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Cyclopeptide Alkaloids from *Hymenocardia acida*

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ABSTRACT

Four cyclopeptide alkaloids (**1-4**) were isolated from the root bark of *Hymenocardia acida* by means of semi-preparative HPLC with DAD and ESIMS detection and conventional separation methods. Compound structure elucidation was performed by spectroscopic data interpretation. Except for the known compound hymenocardine (**1**), the three other alkaloids were isolated for the first time from a natural source. These included a hymenocardine derivative with a hydroxy group rather than a carbonyl that was named hymenocardinol (**2**), as well as hymenocardine *N*-oxide (**3**) and a new cyclopeptide alkaloid containing an unusual histidine moiety that was named hymenocardine-H (**4**). The isolated cyclopeptide alkaloids were tested for their antiplasmodial activity and cytotoxicity. All four compounds showed moderate antiplasmodial activity with IC₅₀ values ranging from 12.2 to 27.9 μM, the most active one being hymenocardine *N*-oxide (**3**), with an IC₅₀ value of 12.2 ± 6.6 μM. Compounds **2-4** were found not to be cytotoxic against MRC-5 cells (IC₅₀ > 64.0 μM), but hymenocardine (**1**) showed moderate cytotoxicity with an IC₅₀ of 51.1 ± 17.2 μM.

Hymenocardia acida Tul. is a shrub or small tree of about 6 m high that grows in the African Savannah. It belongs to the family of the Phyllantaceae, although previously it was classified in the families of the Euphorbiaceae and Hymenocardiaceae.¹⁻³ Extracts of this plant have been used in traditional African medicine. For example, the leaves and roots are used to treat malaria, the roots are used against hypertension, and the plant may be employed as an antiseptic and to treat skin diseases. Another application is the use of decoctions of the leaves or roots to relieve pain.⁴⁻⁷ Previous phytochemical studies on this plant have shown the presence of alkaloids, anthocyanins, anthraquinones, cardiac glycosides, flavonoids, phenols, saponins, steroids, stilbenoids, tannins and triterpenoids.⁵⁻⁷ To date, one cyclopeptide alkaloid, hymenocardine, has been reported.³

The antiplasmodial activity and cytotoxicity of extracts from the leaves of *H. acida* has been shown by Vonthron-Senecheau.⁴ Mahmout et al. reported the compounds lupeol, lupeyl docosanoate, and β -sitosterol to be present in *H. acida* and to have antiplasmodial activity, related to their amphiphilic nature.⁶ Apart from this, little is known about the antiplasmodial constituents of this plant. In view of the traditional use of *Hymenocardia acida* against malaria, the occurrence of the cyclopeptide alkaloid hymenocardine (**1**) in the root bark, and the reported antiplasmodial activity of some cyclopeptide alkaloids such as ziziphines N and Q, mauritine M, nummularine H, and hemsine A,^{8,9} it was decided to investigate in more detail the presence of potentially antiplasmodially active cyclopeptide alkaloids in the root bark of *H. acida*. Cyclopeptide alkaloids are polyamidic bases and they form a relatively rare class of natural products. They are macrocyclic compounds, containing a 13-, 14- or 15-membered ring and they can be classified according to the ring size. They consist of a styrylamine unit and three amino acids as common structural elements.^{10,11}

Root bark of *H. acida* was extracted with 80% methanol and the crude extract was fractionated by liquid-liquid partitioning, followed by flash chromatography. The isolation of single compounds was performed with semi-preparative HPLC with DAD and ESIMS detection and in this way four cyclopeptide alkaloids were obtained (**1-4**). Their structures were elucidated by 1D (^1H , ^{13}C , DEPT 135, DEPT 90) and 2D NMR experiments (COSY, HSQC, HMBC) and comparison to literature data, and confirmed by HRESIMS.

Comparison of the NMR spectra of compound **1** with previously published data showed that this compound is hymenocardine, which was reported to be present in *H. acida* earlier.^{3,12} ¹H and ¹³C NMR chemical shift assignments for **1** are listed in Tables 1 and 2, respectively.

The NMR spectra of compound **2** showed a resemblance to those of compound **1**, but a carbon signal with δ_C 73.4 ppm that correlated with a proton at δ_H 5.01 ppm was present, while these signals were absent from the spectra of compound **1**. Moreover, the signal at 205.1 ppm in the ¹³C NMR spectrum of compound **1**, corresponding to the carbonyl group at position 1 was absent in the ¹³C NMR spectrum of compound **2**. This can be explained by the presence of a hydroxy group instead of a carbonyl group at C-1. Thus, compound **2** is a reduced analogue of compound **1** and has been named hymenocardinol. Although the reduced form of hymenocardine was obtained before, after chemical modification of **1** with sodium borohydride,³ this is the first report of compound **2** as a natural product.

The structure of compound **3** also showed a resemblance to compound **1** according to its NMR spectra, but some of the signals corresponding to the terminal amino acid of the side chain showed more downfield chemical shifts. This could be observed for the two N-methyl groups, which resonated at δ_C 55.3 ppm/ δ_H 3.46 ppm and δ_C 56.9 ppm/ δ_H 3.32 ppm respectively, whereas in compound **1** the values of both methyl groups were δ_C 42.7 ppm/ δ_H 2.69 ppm. Similarly, the CH group substituted by the N-dimethyl group was shifted to δ_C 82.4 ppm/ δ_H 3.90 ppm for compound **3**, compared to δ_C 73.9 ppm/ δ_H 3.60 ppm for compound **1**. A similar pattern was reported by Han et al.¹³ for cyclopeptide alkaloids containing an N-oxide group. Thus, compound **3** was identified as hymenocardine *N*-oxide. 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.

In contrast with the previous two compounds, compound **4** was not closely related structurally to hymenocardine (**1**), as deduced from the NMR spectra. The structure of this compound was elucidated based on the interpretation of its 2D-NMR spectra (COSY, HSQC, and HMBC). Instead of a β -hydroxyvaline unit, present as one of the ring-bound amino acids in compounds **1-3**, this compound was found to contain a β -hydroxyleucine moiety, and in the side chain isoleucine was present instead of valine. Moreover, the 14-membered ring also contained a histidine moiety, whereas a tryptophan moiety was present in compounds **1-3**. The presence of histidine was confirmed by comparison with published NMR data for this amino acid.¹⁵ To the best of our knowledge this is the first report of a cyclopeptide alkaloid containing a histidine moiety. Owing to the presence of this amino acid, compound **4** was named hymenocardine-H.

¹H and ¹³C NMR chemical shift assignments for compounds **1 – 4** are listed in Tables 1 and 2, respectively. For compounds **1-3**, ¹³C NMR spectra were recorded in methanol-*d*₄ and the spectra were recorded in DMSO-*d*₆ for **4**. When NMR spectra in methanol-*d*₄ were recorded for this compound, an artifact (compound **5**) was detected, which was identified as the hemi-acetal form of compound **4**, formed after reaction with the solvent (methanol-*d*₄). This was deduced from the presence of a signal at 105.5 ppm in the ¹³C NMR spectrum, while there was no signal for the carbonyl C-atom at C-1, which would have a chemical shift of more than 200 ppm.

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*.^{Morel, 2005} The configuration of hymenocardine (**1**) has been described in 1968 by Pais et al. and indeed the L-

configuration was found for the tryptophan, the valine and the N-dimethyl-isoleucine present in this cyclopeptide alkaloid.³ For the four compounds that are described in this note, no specific experiments were performed to determine the stereochemistry, but conforming to the already reported configuration of hymenocardine and the occurrence of the L-form in almost all cyclopeptide alkaloids, the L-configuration was adopted for the amino acid moieties present in compounds **1-4**. For hymenocardine-H (**4**) the stereochemistry of the β -hydroxy amino acid, β -hydroxy leucine, was established as *L-erythro* based on the available NMR data. The coupling constant of the doublet corresponding to H-9 was 8.5 Hz, indicative for an *erythro* configuration. In addition, the chemical shifts of C-9 and C-8 were 81.8 ppm and 54.9 ppm, respectively, both indicative for the L-amino acid form. Furthermore, the *J* value of ¹H NMR signal attributed to the methylgroup in position 25 was 6.7 Hz, indicative for a pseudoaxial / equatorial coupling, typical for *L-erythro*- β -hydroxyleucine.^{11,14}

Table 1. ^1H NMR Spectroscopic Data (δ_{H} in ppm, Multiplicity (J in Hz)) for Compounds 1-5^a

position	1	2	3	4	5
	δ_{H} , m (J in Hz)	δ_{H} , m (J in Hz)	δ_{H} , m (J in Hz)	δ_{H} , m (J in Hz)	δ_{H} , m (J in Hz)
1	-	5.01, d (4.0)	-	-	-
2a	3.85, d (17.0)	2.84 ^b	3.81, d (17.0)	3.86 ⁱ	3.21, d (13.8)
2b	3.99, d (17.0)	4.13, dd (14.1, 5.1)	4.00, d (17.0)	3.88 ⁱ	3.82, d (13.8)
3 (N-H)	n.o.	n.o.	n.o.	8.71, t (5.4)	n.o.
5	4.53, m	4.28, m	4.40, dd (10.8, 4.5)	4.38 ^j	4.35, m
6 (N-H)	n.o.	n.o.	n.o.	7.81, d (9.5)	n.o.
8	4.73, s	4.71, s	4.62, s	4.43 ^j	4.43, d (9.4)
9	-	-	-	4.80, d (8.5)	4.81, dd (9.4, 2.0)
12	7.24, d (8.9)	7.14, d (8.4)	7.18, dd (8.3, 2.2)	7.01 ^k	6.90, dd (8.9, 2.7)
13	7.34, d (8.9)	7.32 ^c	7.39, dd (8.2, 2.1)	7.39, d (8.5)	7.34, dd (8.7, 2.2)
15	7.34, d (8.9)	6.95 ^d	7.31 ^f	7.22, dd (8.5, 2.0)	7.21, dd (8.6, 2.2)
16	7.14, dd (9.6, 2.1)	6.97 ^d	7.12, dd (8.4, 2.3)	6.97 ^k	6.81, dd (8.6, 2.5)
17a	2.56, dd (14.1, 4.3)	2.64, dd (14.3, 4.7)	2.58, dd (14.1, 4.6)	2.38, m	2.70, d (7.0)
17b	2.96, dd (14.0, 11.3)	2.97, dd (14.3, 10.4)	2.98, dd (14.0, 10.9)	2.78, dd (15.7, 9.2)	2.82, d (7.0)
20	7.40, d (8.2)	7.44, d (8.0)	7.44, d (7.9)	8.39, m	8.78, d (1.4)
21	6.94, dd (8.0, 7.6)	6.99 ^d	6.99, ddd (8.2, 7.5, 1.0)	8.40 ^l	N-H, n.o.
22	7.05, dd (8.2, 7.6)	7.07, dd (8.0, 7.5)	7.07, ddd (8.0, 7.6, 1.0)	6.99 ^k	7.25, s

23	7.27, d (8.2)	7.30 ^c	7.30 ^f	2.16, m	2.24, m
24	-	-	-	1.09 ^m	1.10, d (7.0)
25	N-H, n.o.	N-H, n.o.	N-H, n.o.	0.85, d (6.7)	0.97, d (7.0)
26	6.89, s	6.95 ^d	6.91, s	8.34, d (9.7)	N-H, n.o.
27	1.92, s	1.91, s	1.44, s	-	-
28	1.49, s	1.46, s	1.92, s	4.27 ^j	4.26, d (9.4)
29	N-H, n.o.	N-H, n.o.	N-H, n.o.	1.63, m	1.80, m
30a	-	-	-	1.06 ^m	1.24 ⁿ
30b	-	-	-	1.33, m	1.54, m
31	4.29, d (9.0)	4.35, d (8.6)	4.25, d (7.7)	0.74, t (7.4)	0.91, t (7.4)
32	1.97, m	2.00, m	1.96, m	0.63, d (6.8)	0.77, d (6.9)
33	0.86, d (6.7)	0.91, d (6.7)	0.92 ^g	8.43 ^l	N-H, n.o.
34	0.93, d (6.7)	0.96, d (6.7)	0.91 ^g	-	-
35	N-H, n.o.	N-H, n.o.	N-H, n.o.	3.57 ^p	3.87, d (4.9)
36	-	-	-	1.93, m	2.16, m
37a	3.60, d (6.6)	3.75, m	3.90, m	1.11 ^m	1.23 ⁿ
37b	-	-	-	1.60, m	1.66, m
38	2.06, m	2.13, m	2.37, m	0.89, t (7.3)	1.04 ^o
39a	1.20, m	1.23, m	1.46, m	0.81, d (6.6)	1.02 ^o
39b	1.65, m	1.67, m	1.71, m	-	-
40	1.02, t (7.4)	1.03 ^e	1.04 ^h	-	-
41	0.97, d (6.6)	1.00 ^e	1.06 ^h	2.59, s	2.90, s
42	-	-	-	2.51, m	2.94, s
43	2.69, s	2.85, s ^b	3.46, s		
44	2.69, s	2.85, s ^b	3.32 ^q		

^aSpectra were recorded at 400 MHz. Solvent for compounds **1-3** and **5**: methanol-*d*₄, compound **4**: DMSO-*d*₆. n.o.: not observed. ^{b-o}Overlapping signals. ^pOverlapping with residual water signal. ^qOverlapping with methanol signal.

Table 2. ^{13}C NMR Spectroscopic Data (δ_{C} in ppm, type) for compounds 1-5^a

position	1	2	3	4	5
	δ_{C} , type	δ_{C} , type	δ_{C} , type	δ_{C} , type	δ_{C} , type
1	205.1, C	73.4, CH	203.6, C	203.2, C	105.5, C
2	50.9, CH ₂	48.8, CH ₂	49.3, CH ₂	50.2, CH ₂	45.5, CH ₂
4	174.0, C	173.3, C	172.3, C	170.3, C	170.8, C
5	54.5, CH	55.6, CH	52.9, CH	50.9, CH	52.7, CH
7	170.3, C	170.5, C	168.8, C	170.2, C	172.3, C
8	59.2, CH	60.6, CH	57.6, CH	54.9, CH	56.6, CH
9	85.5, C	85.4, C	83.8, C	81.8, CH	79.6, CH
11	159.3, C	156.9, C	157.7, C	158.6, C	158.8, C
12	125.5, CH	124.0, CH	123.9, CH	117.2, CH	113.4, CH
13	130.6, CH	128.1, CH	129.0, CH	129.5, CH	130.8, CH
14	134.2, C	139.2, C	132.6, C	130.6, C	132.5, C
15	130.2, CH	128.9, CH	128.5, CH	129.1, CH	129.9, CH
16	123.4, CH	125.2, CH	121.8, CH	121.2, CH	119.9, CH
17	29.4, CH ₂	30.3, CH ₂	27.8, CH ₂	28.3, CH ₂	29.5, CH ₂
18	110.6, C	111.4, C	109.0, C	163.4, C	130.4, C
19	128.6, C	129.3, C	127.0, C	-	-
20	119.2, CH	119.9, CH	117.6, CH	134.3, CH	135.0, CH
21	119.9, CH	120.4, CH	118.3, CH	-	-
22	122.7, CH	123.2, CH	121.0, CH	116.5, CH	118.6, CH
23	112.5, CH	113.0, CH	110.9, CH	28.2, CH	29.9, CH
24	138.2, C	138.7, C	136.6, C	20.8, CH ₃	20.6, CH ₃
25	-	-	-	15.1, CH ₃	15.4, CH ₃
26	124.1, CH	124.7, CH	122.6, CH	-	-

27	25.0, CH ₃	25.4, CH ₃	29.1, CH ₃	169.8, C	172.2, C
28	30.8, CH ₃	31.1, CH ₃	23.3, CH ₃	56.8, CH	59.2, CH
29	-	-	-	36.8, CH	37.8, CH
30	173.0 C	173.6, C	171.5, C	24.9, CH ₂	26.5, CH ₂
31	60.5, CH	61.2, CH	58.7, CH	10.8, CH ₃	10.9, CH ₃
32	32.5, CH	33.1, CH	30.7, CH	15.3, CH ₃	15.9, CH ₃
33	20.0, CH ₃	20.4, CH ₃	18.4, CH ₃	-	-
34	19.8, CH ₃	20.2, CH ₃	17.7, CH ₃	169.9, C	167.0, C
35	-	-	-	70.9, CH	73.7, CH
36	169.7, C	168.4, C	166.6, C	33.2, CH	35.4, CH
37	73.9, CH	74.2, CH	82.4, CH	25.6, CH ₂	27.6, CH ₂
38	35.3, CH	35.9, CH	33.0, CH	11.5, CH ₃	12.3, CH ₃
39	27.2, CH ₂	28.0, CH ₂	28.9, CH ₂	14.1, CH ₃	13.2, CH ₃
40	12.0, CH ₃	12.7, CH ₃	10.5, CH ₃	-	-
41	14.3, CH ₃	14.3, CH ₃	15.7, CH ₃	40.2, CH ₃	43.9, CH ₃
42	-	-	-	41.6, CH ₃	41.6, CH ₃
43	42.7, CH ₃	43.2, CH ₃	55.3, CH ₃		
44	42.7, CH ₃	43.2, CH ₃	56.9, CH ₃		

^aSpectra were recorded at 100 MHz. Solvent for compounds **1-3** and **5**: methanol-d₄, compound **4**: DMSO-d₆.

The antiplasmodial activity against *Plasmodium falciparum* strain K1 and cytotoxicity for MRC-5 cells (human fetal lung fibroblast cells) were determined for compounds **1-4** and the results are displayed in Table 3. All four compounds showed antiplasmodial activity with IC₅₀ values ranging between 12.2 and 27.9 μ M. Compound **1** was the only compound that showed cytotoxicity with an IC₅₀ of 51.1 $\pm$ 17.2 μ M, for the other compounds no cytotoxicity was observed in a concentration up to 64.0 μ M (IC₅₀ > 64.0 μ M).

Table 3. Antiplasmodial activity against *P. falciparum* strain K1 and Cytotoxicity against MRC5-cells (IC₅₀ μ M) for compounds 1 (hymenocardine), 2 (hymenocardinol), 3 (hymenocardine *N*-oxide) and 4 (hymenocardine-H), Obtained from Root Bark of *Hymenocardia acida*.

compound	<i>P. falciparum</i> K1	MRC-5
1	16.4 $\pm$ 6.8	51.1 $\pm$ 17.2
2	17.5 $\pm$ 8.7	> 64.0
3	12.2 $\pm$ 6.6	> 64.0
4	27.9 $\pm$ 16.5	> 64.0
chloroquine	0.2 $\pm$ 0.1	-

From previous results by Suksamrarn et al. and Panseeta et al. it appeared that all cyclopeptide alkaloids with antiplasmodial activity contained a β -hydroxy proline unit, although this amino acid was also present in inactive compounds. Moreover the importance of the methoxy group in the styrylamine moiety and the methylation of the terminal nitrogen atom were suggested.^{8,9} Compounds **1-4** do not contain a β -hydroxy proline moiety nor a methoxy group, but they do have methylated terminal nitrogens. It can be concluded that the presence of the β -hydroxy proline and the methoxy group might be an indication for antiplasmodial activity, but that those functional groups are not indispensable and cyclopeptide alkaloids with other functional groups can also show antiplasmodial activity. It can be hypothesized that the terminal N-methyl groups are crucial for the antiplasmodial activity. In view of the moderate antiplasmodial activity of the isolated constituents, it is not clear to what extent these cyclopeptides could explain the traditional use against malaria.

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 NMR spectrometer (Rheinstetten, Germany) operating at 400 MHz for ^1H 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 the ESI+ 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 the 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. A Grace Reveleris X2 flash chromatography system (Lokeren, Belgium) was used for fractionation of the plant material. TLC was performed on NP F₂₅₄ 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 and iodoplatinate reagent.

Dichloromethane, chloroform, methanol and acetonitrile were of HPLC-quality and hydrochloric acid (37%) and ammonia (25%) were purchased from Fisher Chemical (Loughborough, UK). Formic acid, glacial acetic acid, potassium iodide, and hydrogen hexachloroplatinate (IV) hydrate were supplied by Acros Organics (Geel, Belgium). Methanol-*d*₄ (99.8% D) and DMSO-*d*₆ (99.9% D) were from Sigma-Aldrich (Steinheim, Germany). Bismuth

subnitrate was bought from Merck KGaA (Darmstadt, Germany). Milli-Q water was prepared with a Millipore water purification system (Bedford, MA, USA).

Plant Material. The root bark of *Hymenocardia acida* was collected in December 2012 in Dubréka, Guinea-Conakry. A specimen of this plant is maintained at the Research and Valorization Center on Medicinal Plants in Dubréka (voucher code 62HK530).

Extraction and Isolation. A crude extract was prepared from 2.7 kg of the dried and milled root bark of *H. acida* by means of percolation with 80% CH₃OH/20% H₂O (83 L in total). After evaporation of the solvent and freeze-drying, 356.9 g of crude extract remained. The crude extract was re-dissolved in 50% CH₃OH/50% H₂O and acidified to pH <3 with HCl 2 M. Then, a liquid-liquid partition was performed with CHCl₃. After this, the pH of the CH₃OH/H₂O/H⁺ phase was increased to > 9 by addition of NH₄OH (25%), followed by a second liquid-liquid partition with CHCl₃. Thus, the plant material was divided into three fractions: CHCl₃ (I), CHCl₃ (II), and CH₃OH/H₂O (pH > 9). TLC analysis of these three fractions was performed with CHCl₃-CH₃OH-NH₃ (60:30:10) as the mobile phase and the plates were observed under UV light (254 and 366 nm) and under visible light after spraying with Dragendorff and iodoplatinate reagents.

The TLC analysis indicated that alkaloids were only present in the CHCl₃ (II) phase (8.27 g). Further fractionation of this phase was performed by flash chromatography on a GraceResolv 120 g silica column. The solvents CH₂Cl₂ (A), EtOAc (B) and CH₃OH (C) were used as follows: 0 min 100% A, 0% B, 0% C, changing linearly to 0% A and 100% B at 40 min; then a linear change to 50% B / 50% C in a 20 min time span, which was retained between 60 and 80 min; from 80 to 100 min a linear change to 100% C; finally, this condition was maintained until 115

min. The flow rate was 13 mL/min and 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. Together with TLC analysis, which was performed as described above, test tubes which showed a similar pattern were combined. This resulted in 12 fractions.

Fractions 11 and 12 were selected based on their TLC profiles ($\text{CH}_2\text{Cl}_2\text{-CH}_3\text{OH-NH}_3$ 95:5:2) and were submitted to 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 x 10 mm, particle size, 5 μm) from Grace (Hesperia, CA, USA). Linear gradients with $\text{H}_2\text{O} + 0.1\%$ formic acid (A) and acetonitrile (B) were applied. For fraction 11: 0 to 5 min. 20% B, 25 min. 30% B, 40-45 min. 100% B. For fraction 12: 0 to 5 min. 20% B, 20 min. 25% B, 35 min. 45% B, 40-45 min. 100% B. The flow rate was 3.0 mL/min. Sample concentration 25 mg/mL; injection volume fraction 11: 400 μL , fraction 12: 200 μL . The DAD spectrum was recorded from 200 nm to 450 nm and mass spectra were taken in ESI+ mode, MS scan range: m/z 150 to 750. $V_{\text{capillary}}$ 3.00 kV, V_{cone} 50 V, $V_{\text{extractor}}$ 3 V, $V_{\text{RF Lens}}$ 0.2 V, T_{source} 135 $^{\circ}\text{C}$, $T_{\text{Desolvation}}$ 400 $^{\circ}\text{C}$, desolvation gas flow 750 L/h, cone gas flow 50 L/h. Collection triggers for fraction 11: m/z 659 or 675 with a threshold of 1.1×10^6 resulted in hymenocardine (**1**) and hymenocardinol (**2**). For fraction 12, the automatic collection was triggered by m/z 654; m/z 675 or m/z 691 reaching a threshold of 1.5×10^6 . Again hymenocardine (**1**) was isolated, together with hymenocardine *N*-oxide (**3**) and hymenocardine-H (**4**).

Hymenocardine (**1**): (561 mg) yellowish powder; $[\alpha]_{\text{D}} = -106.8$ (c 0.5, CH_3OH); UV λ_{max} 201, 263 nm; ^1H NMR (methanol- d_4 , 400 MHz) and ^{13}C NMR (methanol- d_4 , 100 MHz), see Tables 1

and 2, respectively, HRESIMS m/z 675.3893 $[M+H]^+$; 697.3705 $[M+Na]^+$ and 713.3444 $[M+K]^+$ (calcd. for $C_{37}H_{51}N_6O_6$, 675.3865)

Hymenocardinol (**2**): (39 mg) yellowish powder; $[\alpha]_D$ -75.6 (c 0.5, CH_3OH); UV λ_{max} 201, 221, 278 nm; 1H NMR (methanol- d_4 , 400 MHz) and ^{13}C NMR (methanol- d_4 , 100 MHz), see Tables 1 and 2, respectively, HRESIMS m/z 677.4079 $[M+H]^+$; 699.3898 $[M+Na]^+$ and 715.3641 $[M+K]^+$ (calcd. for $C_{37}H_{53}N_6O_6$, 677.4027)

Hymenocardine N-oxide (**3**): (5 mg) yellowish powder; $[\alpha]_D$ -100.7 (c 0.4, CH_3OH); UV λ_{max} 204, 220, 263 nm; 1H NMR (methanol- d_4 , 400 MHz) and ^{13}C NMR (methanol- d_4 , 100 MHz), see Tables 1 and 2, respectively, HRESIMS m/z 691.3855 $[M+H]^+$ and 713.3605 $[M+Na]^+$ (calcd for $C_{37}H_{52}N_6O_7$, 691.3814).

Hymenocardine-H (**4**): white powder; $[\alpha]_D$ -55.5 (c 0.9 CH_3OH); UV λ_{max} 206, 266 nm; 1H NMR (DMSO- d_6 , 400 MHz) and ^{13}C NMR (DMSO- d_6 , 100 MHz), see Tables 1 and 2, respectively. HRESIMS m/z 654.4018 $[M+H]^+$; 676.3837 $[M+Na]^+$ and 692.3620 $[M+K]^+$ (calcd for $C_{34}H_{51}N_7O_6$, 654.3974).

Antiplasmodial and Cytotoxicity Activities. The antiplasmodial and cytotoxicity activity determinations of the isolated components were performed as reported before.^{16,17} The antiplasmodial activity of the isolated compounds was tested against the chloroquine-resistant strain *Plasmodium falciparum* K1. The parasite was maintained in continuous log phase growth in RPMI-1640 medium supplemented with 2% P/S solution, 0.37mM hypoxanthine, 25mM HEPES, 25mM $NaHCO_3$ and 10% O+ human serum together with 4% human O+ erythrocytes. All cultures and assays were conducted at 37 °C under micro-aerophilic atmosphere (4% CO_2 , 3% O_2 and 93% N_2). The in vitro antimalarial activity was assessed using the lactate

dehydrogenase assay. Stock solutions of test compounds were prepared in DMSO at a concentration of 20 mM and diluted with culture medium before being added to asynchronous parasite cultures. Assays were performed in 96-well tissue culture plates, each well containing 10 μ L of the test solution containing the test compound together with 190 μ L of the parasite inoculum (1% parasitaemia, 2% haematocrit). After 72 h of incubation at 37 °C, plates were stored at –20 °C until further processing. After thawing, 20 μ L of haemolysed parasite suspension from each well was transferred into another plate together with 100 μ L MalstatTM reagent and 10 μ L of a 1/1 mixture of PES (phenazine ethosulfate, 2 mg/mL) and NBT (nitro blue tetrazolium grade III, 0.1 mg/mL). The plates were kept in the dark for 2 h and change in colour was measured spectrophotometrically at 655 nm. The results were expressed as percentage reduction in parasitaemia compared to control wells. The IC₅₀ was calculated from the drug concentration–response curves. Chloroquine diphosphate was used as an antiparasmodial reference drug. The cytotoxicity was determined on MRC-5 cells (human lung fibroblast), which were cultured in MEM medium, supplemented with 20 mM L-glutamine, 16.5 mM NaHCO₃, 5% foetal calf serum and 2% P/S solution. Cultures were kept at 37 °C and 5% CO₂. Assays were performed in sterile 96-well tissue culture plates, each well containing 10 μ L of test solution containing the test compound, together with 190 μ L of cell suspension (2.5×10^4 cells/mL). After 7 days incubation, cell proliferation/viability was assessed after addition of MTT (50 μ L of a 1/2.5 solution per well). After 4 h of incubation at 37 °C, the % absorbance reduction at 540 nm for the treated cultures and untreated control cultures were obtained and compared, and IC₅₀ values were determined. The means and standard deviations of six and four experiments were calculated for compounds **1**, **2** and **3**, **4** respectively.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

^1H - and ^{13}C -NMR spectra of compounds **1-3** and **5**; ^1H -NMR, COSY, HSQC and HMBC spectra for compound **4** (PDF)

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Notes

The authors declare no competing financial interest.

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