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Antimicrobial Metabolites from the Paracel Islands Sponge *Agelas mauritiana*

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Abstract

Four new alkaloids, (–)-8'-oxo-agelasine D (**2**), ageloxime B (**3**), (+)-2-oxo-agelasidine C (**4**), 4-bromo-*N*-(butoxymethyl)-1*H*-pyrrole-2-carboxamide (**5**), and the known compound (–)-ageloxime D (**1**) were isolated from the marine sponge *Agelas mauritiana*. Their chemical structures were established on the basis of spectroscopic analysis. Compounds **1** and **3** both showed antifungal activity against *Cryptococcus neoformans* and antileishmanial activity against *Leishmania donovani* *in vitro*. Compound **3** also exhibited antibacterial activity against *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRS) *in vitro*.

Graphical Abstract

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Supporting Information Available: NMR spectra and HRESIMS data for compounds **2-5** are available free of charge via the Internet at <http://pubs.acs.org>.

Marine sponges of the genus *Agelas* (order Agelasida, family Agelasidae) have proven to be an excellent source of structurally novel natural products, ranging from diterpene alkaloids¹ to bromopyrrole alkaloids² and glycosphingolipids.³ The diterpene alkaloids derived from this genus include agelines,^{1a} agelasines,^{1b-e} agelasimines⁴ and agelasidines.⁵ They and their analogs have attracted a great deal of attention for their wide range of biological activities such as antimicrobial,^{1a, 5b, 6} antimalarial,^{1d} antileukemic,^{1c} cytotoxic,^{1e, 6} and anti-fouling activities,^{1e, 7} as well as inhibitory effects on Na⁺/K⁺-ATPase.^{5a}

As part of an ongoing investigation of the chemical constituents from marine sponges collected off the Paracel Islands in the South China Sea, studies on the marine sponge *Agelas mauritiana* led to the isolation and determination of the known compound (–)-ageloxime D (**1**) and four new alkaloids (**2–5**). Herein, we report the details of the isolation and structure elucidation of the new compounds and the evaluation of their antimicrobial and antileishmanial activities.

The EtOH extract of the marine sponge *A. mauritiana* was subjected to solvent partitioning, column chromatography (on silica gel, ODS and Sephadex LH-20) and HPLC, to afford compounds **1–5**. Their structures were elucidated by MS and 1D and 2D NMR techniques including ¹H-¹H COSY, HSQC, HMBC, and NOESY. The known compound, (–)-ageloxime D (**1**), was elucidated by comparison of its NMR, MS, and specific rotation data with those in the literature.^{1e}

Compound **2** was obtained as a white amorphous solid. The similarity of the UV absorption pattern (λ_{max} 220, 269 nm, MeOH) to those of agelasines^{1d, e} suggested that compound **2** was a related metabolite. The molecular formula C₂₆H₃₉N₅O was deduced from the HRESIMS, ¹³C NMR and HSQC data. The ¹H NMR and ¹³C NMR spectra of compound **2** (Table 1) were similar to those of agelasine D. Comparison of the NMR data for compound **2** with those of agelasine D^{1b, 1e} revealed that the significant differences were apparent in the adeninium moiety. There was only one aromatic resonance at δ_{H} 8.18 in the ¹H NMR spectrum of compound **2**. Instead of the one sp² methine group (δ_{C} 142.0) in agelasine D,^{1e} one quaternary carbon was detected at δ_{C} 153.0 in the ¹³C NMR spectrum of compound **2**. These results were confirmed by the HMBC correlations from 9'-NCH₃ (δ_{H} 3.45) to C-4' (δ_{C} 148.5) and C-8' (δ_{C} 153.0), H-2' (δ_{H} 8.18) to C-4' (δ_{C} 148.5) and C-6' (δ_{C} 146.0), together with the correlation from 6'-NH₂ (δ_{H} 5.07) to C-5' (δ_{C} 106.1) (Figure 1). An extensive inspection of the ¹H NMR, ¹³C NMR, and HMBC spectra allowed the establishment of the same labdane skeleton for the diterpene moiety as is seen for agelasine D.^{1b} The diterpene moiety was connected to the N-7' atom of the adeninium unit as commonly occurs in agelasine derivatives, which was verified by the observation of the HMBC cross-peaks of H₂-15 (δ_{H} 4.59–4.68) with C-8' (δ_{C} 153.0), C-13 (δ_{C} 141.9), C-14 (δ_{C} 120.8), and C-5' (δ_{C} 106.1). Interestingly, H₂-15 (δ_{H} 4.59–4.68, 2H, dd each, J = 16.6, 5.9 Hz) appears like a quartet of doublets in the ¹H NMR spectrum due to the roof effect.⁸

The relative configuration of compound **2** was deduced from a NOESY experiment. NOE correlations were detected between CH₃-19 (δ_{H} 0.79)/CH₃-20 (δ_{H} 0.66), CH₃-20/H-11b (δ_{H} 1.43), CH₃-18 (δ_{H} 0.87)/H-5 (δ_{H} 1.04), and H-5/H-9 (δ_{H} 1.54), which were identical to those

observed for the co-isolated (–)-ageloxime D (**1**).^{1e} On the basis of the foregoing analysis, the structure of compound **2** was named as (–)-8'-oxo-agelasine D.

Compound **3** was isolated as a white amorphous solid. The molecular formula was established as C₂₆H₄₁N₅O from HRESIMS and ¹³C NMR data. Comparison of the NMR data for compound **3** with those of (–)-ageloxime D (**1**)^{1e} suggested that changes were in the diterpene moiety. The ¹H NMR and ¹³C NMR spectra demonstrated that compound **3** possessed a clerodane skeleton, which was confirmed by the HMBC correlations from the five methyl groups (CH₃-16, 17, 18, 19, and 20) to the associated carbons (Figure 1). Similarly to (–)-ageloxime D, protonation occurred at N-9' when using the CDCl₃ as a solvent which can stabilize the tautomer (imino form) of the adeninium moiety of compound **3**.^{1e} This fact was supported by the observation of a methyl proton doublet at δ_{H} 2.97 (J = 4.9 Hz).

In the NOESY spectrum, correlations between CH₃-20 (δ_{H} 0.70)/CH₃-19 (δ_{H} 0.98), CH₃-20/CH₃-17 (δ_{H} 0.77), CH₃-19/CH₃-17, H-8 (δ_{H} 1.41)/H-11b (δ_{H} 1.23), and H-10 (δ_{H} 1.29)/H₂-11a (δ_{H} 1.37) suggested the three methyl groups were on the same face of the ring system. Further comparison of ¹³C NMR data for **3** and the reported agelasine B revealed the diagnostic high field signal of CH₃-19 (δ_{C} = 19.9), confirming the *trans* ring juncture. For the *cis* isomer, the carbon chemical shift of CH₃-19 resonates at ca. 32–33 ppm.^{1b, 9} Strong NOE correlations between 6'-NOH (δ_{H} 4.78) and H₂-15 (δ_{H} 4.12–4.14) were also detected, which indicated the oxime group was *E* configuration. Therefore, compound **3** was elucidated as the oxime derivative of agelasine B which we named as ageloxime B.

Compound **4** was obtained as a light yellow oil. The molecular formula was established as C₂₃H₃₉N₃O₃S from the HRESIMS peak at m/z 438.2787 [M+H]⁺ and the ¹³C NMR data. It was determined to be a guanidine derivative by the characteristic ¹³C NMR signal (C-4', δ_{C} 157.3) and positive coloration with Sakaguchi reagents.⁵ Its ¹H NMR and ¹³C NMR spectra were similar to those of agelasidine C, except for a ketone group at δ_{C} 199.6 in **4** replacing the methylene group at C-2 (δ_{C} 28.0) in agelasidine C.⁵ The HMBC correlations from H₂-1 (δ_{H} 2.34, 2.25) to C-2, H-3 (δ_{H} 5.86) to C-1 and C-16, CH₃-16 (δ_{H} 1.92) to C-3, C-4, and C-5 suggested the presence of an α,β -unsaturated carbonyl group and the ketone group was located at C-2. High field olefinic methyl resonances at δ_{C} 16.2 (C-19) and 17.1 (C-20) indicated the 9, 10- and 13, 14-double bonds were both *E*-configuration.^{1b}

The relative configuration of compound **4** was found to be the same as in agelasidine C based on comparison of their NMR data. In the NOESY spectrum, correlations between CH₃-17 (δ_{H} 1.02)/CH₃-18 (δ_{H} 0.95) and CH₂-7 (δ_{H} 1.95, 1.67)/H-6 (δ_{H} 2.24) were observed, which revealed that the two methyl groups were co-facial. The absolute configuration of compound **4** can be assigned by comparing its circular dichroism (CD) curve with those of the known compounds. The signs of the short wavelength region (200–220 nm) of the CD spectra for this type of α,β -unsaturated cyclohexenones depend mainly on the configuration at the C-6 position of compound **4**.¹⁰ The CD spectrum of **4** showed a positive Cotton effect (ϵ +49) around 212 nm due to the overlap of Cotton effects attributed to π - π^* and n- σ^* transitions, which was opposite to the negative Cotton effect of (–)-(5*R*)-methyl-2-cyclohexenone¹⁰ in the similar spectral region. This would suggest the *S*

configuration at C-6 of **4**, but due to the more highly substituted ring, a *6R* configuration is assigned for **4**. In this case, the positive specific rotation for the *5S,6R*-compound **4**

$([\alpha]_D^{26} + 26.0, \text{ MeOH})$ correlates with the sign of rotation for (+)-*5S,6R*-agelasidine C $([\alpha]_D^{25} + 8.5, \text{ MeOH})$ and is opposite that for (–)-*5R,6S*-agelasidine C $([\alpha]_D^{29} - 5.6, \text{ MeOH})$.⁵ Accordingly, the new compound was named as (+)-2-oxo-agelasidine C.

Compound **5** was obtained as a white amorphous solid. The ESI mass spectrum showed two pseudomolecular ion peaks at m/z 273 and 275 $[\text{M}-\text{H}]^-$ in the ratio 1:1, suggesting the presence of one bromine atom in the molecule. The molecular formula of compound **5** was determined to be $\text{C}_{10}\text{H}_{15}^{79}\text{BrN}_2\text{O}_2$ by HRESIMS and ^{13}C NMR data. The ^{13}C NMR and DEPT spectra displayed 10 signals including three quaternary carbons, two methines, four methylenes (of which two were oxygenated) and one methyl. The presence of a 4-bromopyrrole-2-carboxamide moiety was indicated by the aromatic resonances at δ_{H} 6.95 (1H, br s) and 6.60 (1H, s) in the ^1H NMR spectrum and the characteristic pattern of resonances (δ_{C} 122.0, 97.1, 111.6, 125.5, and 160.2), which was similar to the values of reported bromopyrrole alkaloids.¹¹ The partial structure of C6–C13 was assigned on the basis of ^1H - ^1H COSY and HMBC spectrometric data. The connection of C10–C13 was deduced from the COSY correlations between H₂-10 (δ_{H} 3.53) and H₂-11 (δ_{H} 1.56), H₂-11 and H₂-12 (δ_{H} 1.35), and H₂-12 and CH₃-13 (δ_{H} 0.91). The HMBC correlations from H₂-8 (δ_{H} 4.88) to C-6 (δ_{C} 160.2) and C-10 (δ_{C} 68.5) and from H-4 (δ_{H} 6.60) to C-5 (δ_{C} 125.5) completed the assignment of the structure of compound **5** as depicted. Therefore, compound **5** was elucidated as 4-bromo-*N*-(butoxymethyl)-1*H*-pyrrole-2-carboxamide.

Compounds **1–4** were assessed for antimicrobial activity against 10 organisms. The agelasine oxime derivatives (**1** and **3**) both showed activity against *C. neoformans* with $\text{IC}_{50}/\text{MIC}$ values of 5.94/10.00 and 4.96/10.00 $\mu\text{g}/\text{mL}$, respectively. Compound **3** also exhibited antibacterial activity against *S. aureus* ($\text{IC}_{50}/\text{MIC} = 7.21/10.00 \mu\text{g}/\text{mL}$) and MRS ($\text{IC}_{50}/\text{MIC} = 9.20/20.00 \mu\text{g}/\text{mL}$). The antileishmanial activity of compounds **1–4** was also tested *in vitro*. Only compounds **1** and **3** exhibited antileishmanial activity against *Leishmania donovani* with $\text{IC}_{50}/\text{IC}_{90}$ values of 29.28/33.96 and 28.55/33.19 $\mu\text{g}/\text{mL}$, respectively.

Experimental Section

General Experimental Procedures

Optical rotation data were obtained on a JASCO P-1030 polarimeter. CD spectrum was obtained on a JASCO J-715 spectropolarimeter. UV spectra were acquired using a Shimadzu UV-240 spectrophotometer (Shimadzu Corp., Kyoto, Japan). NMR experiments were performed on Bruker AVANCE-500 spectrometers. HRESIMS and ESIMS spectra were acquired using a Q-ToF micro YA019 mass spectrometer. HPLC purifications were carried out on a Waters 1525/2996 liquid chromatography. Column chromatography (CC) was performed on Sephadex LH-20 (Pharmacia) and YMC ODS-A (50 μm). Fractions were

monitored by TLC (HSGF 254, Yantai, China) and spots were visualized by heating silica gel plates sprayed with 10% H₂SO₄ in EtOH.

Animal Material

The specimen of *Agelas mauritiana* was collected around Yongxing Island and Seven Connected Islets in the South China Sea in June, 2007. The sponge was identified by Prof. Jin-He Li (Institute of Oceanology, Chinese Academy of Sciences, P. R. China). A voucher sample (No. JNF07) was deposited in the Laboratory of Marine Drugs, Department of Pharmacy, Changzheng Hospital, Second Military Medical University, Shanghai, P. R. China.

Extraction and Isolation

The sponge (6.7 kg, wet weight) was extracted with 95% EtOH at room temperature. The EtOH extract was suspended in H₂O and extracted with EtOAc. The EtOAc-soluble extract was partitioned between MeOH-H₂O (9:1) and petroleum ether. The MeOH-H₂O (9:1) phase was diluted to 3:2 with H₂O and extracted with CH₂Cl₂ to afford the CH₂Cl₂-soluble extract (100.6 g). This CH₂Cl₂-soluble extract was subjected to VLC on silica gel using CH₂Cl₂/MeOH (50:1, 20:1, 10:1, 0:100) as eluent to give four fractions (A–D). Fraction A (23.3 g) was chromatographed on a Sephadex LH-20 column with CH₂Cl₂/MeOH (1:1) as eluting solvent to afford five fractions (A1–A5). Fraction A4 (6.4 g) was subjected to column chromatography (CC) on YMC ODS-A (50 μ m) using MeOH/H₂O (1:1–1:0) to give 13 fractions (A401–A413). Fraction A411 (596.7 mg) was separated by repeated CC on silica gel and followed by HPLC (SunFire Silica, 5 μ m, 10 $\times$ 250 mm, 2 mL/min, UV detection at 220 and 269 nm) using *n*-hexane/2-propanol (82:18) as eluent to yield compound **2** (15.6 mg). Fraction A410 (1.84 g) was subjected to chromatography repeatedly on silica gel and purified by HPLC (YMC Pack B C-18, 5 μ m, 10 $\times$ 250 mm, 1.5 mL/min, UV detection at 225 and 259 nm), eluting with CH₃CN/H₂O (54:46) to afford compound **1** (16.2 mg) and **3** (12.7 mg). The separation of fraction A405 (109.0 mg) was performed by using HPLC (YMC Pack B C-18, 5 μ m, 10 $\times$ 250 mm, 1.5 mL/min, UV detection at 220 nm), eluting with CH₃CN/H₂O (30:70) to obtain compound **5** (1.2 mg). Similarly, fraction B (14.4 g) was subjected to CC on silica gel repeatedly and further purified by HPLC (YMC Pack B C-18, 5 μ m, 10 $\times$ 250 mm, 1.5 mL/min, UV detection at 240 nm) with CH₃CN/H₂O (25:75) as the elute, to yield compound **4** (5.8 mg).

(–)-8'-oxo-agelasine D (**2**)

white amorphous solid; $[\alpha]_D^{26} - 18$ (c 0.05, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 269 (3.47) nm; ¹H NMR (CDCl₃, 500 MHz) and ¹³C NMR (CDCl₃, 125 MHz) data, see Table 1; HRESIMS m/z 438.3235 [M+H]⁺ (calcd for C₂₆H₄₀N₅O, 438.3233).

(–)-Ageloxime B (**3**)

white amorphous solid; $[\alpha]_D^{26} - 110$ (c 0.05, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 259 (2.31) nm; ¹H NMR (CDCl₃, 500 MHz) and ¹³C NMR (CDCl₃, 125 MHz) data, see Table 1; HRESIMS m/z 440.3392 [M+H]⁺ (calcd for C₂₆H₄₂N₅O, 440.3389).

(+)-2-oxo-agelasidine C (4)

light yellow oil; $[\alpha]_D^{26} +26$ (c 0.1, $MeOH$); UV (MeOH) λ_{max} (log ϵ) 240 (3.18) nm; CD (6.86×10^{-4} M, EtOH) λ_{max} (ϵ) 212 (+49), 247 (−1.37), 317 (−0.07) nm; 1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) data, see Table 1; HRESIMS m/z 438.2787 $[M+H]^+$ (calcd for $C_{23}H_{40}N_3O_3S$, 438.2790).

4-bromo-N-(butoxymethyl)-1H-pyrrole-2-carboxamide (5)

white amorphous solid; UV (MeOH) λ_{max} (log ϵ) 220 (3.05) nm; 1H NMR ($CDCl_3$, 500 MHz) δ_H 6.95 (1H, br s, H-2), 6.60 (1H, s, H-4), 4.88 (2H, d, J = 7.2 Hz, H₂-8), 3.53 (2H, t, J = 6.5 Hz, H₂-10), 1.56 (2H, m, H₂-11), 1.35 (2H, m, H₂-12), 0.91 (3H, t, J = 7.0 Hz, H-13); ^{13}C NMR ($CDCl_3$, 125 MHz) δ_C 160.2 (C, C-6), 125.5 (C, C-5), 122.0 (CH, C-2), 111.6 (CH, C-4), 97.1 (C, C-3), 70.1 (CH₂, C-8), 68.5 (CH₂, C-10), 31.7 (CH₂, C-11), 19.2 (CH₂, C-12), 13.9 (CH₃, C-13); HRESIMS m/z 297.0214 $[M+Na]^+$ (calcd for $C_{10}H_{15}^{79}BrN_2O_2Na$, 297.0215) and 299.0200 $[M+Na]^+$ (calcd for $C_{10}H_{15}^{81}BrN_2O_2Na$, 299.0194).

Antimicrobial Assays

All organisms were obtained from the American Type Culture Collection (Manassas, VA), including the fungi *Candida albicans* ATCC 90028, *Candida glabrata* ATCC 90030, *Candida krusei* ATCC 6258, *Cryptococcus neo formans* ATCC 90113, and *Aspergillus fumigatus* ATCC 204305 and the bacteria *Staphylococcus aureus* ATCC 29213, methicillin-resistant *S. aureus* ATCC 33591 (MRS), *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, and *Mycobacterium intracellulare* ATCC 23068. All organisms were tested using modified versions of the CLSI (formerly NCCLS) methods as described previously.¹² The control drugs ciprofloxacin for bacteria and amphotericin B for fungi were included in each assay.

Antileishmanial Assay

Antileishmanial activities of the compounds were assessed *in vitro* against a culture of *Leishmania donovani* promastigotes. In a 96 well microplate assay, compounds with appropriate dilution were added to the leishmania promastigotes culture (2×10^6 cell/mL). The plates were incubated at 26 °C for 72 h and growth of the leishmania promastigotes was determined by Alamar blue assay.¹³ Pentamidine and amphotericin B were used as the standard antileishmanial drugs. IC₅₀ and IC₉₀ values for each compound were computed from the growth inhibition curve.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

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Figure 1.
Key HMBC correlations for compounds **2** and **3**.

Table 1¹H (500 MHz) and ¹³C NMR (125 MHz) Data for **2-4** in CDCl₃

position	2			3			4		
	δ_C , mult.	δ_H , mult. (J in Hz)	HMBC (H → C)	δ_C	δ_H	HMBC (H → C)	δ_C	δ_H	HMBC (H → C)
1	39.1, CH ₂	1.71, br d (11.9) 0.96, br d (11.4)	2,9,10,20	18.3, CH ₂	1.48, m 1.42, m	2,3,9	42.0, CH ₂	2.34, dd (13.8, 10.1) 2.25, ov ^a	2,5,6
2	19.3, CH ₂	1.54, m 1.44, m	1,10	26.9, CH ₂	2.00, ov ^a 2.02, ov ^a		199.6, C		
3	42.1, CH ₂	1.38, m 1.15, br t (13.3)	4,5,18,19	120.4, CH	5.18, br s	4,18	128.1, CH	5.86, s	5,16
4	33.5, C			144.5, C			169.4, C		
5	55.5, CH	1.04, br d (12.3)	4,6,7,9,10,19,20	38.2, C			42.3, C		
6	24.4, CH ₂	1.71, br d (12.6) 1.30, dd (13.1, 4.1)	5,7,8,10	36.8, CH ₂	1.70, br d (12.6) 1.14, m	10,19	33.7, CH	2.24, ov ^a	7
7	38.3, CH ₂	2.36, br d (12.1) 1.90, m	5,6,8,9,17	27.4, CH ₂	1.40, m 1.40, m	8	34.3, CH ₂	1.95, m 1.67, ov ^a	
8	148.4, C			36.3, CH	1.41, m	7,10	35.1, CH ₂	1.65, ov ^a	7,9,10
9	56.2, CH	1.54, m	5,7,8,10,12,17,20	38.6, C			135.6, C		
10	39.6, C			46.4, CH	1.29, br d (11.8)	2,9,19,20	123.6, CH	5.09, br s	11
11	21.6, CH ₂	1.60, m 1.43, m	9,12	36.7, CH ₂	1.37, m 1.23, m	8,10	26.1, CH ₂	2.12, br s	9,12,13
12	38.3, CH ₂	2.20, br t (12.3) 1.88, m	11,16	32.9, CH ₂	1.80, m 1.80, m	11,13,14	39.7, CH ₂	2.12, br s	10,11,14
13	141.9, C			144.3, C			147.4, C		
14	120.8, CH	5.32, br t (5.2)	12,15,16	117.1, CH	5.33, t (7.7)	12,15,16	109.1, CH	5.25, t (7.2)	15,20
15	40.2, CH ₂	4.65, dd (16.6, 5.9) 4.61, dd (16.6, 5.9)	13,14,5',8'	41.5, CH ₂	4.14, br d (3.8) 4.12, br d (4.2)	13,14,5'	53.9, CH ₂	3.92, br s	
16	16.8, CH ₃	1.81, s	12,13,14	16.5, CH ₃	1.61, s	12,13,14	20.4, CH ₃	1.92, s	3,4,5
17	106.2, CH ₂	4.80, s 4.45, s	7,8,9	16.0, CH ₃	0.77, br s	7,8,9	19.7, CH ₃	1.02, s	4,5,6,7

position	2			3			4		
	δ_C , mult.	δ_H , mult. (<i>J</i> in Hz)	HMBC (H $\rightarrow$ C)	δ_C	δ_H	HMBC (H $\rightarrow$ C)	δ_C	δ_H	HMBC (H $\rightarrow$ C)
18	33.5, CH ₃	0.87, s	3,4,5,19	18.0, CH ₃	1.58, s	3,4,5	15.5, CH ₃	0.95, d (5.9)	1,5,6
19	21.7, CH ₃	0.79, s	3,4,5,18	19.9, CH ₃	0.98, s	4,5,10	16.2, CH ₃	1.61, s	8,9,10
20	14.5, CH ₃	0.66, s	5,9,10	18.4, CH ₃	0.70, s	8,9,10,11	17.1, CH ₃	1.75, s	12,13,14
1'							50.7, CH ₂	3.41, br s	
2'	151.3, CH	8.18, s	4',6'	157.6, CH	8.15, s	4'	35.1, CH ₂	3.82, br s	
3'									
4'	148.5, C			160.6, C			157.3, C		
5'	106.1, C			99.6, C					
6'	146.0, C			159.8, C					
8'	153.0, C			164.5, CH	7.97, s	15			
9'-NMe	26.4, CH ₃	3.45, s	4',8'	28.0, CH ₃	2.97, d (4.9)	4'			
6'-NH ₂		5.07, br s	5'						
1'-NH					4.78, ov ^a	5'			
6'-NOH					4.78, ov ^a				

^a ov = overlapped by other signals.