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Isolation and antimalarial activity of new morphinan alkaloids on *Plasmodium yoelii* liver stage

Maëlle Carraz,[a,c,*] Akino Jossang,[a] Philippe Rasoanaivo,[b] Dominique Mazier[c] and François Frappier[a]

[a]*MNHN, USM 0502, CNRS, UMR 5154, Laboratoire de Chimie et Biochimie des Substances Naturelles, Muséum National d'Histoire Naturelle, 75005 Paris, France*
[b]*Laboratoire de Pharmacognosie Appliquée aux Maladies Infectieuses, Institut Malgache de Recherches Appliquées, 101 Antananarivo, Madagascar*
[c]*Inserm, UMR S 511, Immuno-Biologie Cellulaire et Moléculaire des Infections Parasitaires, Centre Hospitalier Universitaire Pitié-Salpêtrière, Université Paris 6, 75013 Paris, France*

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This paper is dedicated to Dr. François Frappier who tragically died while the work was in progress.

Abstract—Decoction of *Strychnopsis thouarsii* is used in the Malagasy traditional medicine to combat malaria. We have shown that this traditional remedy prevents malaria infection by targeting *Plasmodium* at its early liver stage. Bioassay-guided fractionation of *S. thouarsii* stem barks extracts, using a rodent *Plasmodium yoelii* liver stage parasites inhibition assay, led to isolate the new morphinan alkaloid tazopsine (**1**) together with sinococuline (**2**) and two other new related morphinan analogs, 10-*epi*-tazopsine (**3**) and 10-*epi*-tazoside (**4**). Structures were characterized by 2D NMR, MS, and CD spectral analysis. Compounds **1**–**3** were found to fully inhibit the rodent *P. yoelii* liver stage parasites in vitro.

1. Introduction

Malaria still remains the most prevalent parasitic infection with 300–500 million people exposed worldwide and an annual death rate of approximately 2 million people.[1] The infection is initiated when *Plasmodium* sporozoites are injected into a host during the bite of an infected mosquito. In the host, sporozoites first infect the liver where they generate the parasite stage that further invades red blood cells. Most antimalarial drugs aim at inhibiting the blood development of the parasite, since it is responsible for the symptoms of the disease. However, the rising resistance of *Plasmodium* species to a large number of blood schizonticides makes it urgent to develop new drugs to treat malaria.[2]

In the course of new antimalarial leads identification, an ethno-pharmacological survey of plants used traditionally to combat malaria in the Eastern rainforests and the Southern endemic regions of Madagascar had been carried out.[3] One of the selected plants, *Strychnopsis thouarsii* Baillon, an endemic Menispermaceae genus to Madagascar represented by one species,[4,5] was deemed interesting because of its reported efficacy specifically against malaria, when used alone as a decoction or in association with chloroquine.[3,6] Primary chemical investigations on ethanolic extracts of the leaves, roots and stem barks of *S. thouarsii* led to the isolation of fangchinoline and tetrandrine, two *bis*-benzylisoquinolines alkaloids with potent in vitro antimalarial activity against the blood stage of *Plasmodium falciparum*.[7] Moreover, when combinate with chloroquine, they were able to enhance its efficiency on *P. falciparum* resistant strains.[8,9]

As *S. thouarsii* stem barks are prepared traditionally in decoction, simply by boiling the plant in water, we also assessed this preparation against the blood stage of *P. falciparum*. In contrast to the EtOH extract (IC_{50} 2.2 ± 0.3 μg/mL) and the MeOH extract (IC_{50} 5.3 ± 0.9 μg/mL), the decoction showed a weak activity

Keywords: *Strychnopsis thouarsii*; Morphinan; Tazopsine; *Plasmodium yoelii* liver stage.
* Corresponding author. Tel.: +331 39 65 0372; fax: +492 31 97 42 6479; e-mail: maelle_carraz@yahoo.fr

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against the blood development of the Fcb1 strain of *P. falciparum* (IC_{50} 34.0 ± 9.4 μg/mL). Since the liver stage of *Plasmodium yoelii*, a parasite of rodent extensively used as an experimental model for studying the initial phase of the malaria infection[10] was available in our laboratory, we extended the study of the organic and aqueous extracts of *S. thouarsii* stem barks to the liver stage of *P. yoelii*. The extracts were co-incubated with *P. yoelii* infectious sporozoites in cultures of primary mouse hepatocytes and their inhibitory effect on the number of parasites was quantified 48 h after the infection. We observed that the decoction and the methanolic extract significantly decreased the number of the parasites developing in the hepatocytes showing IC_{50} values of 8.5 ± 0.7 μg/mL and 12.5 ± 3.5 μg/mL, respectively, while the ethanolic extract exhibited a weak activity (IC_{50} 79.6 ± 4.6 μg/mL). Furthermore, fangchinoline and tetrandrine bisbenzylisoquinolines, the main components of the ethanolic extract, did not show any activity at this stage. These results supported the fact that the decoction and the methanolic extracts prepared from *S. thouarsii* stem barks should contain compounds active against the liver stage of *P. yoelii* and different from fangchinoline and tetrandrine previously isolated. As a very few number of drugs are known to inhibit the liver stage of *Plasmodium*, we decided to isolate from *S. thouarsii* stem barks the natural-occurring compounds active on this stage.

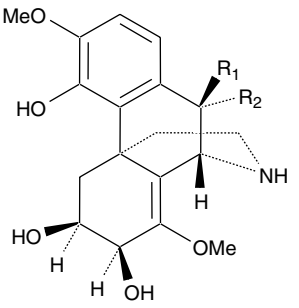
We describe in this report the bioassay-guided fractionation of the MeOH and aqueous plant extracts on *P. yoelii* liver stage leading to the isolation of three active compounds **1**–**3** and their chemical structures solved by Nuclear Magnetic Resonance, Mass Spectroscopy, and Circular Dichroism spectral analysis.

2. Results and discussion

The bioassay-guided fractionation of the crude methanolic extract of *S. thouarsii* stem barks consisted first to partition this extract between water and CH_2Cl_2. The corresponding layers were tested for their inhibition of *P. yoelii* liver stage in cultured mouse hepatocytes. Only the aqueous layer exhibiting the activity was subjected to a reversed phase silica gel RP2 chromatography. From the most active fraction eluted with H_2O–MeOH (60:40) and further separated on a silica gel column, three of the nine fractions obtained (F6–F8), completely inhibited *P. yoelii* sporozoites development at a concentration of 10 μg/mL. Fraction F7 clearing cultures from any parasite with a concentration of 5 μg/mL was the most active. This fraction was submitted to successive bioassay-guided chromatographies until the isolation of the pure active compounds **1** and **2** (Fig. 1) with an extraction yield from the starting plant material of 0.016% (w/w) and 0.005% (w/w) for **1** and **2**, respectively.

2.1. Structure and absolute stereochemistry determination of tazopsine (1)

Compound **1** was an optically active solid, $[\alpha]_D^{22}$ −46 (*c* 0.5, MeOH), whose molecular formula $C_{18}H_{23}NO_6$,



tazopsine (**1**)	R_1 = OH, R_2 = H
sinococuline (**2**)	R_1 = R_2 = H
10-*epi*-tazopsine (**3**)	R_1 = H, R_2 = OH
10-*epi*-tazoside (**4**)	R_1 = H, R_2 = O-β-Glc

Figure 1. Chemical structures of the isolated morphinan alkaloids.

was established from HRCIMS protonated ion $[M+H]^+$ at *m/z* 350.1608 (calcd 350.1604) which indicated eight degrees of unsaturation. The UV spectrum of **1** in MeOH showed three absorption maxima at $\lambda_{max}(\varepsilon)$ 207 (57483), 242sh (9926), 282 (3318). ^1H and ^{13}C NMR spectra of **1** (Table 1) showed signals due to two *ortho*-coupled aromatic protons (H-1 and H-2, J = 8.4 Hz), two methoxyl groups (H_3-3 and H_3-8), four methines (H-6, H-7, H-9, H-10) and three methylenes (H_2-5, H_2-15 and H_2-16). The HSQC and COSY 2D spectra analysis led to design four spin systems **a** (H-1 to H-2), **b** (H-9 to H-10), **c** (H_2-5 to H-6 and H-7), and **d** (H_2-15 to H_2-16). The HMBC spectrum analysis allowed to connect these four spin systems via six sp^2, one sp^3 quaternary carbons, and one amine (Fig. 2). Indeed, the HMBC cross-peaks between protons of system **a** and four sp^2 quaternary aromatic carbons formed a tetrasubstituted benzene ring *A*. The connection between **a** and **b** through C-11 was deduced from ^3J correlations of H-10 to C-1 and C-12. The connections between **b** and **c** through the vinyl group C-14═C-8 were depicted in one hand by HMBC cross-peaks between H-10 and C-14, H-9 and both C-8 and C-13 and in the other hand by those between H_2-5 and C-13, H-7 and C-14, thus forming the ring *C*. The ^{13}C chemical shifts of C-9 (δ 53.1) and C-16 (δ 40.9) indicated their attachment to a nitrogen atom. The ^3J correlations of H-9 to C-16 and H_2-5 to C-15 linked the systems **b** and **c** to the system **d** and formed the ring *D* adjacent to ring *C* via the C-13–C-14 bond. The HMBC correlations of both Hb-5 and Ha-15 to C-12 fixed the ring *A* to the ring *C* through the C-12–C-13 bond and formed the ring *B*. Finally, the HMBC correlations of H_3-3 to C-3 (δ 149.2) and of H_3-8 to C-8 (δ 148.5) allowed to link the two methoxyl groups on positions 3 and 8. With respect to the molecular formula, an hydroxyl group was located on carbon C-4 (δ 145.1) and three others on carbons 6, 7, and 10 (δ 66.9 to δ 73.3), in agreement with their chemical shifts. Thus, the structure of **1** defined a new morphinan skeleton, corresponding to the 4,6,7,10-tetrahydroxy-8,14-didehydro-3,8-dimethoxymorphinan, that we named tazopsine.

Table 1. NMR data for compounds **1**, **3**, and **4** (^{13}C: 75.47 MHz, ^{1}H: 400.13 MHz, 298 K, CD_3OD)

Position		1		3		4	
		δC	δH, m (*J* in Hz)	δC	δH, m (*J* in Hz)	δC	δH, m (*J* in Hz)
1		121.9	6.86, d (8.4)	118.4	7.01, d (8.5)	120.2	7.19, d (8.5)
2		110.7	6.90, d (8.4)	110.7	6.90, d (8.5)	110.5	6.84, d (8.5)
3		149.2	—	148.5	—	148.9	—
4		145.1	—	144.8	—	144.3	—
5	a	36.5	2.95, dd (3.3, 13.4)	36.6	2.93, dd (3.0, 13.7)	36.5	2.93, dd (3.1, 13.7)
	b		2.19, dd (13.4, 13.4)		2.17, dd (13.7, 13.7)		2.17, dd (13.7, 13.7)
6		68.6	3.86, ddd (2.7, 3.3, 13.4)	68.4	3.86, ddd (2.6, 3.0, 13.7)	68.3	3.84, ddd (3.1, 3.3, 13.7)
7		66.9	4.28, d (2.7)	66.1	4.32, d (2.6)	65.8	4.35, d (3.3)
8		148.5	—	148.6	—	149.5	—
9		53.1	4.33, d (2.2)	51.9	4.37, d (5.6)	48.4	4.67, d (5.7)
10		73.3	4.53, d (2.2)	69.7	4.73, d (5.6)	76.9	4.95, d (5.7)
11		132.9	—	134.5	—	131.2	—
12		129.8	—	129.6	—	129.6	—
13		40.1	—	40.0	—	39.6	—
14		121.1	—	120.2	—	118.3	—
15	a	37.5	1.84, ddd (4.7, 12.5, 12.5)	35.9	1.95, ddd (5.0, 12.3, 12.3)	35.7	1.97, ddd (4.6, 12.5, 12.5)
	b		1.99, dd (3.6, 12.5)		2.04, dd (3.8, 12.3)		2.03, dd (3.0, 12.5)
16	a	40.9	2.64, dd (4.7, 13.1)	40.8	2.72, dd (5.0, 12.3)	40.8	2.82, dd (4.6, 12.3)
	b		2.42, ddd (3.6, 12.5, 13.1)		2.66, ddd (3.8, 12.3, 12.3)		2.69, ddd (3.0, 12.3, 12.5)
3-OCH_3		56.6	3.86, s	56.8	3.85, s	56.6	3.85, s
8-OCH_3		57.1	3.69, s	56.6	3.71, s	56.6	3.73, s
Glc-1′						102.8	4.61, d (7.8)
2′					75.2		3.31, dd (7.8, 8.6)
3′			—		—	78.2	3.46, dd (7.8, 8.6)
4′						71.6	3.38, dd (7.8, 9.3)
5′						78.3	3.38, ddd (4.9, 7.1, 9.3)
6′	a					62.8	3.92, dd (4.9, 11.8)
	b						3.76, dd (7.1, 11.8)

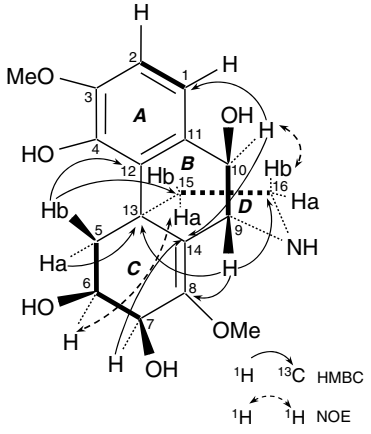


Figure 2. Selected HMBC and NOE correlations of tazopsine (**1**).

The coupling constants between protons H-6 and H-7 ($J = 2.7$ Hz) and between protons H-10 and H-9 ($J = 2.2$ Hz) indicated an axial-equatorial conformation of these protons on the *C* and *B* rings, respectively.[11,12] The NOESY correlations observed between axial H-6 and Ha-15 and those between axial H-10 and Hb-16 (Fig. 2) defined the relative configuration of **1** as 6*S**,7*S**, 9*R**, 10*R**, and 13*S** and the chair conformation for the piperidine *C* ring.

The absolute configuration of **1** was determined by the circular dichroic (CD) exciton chirality method.[13] Indeed, the CD spectrum of **1** (Fig. 3) showed a positive Cotton effect at longer wavelength, $[\theta]^{18}$ (nm): +35117 (243) (*c* 0.2 mM) which indicated that directions of the two interacting chromophores formed by the benzene ring *A* (C-2–C-13 axis) and the vinyl bond (C-8═C-14)

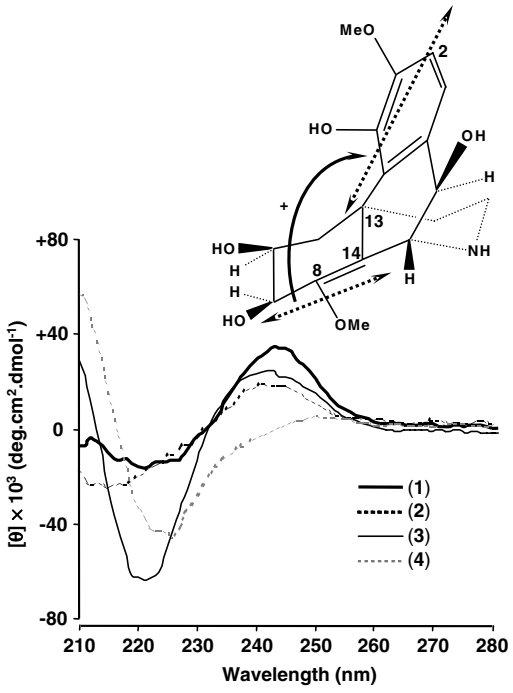


Figure 3. CD spectra of tazopsine (**1**), sinococuline (**2**), 10-*epi*-tazopsine (**3**), and 10-*epi*-tazoside (**4**). Arrows indicate the electric transition dipole of the two chromophores of tazopsine (**1**).

formed a right-handed screw (Fig. 3). This positive chirality required a $C_{13}-S$ stereochemistry. This conclusion was supported by the CD curve of **1** which was similar to that of known morphinan sinococuline (**2**), $[\theta]^{18}$ (nm): +19,448 (241) (*c* 0.2 mM) (Fig. 3), also isolated from *S. thouarsii* stem barks and whose chirality had been previously determined by both circular dichroism exiton chirality method[14,15] and asymmetric synthesis[12] Thus, we established the absolute configuration of **1** as 6*S*, 7*S*, 9*R*, 10*R*, 13*S*.

Comparative thin layer chromatographies between *S. thouarsii* stem barks decoction as they are prepared traditionally and tazopsine (**1**) previously purified showed that the decoction should contain a large amount of **1**. In order to enhance the tazopsine extraction yield, we decided to purify it straight from the decoction. The decoction was subjected to a resin amberlite XAD-4 column chromatography and the second fraction eluted with H_2O–MeOH (50:50) and the most active, was further purified on silica gel to give tazopsine (**1**), with a yield of 0.56% (w/w) from the start plant material as well as two more polar and minor compounds **3** and **4**.

2.2. Structure elucidation of 10-*epi*-tazopsine (3)

Compound **3** was isolated as an optically active amorphous solid, $[\alpha]_D^{22}$ −92 (*c* 0.5, MeOH). The molecular formula $C_{18}H_{23}NO_6$ was deduced from HRCIMS protonated ion $[M+H]^+$ at *m*/*z* 350.1606 suggesting it was an isomer of **1**. ^1H and ^{13}C and 2D NMR spectra were similar to those of **1** except slight deshielding of protons H-1 (*δ* 7.01), H-10 (*δ* 4.73), and Hb-16 (*δ* 2.66) and a higher coupling constant value between H-9 and H-10 (*J* = 5.6 Hz) than **1** (*J* = 2.2 Hz). Moreover, on the contrary to **1**, H-10 and Hb-16 were not correlated on the NOESY spectrum. All these data indicated an epimerization at C-10, conducting to an equatorial–equatorial conformation of H-9 and H-10 on the *C* ring. The super imposable CD spectrum of **3**, $[\theta]^{18}$ (nm): +24930 (242) (*c* 0.2 mM) (Fig. 3), with that of **1** established a C_{13}-*S* stereochemistry. Compound **3** with the absolute configuration 6*S*, 7*S*, 9*R*, 10*S*, 13*S*was named 10-*epi*-tazopsine.

2.3. Structure elucidation of 10-*epi*-tazoside (4)

The molecular formula of the optically active amorphous solid **4**, $[\alpha]_D^{22}$ −7 (*c* 0.1, MeOH), was determined as $C_{24}H_{33}NO_{11}$ by HRCIMS protonated ion $[M+H]^+$ at *m*/*z* 512.2128 (calcd 512.2132), corresponding to nine degrees of unsaturation. The ^{13}C NMR spectrum of **4** showed the signals of the eighteen carbons of 10-*epi*-tazopsine (**3**) and six supplemental signals due to four CH (*δ* 71.6 to *δ* 78.3), one CH_2 (*δ* 62.8), and one CH (*δ* 102.8). These carbons chemical shifts together with 2D NMR data and large vicinal couplings between ring protons due to *trans*-diaxial orientations suggested a glucose unit[16] The large coupling (*J* = 7.8 Hz) of the anomeric proton H-1′ at *δ* 4.61 indicated a β-anomeric configuration for the glucose. HMBC cross-peaks observed between H-1′ and C-10 as well as the low-field shift of C-10 (*δ* 76.9) indicated that the glucose unit was linked to the carbon C-10 of the aglycone. Similar ^1H and ^{13}C NMR data between the aglycone moiety of **4** and **3**, the same coupling constant (*J* = 5.7 Hz) between H-9 and H-10, and the lack of NOESY correlation between H-10 and Hb-16 strongly suggested that the aglycone moiety corresponded to 10-*epi*-tazopsine (**3**). This was confirmed by the acid hydrolysis of **4** with 1 N HCl which led to the 10-*epi*-tazopsine aglycon and glucose. The CD spectrum of **4**, $[\theta]^{18}$ (nm): +5879 (252) (*c* 0.2 mM) (Fig. 3), was similar to the previously described morphinans. On the basis of the above data, the structure of **4** was determined as 10-*O*-β-glucopyranosyl-10-*epi*-tazopsine that was named 10-*epi*-tazoside. To the best of our knowledge, this compound is the first example of a naturally occurring morphinan glycoside.

2.4. Antimalarial activity evaluation on the liver stage of *Plasmodium yoelii*

Tazopsine (**1**), sinococuline (**2**), 10-*epi*-tazopsine (**3**), and 10-*epi*-tazoside (**4**) had the same morphinan skeleton but differed by the nature or the spatial position of the substituent (–H, –OH or –Glc) at C-10. This substituent seemed to play an important role for the antimalarial activity targeted as we observed substantial differences within the morphinan analogs when assessed on the *P. yoelii* liver stage in vitro (Table 2). As activities might had been correlated to their effect on the host cells, we also assessed the cell-toxicity of these compounds on primary mouse hepatocytes.[17]

Except for 10-*epi*-tazoside (**4**) which did not show any antimalarial activity ($IC_{50} > 390$ μM), compounds **1**–**3** exhibited a significant and selective inhibitory activity (*SI* > 13.8) against *P. yoelii* liver stage in vitro. Tazopsine (**1**) showed the most potent inhibitory activity[18] with an IC_{50} value of 3.1 μM. Sinococuline (**2**) was both slightly less active (IC_{50} 4.5 μM) and less toxic on the primary mouse hepatocytes (TC_{50} 62.2 μM) while 10-*epi*-tazopsine (**3**) was 5-fold less active and less toxic on host cells than tazopsine (**1**), giving the better selectivity index *SI* of 24.1. These results allowed to conclude that on one hand, substitution at C-10 position was not necessary for the antimalarial activity but on the other hand, the presence of a free hydroxyl group with a *R* stereochemistry enhanced the inhibitory effect against *P. yoelii* liver stage and with a *S* stereochemistry decreased significantly the toxicity on host mouse primary hepatocytes. The linkage of this hydroxyl group with a

Table 2. In vitro activities on *P. yoelii* 265 BY liver stage and cytotoxicities on host primary mouse hepatocytes

Compounds	IC_{50} *P. yoelii* (μM)	TC_{50} mouse hepatocytes (μM)	Selectivity index[a] (*SI*)
Primaquine	0.64 ± 0.1	70.5 ± 4.3	110
Tazopsine (**1**)	3.1 ± 0.1	43.7 ± 1.6	14.0
Sinococuline (**2**)	4.5 ± 0.4	62.2 ± 5.3	13.8
10-*epi*-Tazopsine (**3**)	16.1 ± 1.9	388.3 ± 6.9	24.1
10-*epi*-Tazoside (**4**)	>390	>390	—

[a] Selectivity index is calculated as the ratio TC_{50}/IC_{50}.

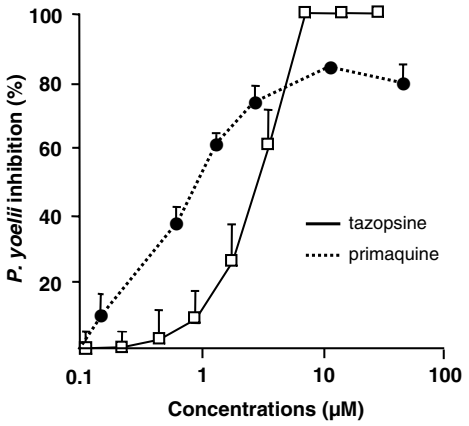


Figure 4. In vitro dose-dependent inhibitions of tazopsine (■) and primaquine (○) on the number of *P. yoelii* liver stage parasites, 48 h after the infection.

glucose unit via a β-glucosidic bond led to a loss of the activity. This result could be explained by the steric bulk created by the glucose unit, reducing the accessibility to the molecule and confirming the importance of this region for the antimalarial activity.

Although a similar effect on the viability of the primary mouse hepatocytes was observed with the reference drug in this model primaquine[19] (TC_{50} 70.5 μM) and tazopsine (**1**) (TC_{50} 43.7 μM), primaquine (IC_{50} 0.64 μM) was 5-fold more active than **1** (Table 2). Dose-dependent decrease of the number of *P. yoelii* parasites in mouse hepatocytes was observed for both compounds (Fig. 4); however the IC_{90}/IC_{50} ratio of 2 for **1** suggested a specific target inhibition as compared to the IC_{90}/IC_{50} ratio >50 for primaquine for which the molecular target is still unknown. Moreover, a total inhibition was observed for tazopsine (**1**) at a concentration as low as 7.1 μM, whereas *P. yoelii* parasites could still be observed in cultures treated with high concentrations of primaquine (38.6 μM of primaquine only inhibited 80% of the number of parasites). We concluded that although the selectivity index calculated on the basis of TC_{50}/IC_{50} ratio was higher for the reference drug primaquine, tazopsine, sinococuline and, 10-*epi*-tazopsine are worth of further detailed studies since they completely and specifically inhibit *P. yoelii* liver stage in vitro.

3. Conclusion

This work is the first report of a plant fractionation guided by a *Plasmodium* liver stage inhibition assay. Though plant extracts and natural-derived compounds are usually assessed on the blood stage of the parasite, we report here the proof of principle that plants can contain compounds effective against the liver stage, the first and obligatory step of the malaria parasites infection. Very few drugs are known to inhibit the malaria parasites at this stage and the identification of tazopsine and related analogs presents a great interest in the context of search for new antimalarial drugs. Moreover, tazopsine was found in large amount in the stem barks decoctions of *S. thouarsii* following the Malagasy folk medicine traditional preparation. These results might explain the continued ethno-pharmacological use of *S. thouarsii* decoctions against malaria.

Tazopsine, 10-*epi*-tazopsine, and sinococuline are the first antimalarials reported to belong to the morphinan class. These structures differ from those of all commercially available antimalarials for which most *P. falciparum* strains have now developed resistances.[20] Moreover, because there are no 8-aminoquinoline-based drugs other than primaquine that are used specifically against the *Plasmodium* liver stage, compounds **1**–**3** can undoubtely constitute an useful chemotherapeutic tool for the causal prophylaxy of malaria.[21] Moreover, as they do not belong to the 8-aminoquinoline class, these morphinan alkaloids are not expected to enhance the risk of haemolysis in G6PD-deficient people.[22]

In the course of the pharmacophore understanding of tazopsine, semi-synthetized analogs will be generated and it is hope that tazopsine chemical derivatizations would lead to more final molecules of the same class that have better selectivity index.

4. Experimental

4.1. General experimental procedures

Optical rotations were measured in MeOH at 22 °C on a Perkin-Elmer model 341 polarimeter. ^{13}C NMR spectra were recorded in CD_3OD on a Bruker AC 300 spectrometer operating at 75.47 MHz. ^{1}H 1D and 2D NMR spectra were recorded in CD_3OD on a Bruker Advance 400 spectrometer operating at 400.13 MHz. Mass spectra were recorded on an API Q-STAR PULSAR *i* of Applied Biosystem. CD spectra were recorded in MeOH at 18 °C on a Jasco J-710 spectropolarimeter with a compounds concentration of 0.2 mM; molar ellipticity [θ] calculations were performed with MS Excel 2000.

4.2. Plant material

Stem barks of *S. thouarsii* Baillon were collected at Andasibe (Eastern rain forest of Madagascar). Botanical identification was performed by A. Rakotozafy and confirmed by comparison with voucher specimens deposited at the Department of Botany, Parc Botanique et Zoologique de Tsimbazaza, Antananarivo.

4.3. Crude methanolic extract fractionation

Dried and powdered *S. thouarsii* stem barks (500 g) were macerated in MeOH. The crude methanolic extract (22 g) dissolved in 2 L of H_2O was partitioned three times with 500 mL of CH_2Cl_2. The resulting aqueous extract (11.7 g) was subjected to a reversed phase silica gel column (RP-2) eluted with a H_2O–MeOH gradient. Fraction F2 (9.4 g) eluted with H_2O–MeOH (60:40) was then separated on a silica gel column eluted with

CH_2Cl_2–MeOH–NH_4OH (85:15:0.5). Fraction F7 (1.31 g) was further purified on a Sephadex LH-20 gel column eluted with MeOH. Fraction F4 (368 mg) was purified on a silica gel column eluted with CH_2Cl_2–MeOH–NH_4OH. Fraction F6 (122 mg) eluted with (87:13:1) contained a mixture of tazopsine (**1**) and sinococuline (**2**) that were separated by preparative TLC eluted with $CHCl_3$–Et_2N (70:30), providing 80 mg of **1** (R_f 0.24) and 27 mg of **2** (R_f 0.61).

4.4. Sinococuline (2)

Amorphous powder, $[\alpha]_D^{22}$ −143 (*c* 0.25, MeOH), ^1H NMR and ^{13}C NMR data were similar to those of the literature.[12,14] HRCIMS *m*/*z* 334.1652 $[M+H]^+$ (calcd for $C_{18}H_{24}NO_5$, 334.1654).

4.5. Decoction fractionation

Dried *S. thouarsii* stem barks (1.1 kg) were prepared by decoction: 300 g of the plant material was poured in 3 L of boiling water and stirred for 30 min. The aqueous extract was cooled and then mixed with an amberlite XAD-4 resin for 12 h. The resin was washed with 3 L of a gradient of H_2O–MeOH. Fraction F2 (26.1 g) eluted with H_2O–MeOH (50:50) was purified on a silica gel column eluted with CH_2Cl_2–MeOH–NH_4OH which gave in fraction F4 eluted with (85:15:2), 6.2 g of pure tazopsine (**1**) (R_f 0.4). Fraction F5 (1.4 g) also eluted with (85:15:2) was further purified on a silica gel column eluted with CH_2Cl_2–MeOH–NH_4OH (80:20:3) and gave 435 mg of 10-*epi*-tazopsine (**3**) (R_f 0.19). Fraction F7 (128 mg) eluted with (50:50:2) gave after preparative TLC purification in (70:30:5) 8 mg of 10-*epi*-tazoside (**4**) (R_f 0.22).

4.6. Hydrolysis of 10-*epi*-tazoside

A solution of 10-*epi*-tazoside (**4**, 2 mg) in 1 N HCl (1 mL) was stirred at 90 °C for 12 h. After evaporation, the residue was purified by TLC using CH_2Cl_2–MeOH–NH_4OH (80:20:3) providing 10-*epi*-tazopsine (**3**) (1 mg, R_f 0.2). Analytical TLC of the residue and commercial pyranoses (Merck) performed with CH_2Cl_2–MeOH (50:50) and then sprayed with thymol revealed the presence of glucose (R_f 0.54, purple spot).

4.7. In vitro assay for *P. yoelii* liver stage inhibition

Plasmodium yoelii yoelii (265 BY strain) sporozoites were obtained by the dissection of infected *Anopheles stephensi* salivary glands. Primary mouse hepatocytes were isolated from mice liver biopsies as previously described[23] and seeded in 8-well Lab-Tek plastic chamber slides (VWR, Fontenay-sous-Bois, France) previously coated with rat tail collagen I (BD Biosciences, Le Pont de Claix, France) at a density of 10^5 cells per well. Mouse hepatocytes were cultured at 37 °C in 5% CO_2 in William's E medium supplemented with 10% fetal calf serum, 1% L-glutamine, 1% sodium pyruvate, 1% insulin–transferrin–selenium, 1% non-essential amino-acids, 1% penicillin–streptomycin (all from Invitrogen, Cergy-Pontoise, France), for 24 h before inoculation of *P. yoelii* sporozoites (10^5 per well). The plant extracts or the purified compounds were solubilised in DMSO, further diluted in culture medium (equal DMSO concentrations of <0.3% per well), and then added to hepatocyte cultures at the time of sporozoite inoculation. Culture medium containing the appropriate drug concentration was changed daily until 48 h. Parasites were quantified on the last day of incubation by immunofluorescence analysis following fixation of the cultures with cold methanol and parasite-specific staining by a mouse polyclonal serum raised against the *P. falciparum* heat shock protein 70, that also cross-reacts with the hsp70 of *P. yoelii*, and revealed with FITC-conjugated goat anti-mouse immunoglobulin (Sigma). Parasite numbers were counted under a fluorescence microscope with a 25× light microscope objective. IC_{50} values, the drug concentration at which a 50% reduction in the number of parasites was observed, as compared to the number in the DMSO control cultures, were calculated by linear regression using Excel software and derived from three independent experiments, where each concentration was tested in triplicates.

4.8. In vitro assay for cells viability

Primary hepatocytes of mice were prepared as above and seeded in 96-well plates coated with collagen, at a density of 5×10^4. Drugs were added to the culture medium which was changed daily, after 24 h of incubation. After 48 h of drug exposure, 50 µl of neutral red (Sigma) at 0,02% in phosphate-buffered saline (PBS) were added per well and incubated for 24 additional hours. Cultures were then washed with PBS and the neutral red extracted with 1% SDS from viable cells and quantified by measuring the OD_{540} on a microplate autoreader photometer (Bio-Tek instruments). TC_{50} values, the drug concentration at which a 50% reduction of neutral red incorporation was observed, as compared to that incorporated in the DMSO control cultures, were calculated by linear regression using Excel software and derived from three independent experiments, where each concentration was tested in quadruplicates.

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