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Isolation and Structure Elucidation by LC-DAD-MS and LC-DAD-SPE-NMR of Cyclopeptide Alkaloids from the Roots of *Ziziphus oxyphylla* and Evaluation of their Antiplasmodial Activity

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ABSTRACT

Nine cyclopeptide alkaloids (**1**-**9**), of which five (compounds **2**, **3**, **5**, **8** and **9**,) are described herein for the first time, were isolated from roots of *Ziziphus oxyphylla* by means of conventional separation methods as well as semi-preparative HPLC with DAD and ESIMS detection and LC-DAD-SPE-NMR. Structure elucidation was done by spectroscopic means. Nummularine-R (**1**), a previously known constituent from this species, was isolated along with its new derivatives *O*-desmethylnummularine-R (**2**) and *O*-desmethylnummularine-R *N*-oxide (**3**). In addition, the known compounds hemsine-A (**4**) and ramosine-A (**6**), as well as hemsine-A *N*-oxide (**5**) were isolated. Moreover, oxyphylline-C (**7**), a known constituent of *Z. oxyphylla* stems, was obtained, and two new compounds were identified, oxyphyllines-E (**8**) and -F (**9**). Just like oxyphylline-C, oxyphyllines-E and –F belong to the relatively rare class of neutral cyclopeptide alkaloids. The antiplasmodial activity and cytotoxicity of compounds **1**, **2**, **4**, **6** and **9** were evaluated and the most promising activity was found for *O*-desmethylnummularine-R (**2**), which exhibited an IC_{50} value of 3.2 ± 2.6 µM against *Plasmodium falciparum* K1, whereas an IC_{50} value of > 64.0 µM was evident for its cytotoxicity against MRC-5 cells.

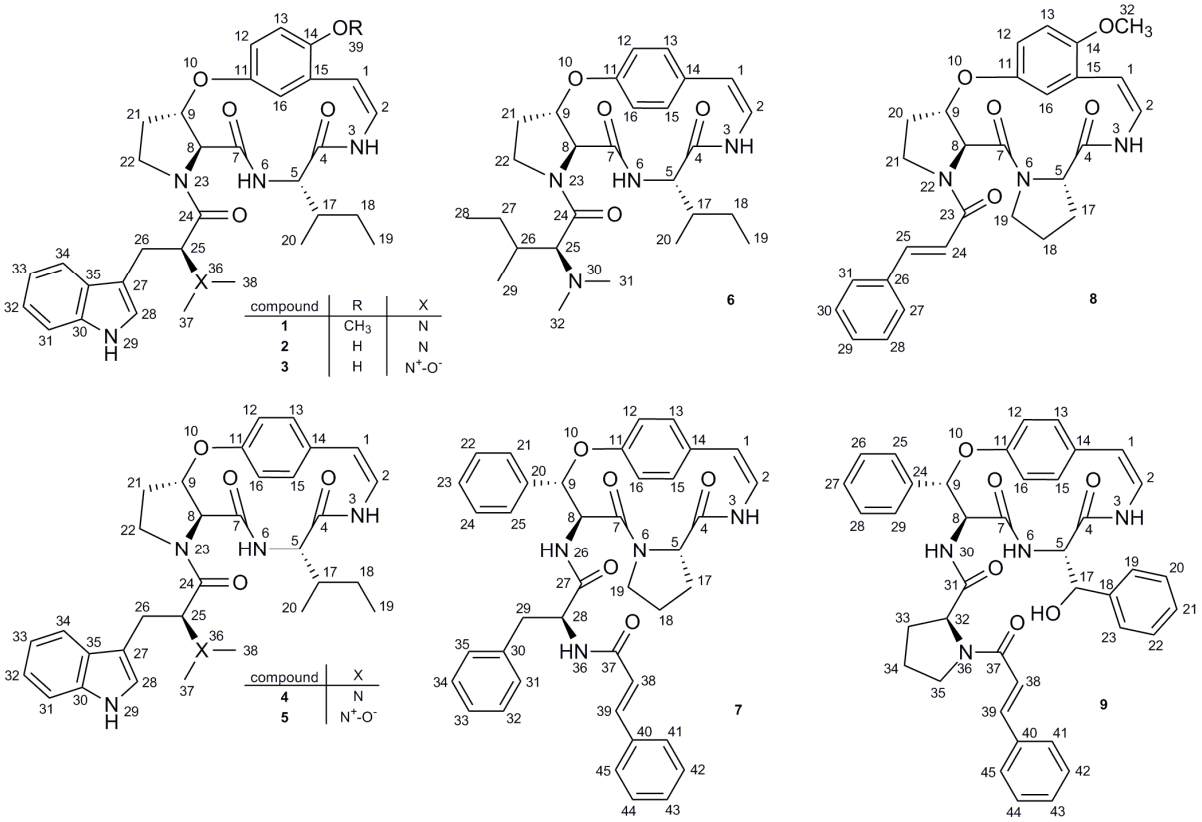
Cyclopeptide alkaloids are polyamide, basic compounds that are distributed widely in various plant families, including the Asteraceae, Celastraceae, Euphorbiaceae, Menispermaceae, Pandaceae, Rubiaceae, Sterculiaceae and Urticaceae, but most prominently in the Rhamnaceae, and especially the genus *Ziziphus*. Typically, their structures contain a 13-, 14- or 15-membered macrocycle, formed by a stryrylamine unit, a common amino acid, and a β-hydroxy-amino acid. Moreover, a side-chain consisting of one (i.e., a total of four building blocks) or two (a total of five building blocks) additional amino acids is attached to this ring. In this manner, the 4(13)-, 4(14)-, 5(13)- and 5(14)-cyclopeptide alkaloid subclasses are distinguished.[1,2]

Ziziphus oxyphylla Edgew. (Rhamnaceae) is a small- to medium-sized tree that grows in the northern regions of Pakistan. The fruits are used as a common food and in traditional medicine different parts of the plant are applied in a wide range of pathological conditions, as, for example, to treat inflammation, microbial infections, fever or pain, allergy, and diabetes. Previous investigations have already shown in vivo antipyretic effect of a methanolic extract of *Z. oxyphylla* leaves and the antinociceptive activity of methanolic extracts of the leaves and roots of this plant,[3,4] but the active compounds are not known. Phytochemical tests revealed the presence of alkaloids, anthraquinones, flavonoids, glycosides, phenols, saponins and tannins[5] and so far seven cyclopeptide alkaloids were isolated and identified from roots and stem (bark) of this species, i.e. hemsine-A, nummularines-C and –R, and oxyphyllines-A, -B, -C and -D.[5-7] Cyclopeptide alkaloids isolated from various sources have been reported to exhibit antibacterial, antifungal, antiplasmodial and sedative activities; antiplasmodial activity more in particular for ziziphines-N and -Q and hemsine-A, mauritine-M and nummularine-H and hymenocardine, hymenocardinol and hymenocardine *N*-oxide.[8-15] Therefore, in the present work, the roots of *Z. oxyphylla* were

selected for the targeted isolation of cyclopeptide alkaloids, to evaluate their antiplasmodial activity, and to establish structure-activity relationships.

RESULTS AND DISCUSSION

Cyclopeptide alkaloids were isolated and identified from two different batches of roots of *Z. oxyphylla*, collected at different locations in Pakistan in different years. Whereas for the first batch a general liquid-liquid partition and fractionation scheme was followed, a more alkaloid-specific fractionation procedure was used for the second batch in order to increase the number of alkaloids detected. The isolation of single compounds was performed by semi-preparative HPLC with DAD and ESIMS detection together with LC-DAD-SPE-NMR and altogether nine different cyclopeptide alkaloids were identified. Their structures were elucidated by a 1D (^{1}H, ^{13}C, DEPT 135, DEPT 90) and 2D NMR experiments (COSY, HSQC, HMBC), mass spectrometry, and by comparison to literature data.



The NMR data of compound **1** were in agreement with previously reported assignments for nummularine-R.[16] ^{1}H and ^{13}C NMR chemical shift assignments for **1** are listed in Tables 1 and 2, respectively. This cyclopeptide alkaloid has been previously isolated from *Z. oxyphylla* and was found to be present in both batches. Compound **2** showed very similar NMR spectra, but the characteristic signals of C-39 (56.7 ppm) and H-39 (3.77 ppm), indicative for the methoxy group attached to the styrylamine moiety of compound **1**, were absent in the spectra of compound **2**. The ESIMS data showed a base peak at *m/z* 611 $[M+Na]^+$ for compound **1** and at *m/z* 597 $[M+Na]^+$ for compound **2**. The accurate mass of compound **2** was found to be 574.3031 daltons $[M+H]^+$, in accordance with a molecular formula of $C_{32}H_{40}N_5O_5$. Taking into account both the NMR and MS data it was concluded that compound **2** contains a hydroxy group instead of a methoxy group at position C-14. Thus, this compound was assigned as *O*-desmethylnummularine-R. Furthermore,

the *N*-oxide of compound **2**, *O*-desmethylnummularine-R *N*-oxide (**3**), also was obtained in the present investigation. Indeed, from the NMR data it was deduced that two *N*-methyl groups were present, indicated by signals at δ_C 57.1 ppm/δ_H 3.49 ppm and δ_C 53.7 ppm/δ_H 3.39 ppm, whereas in compound **2** both *N*-methyl groups occurred at δ_C 42.6 ppm/δ_H 2.92 ppm. Also, C-25 showed a downfield shift of almost 10 ppm when compared to compound **2** (76.3 ppm vs. 67.2 ppm). These findings are in agreement with previously reported NMR assignments for cyclopeptide alkaloid *N*-oxides.[15,17] In addition, the HRESIMS peak at *m/z* 590.3002 $[M+H]^+$ corresponded to the proposed structure ($C_{32}H_{40}N_5O_6$). Compounds **2** and **3** were only detected in the second batch of plant material investigated.

Comparison of the NMR spectra of compound **4** with previously published assignments showed that this compound is hemsine-A, isolated from *Z. oxyphylla* before.[7] Also hemsine-A *N*-oxide was isolated (compound **5**), as deduced from the NMR data. HRESIMS supported the proposed structures. Both of these compounds could only be isolated by following the more alkaloid-specific fractionation method used for the second batch of plant material. Hemsine-A *N*-oxide (compound **5**) is reported here for the first time and although it is not uncommon that cyclopeptide alkaloids are isolated as *N*-oxides, together with tertiary amines,[13] the possibility that the *N*-oxides are artefacts formed during drying, extraction, or isolation cannot completely be excluded.

Compound **6** was identified as ramosine-A, mainly based on the NMR data obtained. This cyclopeptide alkaloid was previously reported from *Paliurus ramosissimus*,[18] but this is the first time it has been isolated from *Z. oxyphylla* (batch 2). Compound **7** was reported in *Z. oxyphylla* by Kaleem et al.[6] and was isolated from the first batch of plant material. The NMR data were in agreement with previously reported assignments for oxyphylline-C.[6]

Compound **8** was identified as a new cyclopeptide alkaloid based on its 1D and 2D NMR spectra in combination with HREIMS measurements. It belonged to the subclass of cyclopeptide alkaloids containing a 13-membered ring composed of a 2-methoxystyrylamine moiety and two proline amino acids. A typical signal in the ^{1}H NMR spectrum of many cyclopeptide alkaloids is the peak due to the β-proton of the β-hydroxylated ring-bound amino acid (H-9). In the case of compound **8**, this signal appeared as a multiplet at 5.37 ppm. In the COSY spectrum, this proton showed cross peaks with a doublet at 4.56 ppm, assigned to H-8, and to a CH_2 group (2.58 ppm/2.38 ppm, H-21). This CH_2 group was connected to another CH_2 group (4.07 ppm/3.85 ppm, H-22) as evident from COSY interactions. Based on these observations and comparison with previously reported NMR assignments, it was concluded that a β-hydroxyproline moiety is present as one of the ring-bound amino acids. In addition, a second proline amino acid unit could be identified. In the COSY spectrum the signal at 4.69 ppm (dd, J = 9.4 Hz; 3.5 Hz), corresponding to the α-proton of this amino acid (H-6), showed clear cross peaks with a CH_2 group at 2.31 ppm/2.05 ppm (H-17). This CH_2 group, in turn, was found to be connected to a second CH_2 unit (2.05 ppm/1.87 ppm, H-18), which again is linked to a third CH_2 group (4.47 ppm/3.38 ppm, H-19), substituted by an *N*-atom closing the five-membered ring of this second proline moiety. The whole ^{1}H NMR spectrum integrated for 28 protons, 13 of which showed a chemical shift value between 5.5 and 9.0 ppm. Only one of these, the doublet at 8.47 ppm, did not show a cross peak in the HSQC spectrum, and was assigned to the NH-proton of the styrylamine moiety (H-3). The styrylamine double bond with $\delta_{C\text{-}1}$ 106.6 ppm /$\delta_{H\text{-}1}$ 5.99 ppm (d, 8.8 Hz) and $\delta_{C\text{-}2}$ 121.7 ppm /$\delta_{H\text{-}2}$ 6.98 ppm was assigned a *cis* conformation, as deduced from the J value of the H-1 doublet (8.8 Hz). HMBC correlations of H-1 with C-16 and H-16 with C-1, H-2 with C-15, H-13 with C-11 and H-12/H-16 with C-14, indicated that the aromatic ring is substituted at positions C-11 and C-

15, bearing a methoxy group (δ_{C-32} 56.0 ppm/δ_{H-32} 3.83 ppm, s) at position C-14. The key HMBC correlations for the structure elucidation of compound **8** are shown in Figure 1. Closure of the macrocyclic ring through 1,3-substitution of the aromatic moiety is typical for 13-membered ring cyclopeptide alkaloids. The fourth and last building block was identified as a cinnamoyl moiety. The aromatic protons occurred at 7.40 ppm (H-28, H-29, H-30) and 7.54 ppm for H-27 and H-31, with δ_{C-29} 130.1 ppm and C-28 and C-30 both at 128.9 ppm, and C-27 and C-31 both at 128.0 ppm. The double bond (δ_{H-24} 6.76 ppm, d (15.6 Hz)/δ_{C-24} 117.1 ppm and δ_{H-25} 7.69 ppm, d (15.5 Hz)/δ_{C-24} 143.4 ppm) showed a *trans* geometry and its linkage to the carbonyl group (C-23) could be established through HMBC-interactions of H-24 and H-25 with C-23; H-25 was also correlated with C-27/C-31. Accordingly, the structure of compound **8** was assigned as shown, and the trivial name oxyphylline-E is proposed. HRESIMS analysis in the positive ion mode resulted in the detection of *m/z* 488.2220 $[M+H]^+$ and 510.2054 $[M+Na]^+$, in agreement with the molecular formula $C_{28}H_{30}N_3O_5$ (calcd *m/z* 488.2180) and $C_{28}H_{29}N_3O_5Na$ (calcd *m/z* 510.1999), respectively.

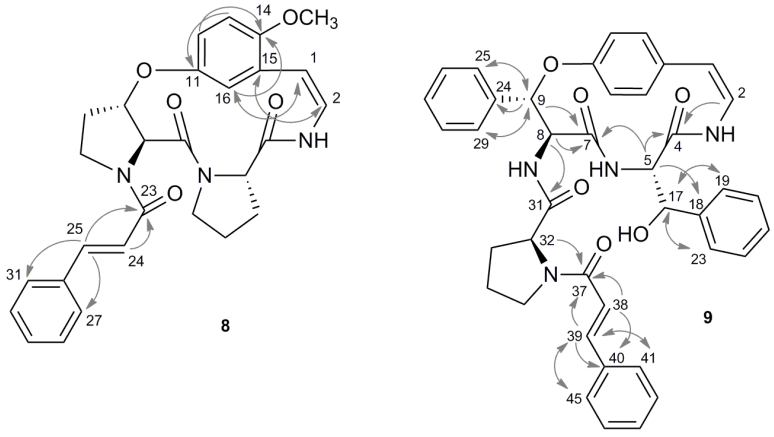


Figure 1. Key HMBC correlations for oxyphylline-E (**8**) and oxyphylline-F (**9**)

From the NMR spectra of compound **9** it was concluded that it contains a 14-membered ring consisting of a styrylamine unit and two β-hydroxy-phenylalanine amino acid moieties. The side-chain consisted of a proline and a cinnamoyl moiety. As for compound **8**, the signal of H-9

appearing at δ_H 5.66 ppm (δ_C 82.5 ppm) could be used as a starting point for the structure elucidation. From COSY interactions, this proton could be linked to H-8 at 4.56 ppm. In contrast to compound **8**, however, no interactions with a CH_2 group were observed. Instead, the HMBC spectrum showed cross peaks with carbon atoms belonging to an aromatic system, $\delta_{C\text{-}24}$ 139.1 ppm, with C-25 and C-29 both resonating at δ_C 129.6 ppm. Further inspection of the COSY and HMBC spectra led to the identification of β-hydroxy-phenylalanine as one of the ring-bound amino acids. Both H-8 and H-9 showed HMBC interactions with a carbon signal at 171.0 ppm, corresponding to the carbonyl group at C-7. A proton at δ_H 4.30 ppm (d, 9.5 Hz) also showed a cross peak in the HMBC spectrum with C-7, and was assigned to the α-proton of the other ring-bound amino acid (H-5). The H-5 proton showed a COSY correlation to a proton at 4.66 ppm (d, J = 9.4 Hz), assigned to H-17. Analysis of the HMBC and COSY spectra then led to the identification of a second phenyl ring. Since the integration of the signal at 4.66 ppm (H-17) accounted for only one proton and given its downfield shift compared to the β-protons in phenylalanine and comparison to literature data,[19,20] it could be concluded that C-17 is hydroxylated. Therefore, this amino acid unit was identified also as β-hydroxy-phenylalanine. Through HMBC correlations of H-5 (at 4.30 ppm) and H-2 (at 6.17 ppm) with the carbonyl group at C-4 (δ_C 171.7 ppm), a link with the stryrylamine moiety could be established. This was supported by the relatively weak long-distance correlation in HMBC of H-1 (δ_H 6.86 ppm) with C-4. As in compound **8**, the double bond was assigned a *cis* conformation given the *J* value of 7 Hz, but, in contrast to compound **8**, the aromatic ring did not contain a methoxy group and showed a *para*-substitution in positions 11 and 14 to close a 14-membered macrocyclic ring.

In addition, H-8 also correlated with a carbonyl group at 170.2 ppm in the HMBC spectrum assigned to C-31. The same carbonyl group also correlated with a proton at 4.10 ppm, assigned to

H-32. The COSY spectrum revealed the connection of H-32 to a CH_2 group (δ_H 2.14 ppm, m/1.55 ppm, m, H-33), which, in turn was connected to a second CH_2 group (δ_H 1.78 ppm, m/1.41 ppm, m, H-34), and finally a third CH_2 group (δ_H 3.00 ppm, t (8.8 Hz)/3.30 ppm, m, H-35), leading to the identification of this amino acid substituent as proline. The H-32 proton correlated with another carbonyl group in the HMBC spectrum (δ_C 168.0, C-37). The latter correlation allowed the position of the terminal cinnamoyl moiety to be established, since it showed two more correlations with two doublets (δ_H 6.65 ppm and 7.60 ppm), coupled to each other with a *J* value of 15.5 Hz, indicative of a *trans* alkene bond. Further inspection of the COSY and HMBC spectra allowed a phenyl ring to be identified, thus completing the cinnamoyl moiety. The key HMBC correlations of compound **9** are shown in Figure 1. HRESIMS analysis in the (+)-ESIMS mode gave *m/z* 671.2885 $[M+H]^+$ and 693.2698 $[M+Na]^+$, which were in agreement with a molecular formula of $C_{40}H_{39}N_4O_6$, (calcd *m/z* 671.3228) and $C_{40}H_{38}N_4O_6Na$ (calcd *m/z* 693.2684), respectively. For compound **9**, the name oxyphylline-F was adopted. Compound **8** was isolated from batch 1, whereas compound **9** was found in both batches of plant material.

Nummularine-R (**1**) and its derivatives **2** and **3** belong to the group of 4(13)-cyclopeptide alkaloids and hemsine-A (**4**), its *N*-oxide (**5**) and ramosine-A (**6**) to the 4(14)-group. Oxyphyllines-C (**7**), -E (**8**) and -F (**9**) are so-called neutral cyclopeptide alkaloids, in which the basic amino acid in the side-chain is missing, and contain a cinnamoyl moiety instead.[2] Neutral cyclopeptide alkaloids are relatively rare. Until now, only fifteen representatives have been reported and all of them were found to contain a 14-membered macrocyclic ring. Oxyphylline-E is thus the first 13-membererd neutral cyclopeptide alkaloid.

Amino acids that occur in Nature are usually present in the L-configuration. Also in cyclopeptide alkaloids, this configuration is found in the vast majority and only rarely the D-

configuration has been reported, for example, in scutianine-E, isolated from *Scutia buxifolia*.[9] The configuration of nummularine-R (**1**) has been described in 2010 by Nisar et al., based on NOESY interactions, and indeed the L-configuration was found for the three amino acid units present in this cyclopeptide alkaloid.[16] The L-configuration was also reported for hemsine-A (**4**) and ramosine-A (**6**).[18] In oxyphylline-C (**8**) the two ring-bound amino acid units were found to be present in the L-configuration as well.[6] Conforming to the already reported configurations of these cyclopeptide alkaloids, the same configuration was adopted here and the L-configuration was also adopted for the closely structurally related compounds, *O*-desmethylnummularine-R (**2**), *O*-desmethylnummularine-R *N*-oxide (**3**) and hemsine-A *N*-oxide (**5**) and for oxyphylline-E (**8**) and oxyphylline-F (**9**). For the β-hydroxy-proline unit in oxyphylline-E (**8**), the same relative configuration was adopted for C-9, as reported for other cyclopeptide alkaloids, based on similar ^{1}H NMR chemical shifts and coupling patterns. The *J* value of the α- and β-proton of the β-hydroxy-phenylalanine unit in compound **7** and the corresponding unit in compound **9** can be used to distinguish the *erythro* form from the *threo* form. In case of the *erythro* form, a *J* value of 8 Hz is typical, while the *threo* form exhibits a smaller *J* value of approximately 2 Hz.[2] For oxyphylline-C, the L-*erythro* form has been reported before ($J_{8\text{-}9}$ 7.2 Hz).[6] Here, both oxyphylline-C (**7**) and the new compound, oxyphylline-F (**9**) were found to contain L-*erythro*-β-hydroxy-phenylalanine, since they showed $J_{8\text{-}9}$ values of 7.2 and 8.4 Hz, respectively. Moreover, $J_{5\text{-}17}$ of 9.5 Hz and $J_{17\text{-}5}$ of 9.4 Hz are indicative for the fact that the other β-hydroxy-phenylalanine unit in compound **9** is also present in the L-*erythro* form.

NMR chemical shift assignments for compounds **1**–**9** were based on 1D and 2D NMR experiments and are listed in Tables 1 and 2, respectively. For compounds **1**-**6** and **9** the NMR spectra were recorded in methanol-d_4. For compounds **7** and **8** chloroform-*d* was used.

Table 1. ^{1}H NMR Spectroscopic Data [δ_H in ppm, Multiplicity (*J* in Hz)] for Compounds 1-9[a]

position	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1	6.03, d (8.9)	6.03, d (8.9)	6.03, d (9.0)	6.70, d (7.6)	6.66, d (7.5)	6.76, d (7.5)	6.43, d (7.5)	5.99, d (8.8)	6.86, d (7.3)
2	6.80[b]	6.80[b]	6.79[b]	6.29, d (7.6)	6.29, d (7.5)	6.16, d (7.5)	6.76, t (8.4)	6.98[b]	6.17, d (7.4)
3 (N-H)	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	6.63,d (9.8)	8.47, d (11.8)	n.o.
5	4.26, d (6.5)	4.27, d (6.5)	4.25, d (6.0)	4.02, d (7.1)	3.95, d (7.0)	3.86, d (8.4)	3.92, d (7.3)	4.69, dd (9.4, 3.5)	4.30, d (9.5)
8	4.42, d (3.2)	4.42[b]	4.45, d (3.0)	4.14, d (6.5)	4.17, d (6.4)	4.17, d (6.8)	4.78, dd (9.7, 7.2)	4.56, d (5.5)	4.56, d (8.4)
9	5.07, m	5.04, m	5.02, m	5.22, m	5.20, m	5.35, m	5.95, d (7.2)	5.37, m	5.66, d (8.4)
12	6.79[b]	6.65[b]	6.62[b]	7.11[b]	7.10[b]	7.13, dd (8.4, 2.4)	7.10[b]	6.87[b]	7.10[b]
13	6.97, d (9.1)	6.79[b]	6.77[b]	7.05[b]	7.03[b]	7.06[b]	7.26[b]	6.93[b]	7.03[b]
15	-	-	-	7.10[b]	7.09[b]	7.08[b]	7.37[b]	-	7.06[b]
16	6.73[b]	6.66[b]	6.65[b]	7.05[b]	7.03[b]	7.02, dd (8.2, 2.4)	7.14[b]	6.88[b]	7.07[b]
17a	1.94, m	1.95, m[b]	1.97, m	1.76, m	1.72, m	1.59[b]	1.46, m	2.05, m[b]	4.66, d (9.4)
17b	-	-	-	-	-	-	2.25, m	2.31, m	-

18a	1.27, m	1.28, m	1.28, m	1.20, m	1.13, m	1.05, m	1.58, m	1.87, m	-
18b	1.57, m	1.56, m	1.51, m	1.54, m	1.49, m	1.51[b]	1.85, m	2.05, m[b]	-
19a	0.93, t (7.4)	0.94, t (7.5)	0.93, t (7.3)	0.87[b]	0.81, t (7.5)	0.78[b]	3.04, t (8.7)	3.38, m	7.25[b]
19b							3.24, m	4.47, m	-
20a	1.06, d (6.9)	1.06, d (6.9)	1.03, d (6.9)	0.88[b]	0.77, d (6.8)	0.81[b]	-	2.38, m	7.17[b]
20b	-	-	-	-	-	-	-	2.58, m	-
21a	2.03, m	2.00[b]	2.04, m	2.04, m	2.04, m	2.22, m	7.37[b]	3.85, m[b]	7.16[b]
21b	2.13, m	2.11, m	2.10, m	2.30, m	2.25, m	2.57, m	-	4.07, t (9.3)	-
22a	2.46, m	2.42, m	2.56, m	2.54, m	2.59, m	3.59, m	7.20[b]	-	7.17[b]
22b	3.67, m	3.65, m	3.88, m	3.73, m	4.00, t (9.5)	4.10[b]	-	-	-
23	-	-	-	-	-	-	7.19-7.28[d]	-	7.25[b]
24	-	-	-	-	-	-	7.20[b]	6.76, d (15.6)	-
25	4.37, m	4.41[b]	4.54, dd (11.8, 2.7)	4.35, dd (9.8, 4.1)	4.50, dd (11.2, 2.7)	4.07[b]	7.37[b]	7.68, d (15.5)	7.29[b]
26a	3.21, dd (13.6, 10.6)	3.21, dd (14.1, 10.6)	3.36[c]	3.19, dd (14.6, 9.6)	3.26[c]	2.08, m	7.55[b]	-	7.16[b]
26b	3.50, dd (14.2, 3.9)	3.52, dd (14.1, 3.7)	3.53[b]	3.44, dd (14.6/4.0)	3.47, dd (13.6, 2.6)		-	-	-

27a	-	-	-	-	-	1.23, m	-	7.54[b]	7.24[b]
27b	-	-	-	-	-	1.57[b]	-	-	-
28	6.92, s	6.93, s	6.86, s	7.01[b]	6.90, s	0.97, t (7.3)	4.60, m	7.40[b]	7.16[b]
29a	N-H, n.o.	NH, n.o.	NH, n.o.	NH, n.o.	NH, n.o.	0.91, d (6.7)	2.79, dd (15.5, 10.6)	7.40[b]	7.29[b]
29b	-	-	-	-	-	-	3.39, d (15.5)	-	-
30	-	-	-	-	-	-	-	7.40[b]	NH, n.o.
31	7.37, d (7.9)	7.37, d (7.8)	7.36, d (7.9)	7.38, d (7.9)	7.33, d (8.0)	2.77, s	7.09[b]	7.54[b]	-
32	7.13, t (7.5)	7.14, t (7.5)	7.13, t (7.1)	7.15[b]	7.09[b]	2.77, s	7.24[b]	3.83, s	4.10, d (7.4)
33a	7.08, t (7.5)	7.08, t (7.4)	7.06, t (7.6)	7.10[b]	7.04[b]		7.14[b]		1.55, m
33b	-	-	-	-	-		-		2.14, m
34a	7.61, d (7.9)	7.61, d (7.8)	7.64, d (7.9)	7.61, d (7.9)	7.62, d (7.7)		7.24[b]		1.41, m
34b	-	-	-	-	-		-		1.78, m
35a	-	-	-	-	-		7.09[b]		3.00, t (8.8)
35b	-	-	-	-	-		-		3.30[d]

36	-	-	-	-	-	5.99, d (7.7)	-
37	2.90, s	2.92, s	3.49, s	2.88, s	3.42, s	-	-
38	2.90, s	2.92, s	3.39, s	2.88, s	3.35, s	6.30, d (15.4)	6.65, d (15.5)
39	3.77, s	OH, n.o.	OH, n.o.			7.53, d (15.3)	7.60, d (15.5)
40						-	-
41						7.56[b]	7.68, d (7.3)
42						7.46[b]	7.45[b]
43						7.45[b]	7.44[b]
44						7.46[b]	7.45[b]
45						7.56[b]	7.68, d (7.3)
46							OH, n.o.

[a]Spectra were recorded at 400 MHz. Solvent for compounds **1**-**6**, **9**: methanol-d_4; solvent for compounds **7** and **8**: chloroform-*d*; n.o.: not observed. [b]Overlapping signals. [c]Overlapping with residual solvent signal. [d]Could not be assigned unequivocally, due to overlapping of aromatic signals.

Table 2. ^{13}C NMR Spectroscopic Data (δ_C in ppm, type) for Compounds 1-9[a]

position	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1	110.5, CH	110.8, CH	109.3, CH	128.1, CH	127.6, CH	131.3, CH	116.1, CH	106.6, CH	133.8, CH
2	122.0, CH	121.6, CH	119.7, CH	126.5, CH	126.5, CH	126.7, CH	125.6, CH	121.7, CH	127.4, CH
4	170.7, C	170.7, C	169.1, C	171.5, C	171.5, C	172.1, C	170.4, C	171.8 or 167.9[c]	171.7, C
5	61.7, CH	61.7, CH	59.8, CH	59.7, CH	59.7, CH	59.8, CH	59.1, CH	62.1, CH	59.0, CH
7	172.1, C	172.2, C	n.o.	172.5, C	172.7, C	172.6, C	170.8, C	171.8 or 167.9[c]	171.0, C
8	67.0, CH	67.1, CH	65.4, CH	66.8, CH	66.9, CH	67.1, CH	55.9, CH	63.2, CH	57.6, CH
9	79.0, CH	78.8, CH	77.4, CH	83.8, CH	84.0, CH	83.4, CH	82.0, CH	78.9, CH	82.5, CH
11	152.4, C	151.6, C	149.7, C	158.6, C	158.6, C	158.7, C	155.3, C	150.9, C	156.5, C
12	119.0, CH	119.2, CH	117.7, CH	119.8, CH	120.2, CH	119.0, CH	130.3, CH	117.0, CH	119.6, CH
13	115.3, CH	119.3, CH	117.9, CH	132.2, CH	132.2, CH	132.2, CH	123.5, CH	113.9, CH	131.3, CH
14	153.3, C	150.8, C	150.0, C	133.5, C	133.4, C	133.6, C	132.4, C	151.4, C	133.1, C
15	125.4, C	123.2, C	121.7, C	131.0, CH	130.9, CH	131.0, CH	123.5, CH	124.2, C	130.2, CH
16	112.6, CH	112.1, CH	110.4, CH	122.4, CH	122.6, CH	122.1, CH	131.9, CH	110.8, CH	122.4, CH
17	37.2, CH	37.3, CH	35.6, CH	38.6, CH	38.6, CH	39.1, CH	25.8, CH_2	29.1, CH_2	74.6, CH
18	26.5, CH_2	26.5, CH_2	24.7, CH_2	25.5, CH_2	25.5, CH_2	25.6, CH_2	24.5, CH_2	25.0, CH_2	142.1, C
19	11.9, CH_3	12.0, CH_3	10.4, CH_3	11.7, CH_3	11.7, CH_3	11.5, CH_3	46.8, CH_2	48.0, CH_2	128.4, CH

20	16.4, CH_3	16.5, CH_3	14.8, CH_3	15.8, CH_3	15.8, CH_3	15.8, CH_3	137.3, C	32.8, CH_2	129.0, CH
21	33.4, CH_2	33.5, CH_2	31.6, CH_2	32.5, CH_2	32.3, CH_2	32.8, CH_2	128.3, CH	45.7, CH_2	128.7, CH
22	47.4, CH_2	47.5, CH_2	46.2, CH_2	46.8, CH_2	47.4, CH_2	47.9, CH_2	128.4, CH	-	129.0, CH
23	-	-	-	-	-	-	128.0-129.0[b]	165.1, C	128.4, CH
24	169.2, C	169.0, C	n.o.	169.4, C	167.8, C	167.9, C	128.4, CH	117.1, CH	139.1, C
25	67.2, CH	67.2, CH	76.3, CH	66.6, CH	77.4, CH	70.0, CH	128.3, CH	143.4, CH	129.6, CH
26	25.6, CH_2	25.8, CH_2	24.5, CH_2	24.6, CH_2	26.0, CH_2	36.6, CH	-	134.8, C	129.0, CH
27	107.4, C	107.2, C	106.0, C	108.1, C	107.8, C	26.7, CH_2	166.9, C	128.0, CH	129.0, CH
28	125.8, CH	125.9, CH	123.7, CH	125.7, CH	125.3, CH	12.2, CH_3	54.1, CH	128.9, CH	129.0, CH
29	-	-	-	-	-	13.3, CH_3	36.1, CH_2	130.1, CH	129.6, CH
30	138.1, C	138.0, C	136.5, C	137.9, C	137.9, C	-	136.4, C	128.9, CH	-
31	112.9, CH	112.9, CH	111.2, CH	112.6, CH	112.5, CH	42.6, CH_3	128.6, CH	128.0, CH	170.2, C
32	123.0, CH	123.0, CH	121.2, CH	122.7, CH	122.7, CH	42.6, CH_3	128.7, CH	56.0, CH_3	60.4, CH
33	120.5, CH	120.5, CH	118.7, CH	120.2, CH	120.1, CH		126.7, CH		26.6, CH_2
34	118.8, CH	118.7, CH	117.2, CH	118.6, CH	118.8, CH		128.7, CH		25.3, CH_2
35	128.4, C	128.4, C	126.4, C	128.3, C	128.0, C		128.6, CH		47.8, CH_2
37	42.7, CH_3	42.6, CH_3	57.1, CH_3	42.3, CH_3	58.4, CH_3		166.0, C		168.0, C
38	42.7, CH_3	42.6, CH_3	53.7, CH_3	42.3, CH_3	55.3, CH_3		117.3, CH		119.6, CH
39	56.7, CH_3						143.4, CH		144.2, CH

40	134.8, C	136.3, C
41	128.0, CH	129.2, CH
42	129.1, CH	130.1, CH
43	130.1, CH	131.3, CH
44	129.1, CH	130.1, CH
45	128.0, CH	129.2, CH

[a]Spectra were recorded at 100 MHz. Solvent for compounds **1**-**6**, **9**: methanol-d_4; solvent for compounds **7** and **8**: chloroform-*d*; n.o.: not observed. [b]Could not be assigned unequivocally, due to overlapping of aromatic signals. [c]May be interchanged.

The antiplasmodial activity against *Plasmodium falciparum* strain K1 and cytotoxicity for MRC-5 cells (human fetal lung fibroblast cells) were determined in triplicate for compounds **1**, **2**, **4**, **6** and **9** and the results are displayed in Table 3. The quantities of the other compounds isolated were insufficient for such testing.

Table 3. Antiplasmodial Activity against *P. falciparum* Strain K1 and Cytotoxicity against MRC5-cells (IC_{50} µM) for Compounds 1, 2, 4, 6 and 9, Obtained from the Roots of *Ziziphus oxyphylla*

compound	*P. falciparum* K1	MRC-5
1	3.2 ± 2.6	30.6 ± 4.0
2	7.1 ± 1.6	> 64.0
4	13.6 ± 9.3	> 64.0
6	> 32.0	> 64.0
9	7.4 ± 3.0	31.2 ± 1.4
chloroquine	0.3 ± 0.2	-

Nummularine-R (**1**), *O*-desmethylnummularine-R (**2**) and oxyphylline-F (**9**) showed the most potent antiplasmodial activity (IC_{50} values of 3.2, 7.1 and 7.4 µM, respectively), but only compound **2** did not show cytotoxicity at the highest test concentration of 64.0 µM and thus seems the most promising. For hemsine-A (**4**), an IC_{50} value of 13.6 µM was found; the antiplasmodial activity of this compound has been reported before (IC_{50} 7.3 µM).[10]

Suksamrarn et al.[10] described the possible importance of the methoxy group in position 2 of the styrylamine unit of cyclopeptide alkaloids for resultant antiplasmodial activity, but from the present study it can be concluded that cyclopeptide alkaloids with a 2-hydroxystyrylamine moiety, like compound **2**, are also active. Moreover it was hypothesized that a mono- or dimethylated

terminal *N*-atom is crucial for the activity,[10,11] but this is in contradiction with the activity that was found herein for the new compound oxyphylline-F (**9)**, which contained a terminal cinnamoyl moiety and no *N*-(di)methyl group. Based on the structural features and IC_{50} values found for nummularine-R (**1**), *O*-desmethylnummularine-R (**2**), hemsine-A (**4**) and ramosine-A (**6**), it might be hypothesized that the tryptophan moiety in the side-chain is important for antiplasmodial activity. Compound **9**, however, does not contain a tryptophan unit and also showed antiplasmodial activity, which led to the conclusion that a tryptophan unit may play a role in mediating antiplasmodial activity, but it is not indispensible. Interestingly, oxyphylline-F (**9**) is the first neutral cyclopeptide alkaloid for which the in vitro antiplasmodial activity has been reported. Apparently, it is not possible yet to determine a clear structure-activity relationship for the antiplasmodial activity of cyclopeptide alkaloids, since only a limited set of compounds has been evaluated so far. Evaluation of more cyclopeptide alkaloids will be necessary before clear structure-activity relationships can be established.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were determined on a JASCO P-2000 spectropolarimeter (Easton, MD, USA) with Spectramanager software and with methanol as blank. NMR spectra were recorded on a Bruker DRX-400 spectrometer (Rheinstetten, Germany) equipped with either a 3-mm inverse broadband (BBI) probe or a 5-mm dual $^{1}H/^{13}C$ probe using standard Bruker pulse sequences and operating at 400 MHz for ^{1}H and at 100 MHz for ^{13}C NMR spectra. The spectra were processed with Topspin version 1.3. An Agilent QTOF 6530 mass spectrometer (Santa Clara, CA, USA) with MassHunter version B.06 software was used to perform accurate mass measurements. The mass spectrometer was operated in (+)-ESIMS mode at a

resolution of 20,000. Calibration was done externally, and the samples were measured after direct infusion.

A semi-preparative HPLC system with DAD and ESIMS detectors was used for isolation of pure compounds and was comprised of a sample manager, injector and collector (2767), a quaternary gradient module (2545), a System Fluidics Organizer, an HPLC pump (515), a photodiode array detector (2998), and a Micromass Quattro mass spectrometer with TQD, all supplied by Waters (Milford, MA, USA). MassLynx version 4.1 was used to process the data. The LC–SPE–NMR configuration consisted of an Agilent 1200 series HPLC system with an in-line solvent degasser, a quaternary pump, an autosampler, a column compartment, and a diode-array detector. Samples were collected using a Bruker/Spark Solid Phase Extraction system using 2-mm GP resin cartridges, and prepared for NMR spectroscopy using a Gilson Liquid Handler 215. A Grace Reveleris X2 flash chromatography system (Lokeren, Belgium) was used for fractionation of the plant material. TLC was performed on NP F_{254} plates (20 cm × 20 cm) from Merck (Darmstadt, Germany) and the spots were observed under UV light (254 and 366 nm) and under visible light after spraying with Dragendorff reagent, iodoplatinate reagent, and cerium sulphate reagent.[21]

Dichloromethane, chloroform, ethyl acetate, methanol and acetonitrile of HPLC quality, hydrochloric acid (37%), sulfuric acid, and ammonia (25%) were purchased from Fisher Chemical (Loughborough, UK). Formic acid, glacial acetic acid, trichloroacetic acid, potassium iodide, and hydrogen hexachloroplatinate (IV) hydrate, and *n*-butanol were supplied by Acros Organics (Geel, Belgium). Methanol-d_4 (99.8% D) and chloroform-*d* (99.8% D) were from Sigma-Aldrich (Steinheim, Germany). Bismuth subnitrate was purchased from Merck KGaA (Darmstadt, Germany) and cerium sulphate was purchased from Roth (Karlsruhe, Germany). Milli-Q water was prepared with a Millipore water purification system (Bedford, MA, USA).

Plant Material. Two different batches of roots from *Ziziphus oxyphylla* were collected in Pakistan. Batch 1 (1.5 kg) was collected in September and October 2009 in the Swat Valley, northern Pakistan. The identification was done by Prof. Dr. Mansoor Ahmad and a voucher specimen (0012-2009/AZ) was deposited at the Laboratory of Pharmacognosy, Research Institute of Pharmaceutical Sciences, University of Karachi, Pakistan. Batch 2 (2.9 kg) was collected in July and August 2012 in the city of Upper Dir, District Upper Dir, KPK Province, Pakistan. This identification was performed by Dr. Muhammad Zafar and a voucher specimen (5698-IK) was deposited at the Herbarium of Pakistan, Quaid-i-Azam University, Islamabad, Pakistan.

Extraction and Isolation. The plant material of both batches was dried and milled before extraction. Batch 1 was macerated in methanol for 10 days and this was repeated three times with fresh solvent. The macerate was filtered and the solvent was evaporated under reduced pressure and at 40 °C until dryness. This resulted in 509 g of crude extract, of which 100 g was suspended in 200 mL 80% methanol and sequentially partitioned with *n*-hexane, chloroform, ethyl acetate, and *n*-butanol. A part of the chloroform fraction (3.3 g of 14 g) was submitted to flash chromatography for further fractionation. A GraceResolv 120 g silica column was used with the following solvents: dichloromethane (A), ethyl acetate (B) and methanol (C) and a flow rate of 40 mL/min. The gradient was as follows: 0 min 100% A, 0% B and C, changed stepwise to 0% A, 100% B and 0% C at 89 min. This condition was kept for 4 min. Then, a stepwise change to 0% A, 25% B and 75% C was applied in 24 min and this final condition was kept for 4 min. For detection, ELSD and UV absorption at 254 nm and 366 nm were used. Throughout the whole experiment the eluent was collected in test tubes, based on the ELSD and UV absorption intensity.

Based on the obtained chromatograms in combination with a TLC analysis of the collected eluates, performed on normal-phase plates with 10% methanol in chloroform as the mobile phase and observation of the results as described before, test tubes that showed a similar pattern were combined. This resulted in 14 fractions.

A crude extract of batch 2 was prepared by means of percolation with 80% methanol (105 L in total). After evaporation of the solvent and freeze-drying, 380 g of crude extract were obtained. The crude extract was dissolved in 50% methanol/50% water and was acidified to pH <3 with 2 M HCl. Then, a liquid-liquid partition was performed with dichloromethane. Next, the pH of the acidified phase was increased to >9 by the addition of NH_4OH (25%), followed by a second liquid-liquid partition with dichloromethane. Thus, the plant material was divided into three fractions: CH_2Cl_2 (I), CH_2Cl_2 (II), and CH_3OH/H_2O (pH >9) (III). TLC analysis of these three fractions was performed as described before. The TLC indicated that alkaloids were only present in the CH_2Cl_2 (II) phase (3.06 g). Further fractionation of this phase was performed by means of flash chromatography. The same type of column and solvents were used as for batch 1. The flow rate and settings of the detector were also identical. The gradient was as follows: 0 min 100% A, 0% B and C, changed stepwise to 0% A, 100% B and 0% C at 39 min. This condition was kept for 3.5 min. Then, a stepwise change to 0% A, 25% B and 75% C was accomplished in 64 min and this final condition was kept for 15 min. The ELSD and UV absorption signal in combination with TLC analysis, which was performed as described above, were used in order to combine test tubes with a similar content. This resulted in 23 fractions.

Based on TLC analysis [mobile phase CH_2Cl_2-CH_3OH (90:10)], fractions 4, 10, 11 and 12 from batch 1 and fractions 11, 12, 16 and 23 from batch 2 were submitted for semi-preparative HPLC. The system was operated with a C_{18} Luna column (250 mm x 10.0 mm, particle size 5 µm) from

Phenomenex (Utrecht, the Netherlands) and a C_{18} guard column (10 mm × 10 mm, particle size 5 µm) from Grace (Hesperia, CA, USA). As mobile phase, H_2O or H_2O + 0.1% formic acid (A) and acetonitrile (B) were used. Linear gradients were applied and the flow rate was set at 3.0 mL/min. More detailed information about the HPLC conditions used for the isolation of each compound is given in Table S1, Supporting Information. The DAD spectrum was recorded from 200 nm to 450 nm and mass spectra were taken in (+)-ESIMS mode, with MS scan range: *m/z* 150 to 850. $V_{capillary}$ 3.00 kV, V_{cone} 50 V, $V_{extractor}$ 3 V, $V_{RF\text{-}Lens}$ 0.2 V, T_{source} 135 °C, $T_{Desolvation}$ 400 °C, desolvation gas flow 750 L/h, cone gas flow 50 L/h. Interesting peaks were selected based on the UV-spectrum and the *m/z*-value of each peak. The collection of the eluate was triggered as long as the intensity of selected *m/z* values exceeded the set threshold. The specific settings for the collection of each cyclopeptide alkaloid are shown in Table S1, Supporting Information.

^{1}H NMR spectra of each collected subfraction were recorded, giving an indication of the purity and the type of compound(s) present. Whenever the ^{1}H NMR spectrum appeared to represent a single compound, additional ^{13}C NMR and/or 2D NMR spectra (COSY, HSQC, HMBC) were recorded. From the ^{1}H NMR spectrum of the two samples isolated from batch 1, fraction 4, it could be concluded that these samples contained more than just one compound, but signals characteristic for cyclopeptide alkaloids could be seen. In order to further purify these two samples, LC-DAD-SPE-NMR was applied. For sample 1-4-1, water (A) and acetonitrile (B) were used as the mobile phase in the following gradient: 0 to 5 min 50% B, 20 min 65% B, 22 to 27 min 100% B. The flow rate was 0.8 mL/min and a Luna C_{18} column (250 x 4.6 mm, particle size, 5µm; pore size, 100 Å) was used as the stationary phase. The sample with a concentration of 10 mg/mL was injected in 7 consecutive runs, 15 µL per injection. For sample 1-4-3, the same HPLC conditions were applied, but the gradient was as follows: 0 to 5 min 56% B, 20 min 66% B, 25 to 30 min, 100% B. The

sample with a concentration of 10 mg/mL was injected in 14 consecutive runs, 20 µL per injection. The eluting compounds were multitrapped on SPE cartridges, which were then dried with nitrogen gas. The adsorbed compounds (**7** and **8** for sample 1-4-3 and sample 1-4-1, respectively) were eluted into 3 mm NMR tubes using methanol-d_4.

Nummularine-R (**1**): yellow powder (46 mg); $[\alpha]_D$ -244.7 (*c* 0.5, CH_3OH); UV λ_{max} 219, 270, 318 nm; ^1H NMR (methanol-d_4, 400 MHz) and ^{13}C NMR (methanol-d_4, 100 MHz), see Table 1 and 2, respectively; (+)-ESIMS *m/z* 589 $[M+H]^+$; 611 $[M+Na]^+$.

O-Desmethylnummularine-R (**2**): yellow powder (28 mg); $[\alpha]_D$ -295.5 (*c* 0.6, CH_3OH); UV λ_{max} 222, 268, 320 nm; ^1H NMR (methanol-d_4, 400 MHz) and ^{13}C NMR (methanol-d_4, 100 MHz), see Table 1 and 2, respectively; HRESIMS *m/z* 574.3031 $[M+H]^+$ (calcd for $C_{32}H_{40}N_5O_5$, 574.3024).

O-Desmethylnummularine-R N-oxide (**3**): yellow powder (1 mg); UV λ_{max} 202, 269, 319 nm; ^1H NMR (methanol-d_4, 400 MHz) and ^{13}C NMR (methanol-d_4, 100 MHz), see Table 1 and 2, respectively; HRESIMS *m/z* 590.3002 $[M+H]^+$ (calcd for $C_{32}H_{40}N_5O_6$, 590.2973).

Hemsine-A (**4**): yellow oil (198 mg); $[\alpha]_D$ -81.3 (*c* 0.5, CH_3OH); UV λ_{max} 221, 280 (shoulder) nm; ^1H NMR (methanol-d_4, 400 MHz) and ^{13}C NMR (methanol-d_4, 100 MHz), see Table 1 and 2, respectively; HRESIMS *m/z* 558.3097 $[M+H]^+$ (calcd for $C_{32}H_{40}N_5O_4$, 558.3097).

Hemsine-A N-oxide (**5**): yellow powder (6 mg); $[\alpha]_D$ -65.7 (*c* 0.6, CH_3OH); UV λ_{max} 218, 280 (shoulder) nm; ^1H NMR (methanol-d_4, 400 MHz) and ^{13}C NMR (methanol-d_4, 100 MHz), see Table 1 and 2, respectively; HRESIMS *m/z* 574.3036 $[M+H]^+$ (calcd for $C_{32}H_{40}N_5O_5$, 574.3024).

Ramosine-A (**6**): yellow powder (4 mg); $[\alpha]_D$ -139.7 (*c* 0.4, CH_3OH); UV λ_{max} low end absorption; ^1H NMR (methanol-d_4, 400 MHz) and ^{13}C NMR (methanol-d_4, 100 MHz), see Table 1 and 2, respectively; (+)-ESIMS *m/z* 508 $[M+Na]^+$.

Oxyphylline-C (**7**): ^{1}H NMR (chloroform-*d*, 400 MHz) and ^{13}C NMR (chloroform-*d*, 100 MHz), see Table 1 and 2, respectively; (+)-ESIMS *m/z* 655 $[M+H]^+$.

Oxyphylline-E (**8**): ^{1}H NMR (chloroform-*d*, 400 MHz) and ^{13}C NMR (chloroform-*d*, 100 MHz), see Table 1 and 2, respectively; HRESIMS *m/z* 488.2220 $[M+H]^+$, 510.2054 $[M+Na]^+$ (calcd for $C_{28}H_{30}N_3O_5$, 488.2180, calcd for $C_{28}H_{29}N_3O_5Na$ 510.1999).

Oxyphylline-F (**9**): yellowish powder (41 mg); $[\alpha]_D$ -117.9 (*c* 0.5, CH_3OH); UV λ_{max} low end, 284 nm; ^{1}H NMR (methanol-d_4, 400 MHz) and ^{13}C NMR (methanol-d_4, 100 MHz), see Table 1 and 2, respectively; HRESIMS *m/z* 671.2885 $[M+H]^+$, 693.2698 $[M+Na]^+$ (calcd for $C_{40}H_{39}N_4O_6$, 671.3228, calcd for $C_{40}H_{38}N_4O_6Na$ 693.2684).

Antiplasmodial and Cytotoxicity Activities. The antiplasmodial and cytotoxicity activity determinations of the selected isolated components were performed as reported before.[15,22,23] The antiplasmodial activity was tested against the chloroquine-resistant strain *Plasmodium falciparum* K1. The cytotoxicity was determined on MRC-5 cells (human lung fibroblast). The means and standard deviations of three experiments were calculated.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 1D (^{1}H, ^{13}C) and/or 2D (COSY, HSQC, HMBC) spectra of compounds **1**-**9** are included as Supporting Information (PDF)

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Notes

The authors declare no competing financial interest.

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