *in vitro* activity of the commercial drug suramine used as positive control in the assay ($IC_{50} = 0.10$ μg/ml). Regarding the *in vitro* antiplasmodial activity, the 16 hybrids exhibited low IC_{50}s, particularly those having a chenodeoxycholic moiety for which the activity was comparable to the free natural alkaloids, albeit their lower selectivity. However, these results were obtained for the chloroquine-sensitive strain 3D7, which means that the compounds should have to be further evaluated on a resistant strain in future studies. This first evaluation of antiparasitic *in vitro* activities of hybrids *Cinchona* alkaloids/bile acids gives a good starting point for the discovery of new antitrypanosomal and antiplasmodial leads, and can be seen as an example of the use of bile acids as drug transporters. The hydroxylation degree of the bile acid seems to have a positive effect on the activity and the preparation of additional hybrids in this sense has already started in our laboratory.

6. Experimental

6.1. General

Bile acids (lithocholic and chenodeoxycholic acids) and *Cinchona* alkaloids (quinine, quinidine, cinchonine, cinchonidine) were obtained from commercial sources and used as such or recrystallized prior to use when necessary. Peracetylated derivatives of bile acids were obtained by standard procedures, using Ac_2O/DMAP/Pyridine. 2-Mercaptopyridine-*N*-oxide, *N*,*N*′-dicyclohexylcarbodiimide DCC and camphorsulfonic acid were purchased from Aldrich. All the solvents were distilled prior to use and CH_2Cl_2 used for reactions was bidistilled from phosphorous pentoxide. NMR spectra were recorded in deuterated chloroform on Bruker AC-200 (200.13 MHz) and Bruker Avance II (500.13 MHz) spectrometers, using the signals of residual non-deuterated chloroform as internal reference (δ_H 7.26, δ_C 77.0). All 2D NMR experiments (COSY, DEPT-HSQC, HMBC, and NOESY) were performed using standard pulse sequences. HRMS were acquired on a Bruker micrOTOF-Q II spectrometer. IR spectra were obtained on an FT-IR Nicolet Magna 550 instrument, optical rotations on a Perkin Elmer Polarimeter 343 (at 589 nm), and melting points on a Fisher-Johns apparatus. TLCs were carried out on Merck Sílicagel 60 F254 plates. TLC plates were sprayed with 2% vanillin in concentrated H_2SO_4 or with Dragendorff's spray reagent (Aldrich). Merck Silicagel (230–400 mesh) and RP-18 (Aldrich) were used for vacuum liquid chromatography.

6.2. Chemistry

6.2.1. General procedure for the formation of hybrids *4a*–*h*

In a flask protected from light with aluminium foil, the peracetate of a bile acid (**2a**–**b**, 80 mg, 0.1–0.2 mmol) and 2-mercaptopyridine-*N*-oxide (1,5 equivalents) were dissolved in 8 mL of dry CH_2Cl_2, and the solution was cooled to 0 °C. Then, 1.5 eq. of DCC were added and the solution was stirred at 0 °C between 2 and 3 h, monitoring the reaction by TLC. The solution was directly

filtered through a cotton plug to remove dicyclohexylurea, into a flask containing a *Cinchona* alkaloid (**1a**–**d**, 10 eq.) and camphorsulfonic acid (20 eq.) dissolved in 4 mL of dry CH_2Cl_2. The solution was set under N_2, and subsequently irradiated using a single 300 W tungsten lamp during 1h15′ while maintaining the temperature at 0 °C with an ice bath. The reaction was quenched with an aqueous solution of NaOH (2N) and extracted 3 times with CH_2Cl_2. The organic layers were combined, washed with water and evaporated leaving a residue. The latter was first rapidly separated in 4 fractions by VLC on silica, eluting with cyclohexane/EtOAc/methanol 1/1/0, 0/1/0, 0/95/5 and 0/9/1 respectively. Fractions 3 and 4 were pooled together to be further purified by VLC on reversed-phase (Rp-18), eluting with mixtures of H_2O/MeOH from 1/0 to 0/1, increasing the proportion of methanol by steps of 10%, leading to the desired compound (**4a**–**h**).

*6.2.2. Deacetylation of hybrids **4a**–**h***

A solution of NaOH in methanol (20%) was added to compounds **4a**–**h** up to a final concentration of 1 mg/mL, and the solution was then refluxed for 3 h in the case of the hybrids with a lithocholic acid moiety and overnight for the hybrids derived from chenodeoxycolic acid. The solvent was removed under reduced pressure and the residue dissolved in H_2O before extraction with dichloromethane. Evaporation of the organic layer led to the desired compound (**5a**–**h**) with a quantitative yield.

*6.2.3. Compound **4a***

White amorphous powder; mp 104–114 °C; IR (KBr, ν_{max}, cm^{-1}): 2933, 2865, 1734, 1622, 1601, 1451, 1380, 1362, 1242, 1032, 758; α_D ($CHCl_3$, *c* 0.440) − 20; ^1H and ^{13}C NMR: see Tables S17 and S19 in Supplementary material. HRMS (ESI+): $[M + H]^+$ $C_{45}H_{65}N_2O_4$, calculated for 697.4939, found 697.4906.

*6.2.4. Compound **4b***

White amorphous powder; mp 87–97 °C; IR (KBr, ν_{max}, cm^{-1}): 2933, 2871, 1733, 1640, 1601, 1451, 1377, 1363, 1247, 1236, 1032; α_D ($CHCl_3$, *c* 0.395) − 29; ^1H and ^{13}C NMR: see Tables S17 and S19 in Supplementary material. HRMS (ESI+): $[M + H]^+$ $C_{47}H_{67}N_2O_6$, calculated for 755.4994, found 755.5026.

*6.2.5. Compound **4c***

White amorphous powder; mp 80–90 °C; IR (KBr, ν_{max}, cm^{-1}): 2927, 2865, 1736, 1621, 1600, 1452, 1380, 1360, 1241, 1028, 833; α_D ($CHCl_3$, *c* 0.285) + 70; ^1H and ^{13}C NMR: see Tables S17 and S19 in Supplementary material. HRMS (ESI+): $[M + H]^+$ $C_{45}H_{65}N_2O_4$, calculated for 697.4939, found 697.4969.

*6.2.6. Compound **4d***

White amorphous powder; mp 100–114 °C; IR (KBr, ν_{max}, cm^{-1}): 2933, 2871, 1733, 1622, 1457, 1375, 1362, 1247, 1234, 1026, 834, 755; α_D ($CHCl_3$, *c* 0.215) + 89; ^1H and ^{13}C NMR: see Tables S17 and S19 in Supplementary material. HRMS (ESI+): $[M + H]^+$ $C_{47}H_{67}N_2O_6$, calculated for 755.4994, found 755.4996.

*6.2.7. Compound **4e***

White amorphous powder; mp 104–112 °C; IR (KBr, ν_{max}, cm^{-1}): 2933, 2867, 1736, 1600, 1452, 1379, 1362, 1243, 1027, 757; α_D ($CHCl_3$, *c* 0.365) +89; ^1H and ^{13}C NMR: see Tables S18 and S19 in Supplementary material. HRMS (ESI+): $[M + H]^+$ $C_{44}H_{63}N_2O_3$, calculated for 667.4833, found 667.4860.

*6.2.8. Compound **4f***

White amorphous powder; mp 105–113 °C; IR (KBr, ν_{max}, cm^{-1}): 2937, 2870, 1733, 1600, 1452, 1377, 1364, 1247, 1024, 756; α_D ($CHCl_3$, *c* 0.365) + 72; ^1H and ^{13}C NMR: see Tables S18 and S19 in Supplementary material. HRMS (ESI+): $[M + H]^+$ $C_{46}H_{65}N_2O_5$, calculated for 725.4888, found 725.4918.

*6.2.9. Compound **4g***

White amorphous powder; mp 84–90 °C; IR (KBr, ν_{max}, cm^{-1}): 2935, 2866, 1737, 1601, 1451, 1380, 1362, 1243, 1028, 757; α_D ($CHCl_3$, *c* 0.360) − 4; ^1H and ^{13}C NMR: see Tables S18 and S19 in Supplementary material. HRMS (ESI+): $[M + H]^+$ $C_{44}H_{63}N_2O_3$, calculated for 667.4833, found 667.4842.

*6.2.10. Compound **4h***

White amorphous powder; mp 165–170 °C; IR (KBr, ν_{max}, cm^{-1}): 2939, 2867, 1733, 1601, 1452, 1377, 1364, 1248, 1025, 759; α_D ($CHCl_3$, *c* 0.375) −17; ^1H and ^{13}C NMR: see Tables S18 and S19 in Supplementary material. HRMS (ESI+): $[M + H]^+$ $C_{46}H_{65}N_2O_5$, calculated for 725.4888, found 725.4876.

*6.2.11. Compound **5a***

White amorphous powder; mp 208–222 °C; IR (KBr, ν_{max}, cm^{-1}): 3290, 2929, 2863, 1622, 1600, 1456, 1363, 1233, 1035, 756; α_D ($CHCl_3$, *c* 0.350) − 18; ^1H and ^{13}C NMR: see Tables S20 and S22 in Supplementary material. HRMS (ESI+): $[M + H]^+$ $C_{43}H_{63}N_2O_3$, calculated for 655.4833, found 655.4850.

*6.2.12. Compound **5b***

White amorphous powder; mp 110–119 °C; IR (KBr, ν_{max}, cm^{-1}): 3329, 2928, 2866, 1622m 1601, 1456, 1363, 1233, 1079, 1036, 833, 757; α_D ($CHCl_3$, *c* 0.275) − 25; ^1H and ^{13}C NMR: see Tables S20 and S22 in Supplementary material. HRMS (ESI+): $[M + H]^+$ $C_{43}H_{63}N_2O_4$, calculated for 671.4782, found 671.4789.

*6.2.13. Compound **5c***

White amorphous powder; mp 214–228 °C; IR (KBr, ν_{max}, cm^{-1}): 3172, 2931, 2866, 1622, 1602, 1455, 1360, 1233, 1034, 834, 756; α_D ($CHCl_3$, *c* 0.250) + 111; ^1H and ^{13}C NMR: see Tables S20 and S22 in Supplementary material. HRMS (ESI+): $[M + H]^+$ $C_{43}H_{63}N_2O_3$, calculated for 655.4833, found 655.4840.

*6.2.14. Compound **5d***

White amorphous powder; mp 105–118 °C; IR (KBr, ν_{max}, cm^{-1}): 3351, 2928, 2865, 1621, 1601, 1454, 1359, 1232, 1078, 1034, 833, 755; α_D ($CHCl_3$, *c* 0.374) +91; ^1H and ^{13}C NMR: see Tables S20 and S22 in Supplementary material. HRMS (ESI+): $[M + H]^+$ $C_{43}H_{63}N_2O_4$, calculated for 671.4782, found 671.4797.

*6.2.15. Compound **5e***

White amorphous powder; mp 245–250 °C; IR (KBr, ν_{max}, cm^{-1}): 3346, 2925, 2860, 1600, 1453, 1263, 1107, 1070, 1046, 955, 755; α_D ($CHCl_3$, *c* 0.255) + 90; ^1H and ^{13}C NMR: see Tables S21 and S22 in Supplementary material. HRMS (ESI+): $[M + H]^+$ $C_{42}H_{61}N_2O_2$, calculated for 625.4728, found 625.4750.

*6.2.16. Compound **5f***

White amorphous powder; mp 105–114 °C; IR (KBr, ν_{max}, cm^{-1}): 3313, 2927, 2857, 1601, 1455, 1109, 1079, 999, 758; α_D ($CHCl_3$, *c* 0.355) + 77; ^1H and ^{13}C NMR: see Tables S21 and S22 in Supplementary material. HRMS (ESI+): $[M + H]^+$ $C_{42}H_{61}N_2O_3$, calculated for 641.4678, found 641.4677.

*6.2.17. Compound **5g***

White amorphous powder; mp 227–235 °C; IR (KBr, ν_{max}, cm^{-1}): 3350, 2930, 2864, 1601, 1451, 1378, 1216, 1041, 912, 758; α_D ($CHCl_3$, *c* 0.195) −8; ^1H and ^{13}C NMR: see Tables S21 and S22 in Supplementary material. HRMS (ESI+): $[M + H]^+$ $C_{42}H_{61}N_2O_2$, calculated for 625.4728, found 625.4753.

*6.2.18. Compound **5h***

White amorphous powder; mp 126–130 °C; IR (KBr, ν_{max}, cm^{-1}): 3354, 2931, 2866, 1601, 1452, 1377, 1078, 1043, 757; α_D ($CHCl_3$, *c* 0.375) − 17; ^1H and ^{13}C NMR: see Tables S21 and S22 in Supplementary material. HRMS (ESI+): $[M + H]^+$ $C_{42}H_{61}N_2O_3$, calculated for 641.4678, found 641.4694.

6.3. Biological assays

6.3.1. Parasites, cells and media

Trypanosoma brucei brucei (strain Lister 427) bloodstream forms were cultured *in vitro* in HMI9 medium containing 10% heat-inactivated foetal bovine serum [24]. Parasites were incubated in a humidified atmosphere with 5% CO_2 at 37 °C.

L. mexicana mexicana promastigotes (MHOM/BZ/84/BEL46) were cultured *in vitro* in a semi-defined medium (SDM-79) [25] supplemented with 15% heat-inactivated foetal bovine serum. Parasites were incubated in a humidified atmosphere with 5% CO_2 at 28 °C.

P. falciparum (3D7, originally isolated from a patient living near Schipol airport, The Netherlands) asexual erythrocytic stages were cultivated continuously *in vitro* according to the procedure described by Trager and Jensen (1976) at 37 °C and under an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 [26]. The host cells were human red blood cells (A or O Rh+). The culture medium was RPMI 1640 (Gibco) containing 32 mM $NaHCO_3$, 25 mM HEPES and L-glutamine. The medium was supplemented with 1.76 g/l glucose (Sigma–Aldrich), 44 mg/ml hypoxanthin (Sigma–Aldrich), 100 mg/l gentamycin (Gibco) and 10% human pooled serum (A or O Rh^+). Parasites were subcultured every 3–4 days with initial conditions of 0.5% parasitaemia and 1% haematocrit.

The human non-cancer fibroblast cell line, WI38, was cultivated *in vitro* in DMEM medium (Gibco) containing 4 mM L-glutamine, 1 mM sodium pyruvate supplemented with 10% heat-inactivated foetal bovine serum (Gibco) and penicillin–streptomycin (100 UI/ml to 100 μg/ml). Cells were incubated in a humidified atmosphere with 5% CO_2 at 37 °C.

6.3.2. In vitro antitrypanosomal activity

The *in vitro* test was performed as described by Hoet et al. (2004) [27]. Suramin (a commercial antitrypanosomal drug) was used as positive control with an initial concentration of 1 μg/ml. First stock solutions of compounds were prepared in DMSO or ethanol at 4 mg/ml. The highest concentration of solvent to which the parasites were exposed was 0.5%, which was shown to have no measurable effect on parasite viability. Compounds were tested in eight serial three-fold dilutions (final concentration range: 20–0.009 μg/ml, three wells/concentration) in 96-well microtiter plates. All tests were performed in duplicate.

6.3.3. In vitro antileishmanial activity

The *in vitro* test was performed as described by Hoet et al. [28]. Amphotericin B (a commercial drug) was used as positive control in all experiments with an initial concentration of 1 μg/ml. First stock solutions of compounds were prepared in DMSO or ethanol at 4 mg/ml. The highest concentration of solvent to which the parasites were exposed was 0.5%, which was shown to have no measurable effect on parasite viability. Compounds were tested in eight serial three-fold dilutions (final concentration range: 20–0.009 μg/ml, three wells/concentration) in 96-well microtiter plates. All tests were performed in duplicate.

6.3.4. In vitro antiplasmodial activity

Parasite viability was measured using parasite lactate dehydrogenase (pLDH) activity according to the methods described by Makler et al. [29]. The *in vitro* test was performed as described by Murebwayire et al. [30]. Artemisinin (Sigma) was used as positive control in all experiments with an initial concentration of 100 ng/ml. First stock solutions of compounds were prepared in DMSO or ethanol at 4 mg/ml. The highest concentration of solvent to which the parasites were exposed was 0.5%, which was shown to have no measurable effect on parasite viability. Compounds were tested in eight serial three-fold dilutions (final concentration range: 20–0.009 μg/ml, two wells/concentration) in 96-well microtiter plates. The parasitaemia and the haematocrit were 2% and 1%, respectively. All tests were performed in triplicate.

6.3.5. Cytotoxicity assay

The cytotoxicity of compounds on WI38 cells was evaluated as described by Stevigny et al. [31], using the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma)) colorimetric method based on the cleavage of the reagent by mitochondrial dehydrogenases in viable cells [32]. Camptothecin was used as positive cytotoxic reference compound. Compounds were prepared in DMSO or ethanol at 4 mg/ml. The highest concentration of solvent to which the cells were exposed was 0.5%, which was shown to be non-toxic. The solutions were tested in eight serial three-fold dilutions in 96-well microtiter plates with a final concentration range of 20–0.009 μg/ml (two wells/concentration). All tests were performed in triplicate.

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Appendix A. Supplementary material

Supplementary material related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.06.004.

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