Pyrinodemins B—D, Potent Cytotoxic *bis*-Pyridine Alkaloids from Marine Sponge *Amphimedon* sp.

Keiko HIRANO,[a] Takaaki KUBOTA,[a] Masashi TSUDA,[a] Yuzuru MIKAMI,[b] and Jun'ichi KOBAYASHI*,[a]

Graduate School of Pharmaceutical Sciences, Hokkaido University,[a] Sapporo 060–0812, Japan and Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University,[b] Chiba 260–8673, Japan.
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New *bis*-pyridine alkaloids, pyrinodemins B—D (1—3), have been isolated together with pyrinodemin A (4) and related 3-alkyl pyridine alkaloids 5—8 from the Okinawan marine sponge *Amphimedon* sp. and the structures were elucidated from spectroscopic data. Pyrinodemins B—D (1—3) showed potent cytotoxicity, while compounds 5—8 exhibited antimicrobial activity.

Key words sponge; *Amphimedon* sp.; *bis*-pyridine alkaloids; cytotoxicity; antimicrobial activity

A number of 3-alkyl pyridine alkaloids have been isolated from marine sponges of several genera.[1)] Almost of them possessed a long aliphatic chain with a various nitrogen-containing terminus,[2—7)] and some had dimeric or polymeric structures of the 3-alkyl pyridine.[8—11)] During our search for bioactive metabolites from Okinawan marine sponges,[12,13)] we previously isolated cytotoxic pyridine alkaloids from sponges of the genera *Theonella*[4)] and *Nyphates*.[14—16)] More recently, potent cytotoxic *bis*-pyridine alkaloids with a unique *cis*-cyclopent[*c*]isoxazolidine moiety, pyrinodemins B—D (**1**—**3**), have been isolated together with pyrinodemin A[17)] (**4**) and its related 3-alkyl pyridine alkaloids **5**—**8** from the Okinawan marine sponge *Amphimedon* sp. Here we describe the isolation and structure elucidation of **1**—**3** and **5**—**8**, and potent cytotoxicity of **1**—**3** against tumor cell lines as well as antimicrobial activity of **5**—**8**.

The sponge *Amphimedon* sp. (SS-955) was collected off Nakijin, Okinawa, and extracted with MeOH. EtOAc-soluble materials of the MeOH extract were subjected to silica gel columns ($CHCl_3$–MeOH and then hexane–EtOAc) followed by reversed-phase HPLC on 6-(phenyl)hexylsilyl (MeOH–H_2O or CH_3CN–H_2O) to afford pyrinodemins B (**1**, 0.00009%, wet weight), C (**2**, 0.00005%), and D (**3**, 0.00004%), and compounds **5** (0.0005%), **6** (0.0004%), **7** (0.0004%), and **8** (0.0003%), together with pyrinodemin A (**4**, 0.00011%).

High resolution (HR) FAB-MS data (*m/z* 562.4758 [M+H]$^+$, Δ +2.2 mmu) of pyrinodemin B[18)] (**1**) established the molecular formula, $C_{38}H_{59}N_3O$. ^{1}H- and ^{13}C-NMR data indicated that **1** was an analogue of pyrinodemin A (**4**). The ^{13}C-NMR spectrum revealed five sp^2 carbon signals [C-2 and C-2′ δ_C 146.5 (2C, d); C-3 and C-3′, δ_C 137.5 (2C, s); C-4 and C-4′, δ_C 122.5 (2C, d); C-5 and C-5′, δ_C 135.4 (2C, d); C-6 and C-6′, δ_C 150.0 (2C, d)] due to two pyridine rings, sp^3 carbon signals due to three methines (C-15, δ_C 77.2; C-16, δ_C 49.3; C-20, δ_C 72.2), one methylene (C-19′, δ_C 57.3) at relatively lower field, and methylenes in a long alkyl chain (δ_C 26—34). Aromatic proton signals [H-2 and H-2′, δ_H 8.42 (2H); H-4 and H-4′, δ_H 7.48 (2H); H-5 and H-5′, δ_H 7.19 (2H); H-6 and H-6′, δ_H 8.44 (2H)] in the ^{1}H-NMR spectrum suggested the presence of two 3-alkyl-substituted pyridine rings. Proton and carbon chemical shifts of three methines at C-15 (δ_H 4.05; δ_C 77.2, d), C-16 (δ_H 2.83; δ_H 49.3, d), and C-20 (δ_H 3.46; δ_C 72.2, d) corresponded well to those of **4**, suggesting the presence of an isoxazolidine ring. The presence of a cyclopent[*c*]isoxazolidine moiety was deduced from the intense fragment ion peak at *m/z* 270 ([$C_{19}H_{28}N$]$^+$) in the electron impact mass spectrum (EI-MS), which might be generated from **1** through Hoffmann-like elimination of the isoxazolidine ring.[19)] Detailed analysis of the EI-MS fragmentation pattern (Fig. 1) suggested the presence of the two alkyl chains from C-7 to C-14 and from C-7′ to C-19′. In the ^{1}H-NMR spectrum of **4**, two olefin proton signals (H-16 and H-17) were observed at δ 5.34 (2H), while such olefin signals were not observed for **1**. The *cis*-ring junction of the bicyclic system was deduced from the nuclear Overhauser effect spectroscopy (NOESY) correlation for H-16/H-20. NOESY correlations of H-15/H-16 and H-15/H-20 indicated that the relative stereochemistry of H-15 and H-16 was *cis*. Therefore the structure of pyrinodemin B was concluded to be **1**.

Pyrinodemins C (**2**) and D (**3**) were revealed to have the molecular formulae, $C_{37}H_{57}N_3O$ and $C_{36}H_{57}N_3O$, respectively, by the HR-FAB-MS data. The structures of **2** and **3** were elucidated to be analogues lacking one of CH_2 units from C-7′ to C-16′ in the alkyl side chain of pyrinodemin A (**4**) and from C-7′ to C-19′ in that of pyrinodemin B (**1**), respectively, by analyses of ^{1}H-NMR and EI-MS data. The position of the disubstituted olefin in **2** was assigned to C-15′ on the basis of EI-MS fragment ions at *m/z* 190 [($C_{14}H_{22}N$)$^+$] and 217 [($C_{16}H_{25}N$)$^+$], and the *Z*-geometry of the olefin was implied by the chemical shifts of the allylic carbons (C-14′ and C-17′, δ_C *ca.* 27),[20)] which were deduced from HMQC cross-peaks.

Compound **5** was shown to have the molecular formula, $C_{19}H_{30}N_2O$, by HREIMS (*m/z* 302.2352 [M]$^+$, Δ −0.6 mmu). The ^{1}H- and ^{13}C-NMR spectra suggested that **5** was a monomer 3-alkyl pyridine alkaloid with a disubstituted double bond. In the ^{1}H- and ^{13}C-NMR spectra, a pair of sp^2 methine signals [δ_H 6.71 (0.4H, t, H-20) and 7.43 (0.6H, t, H-20); δ_C 152.3 (0.6C, d, C-20) and 152.8 (0.4C, d, C-20)] were observed, corresponding to *E* and *Z*-type oxyimino groups.[14)] The ratio of *E* and *Z*-forms was estimated to be 3 : 2 by the intensity of the imino proton signals. The EI-MS fragment ions at *m/z* 285 and 244 indicated the presence of an oxime terminus (Fig. 2). The position of the double bond was determined to be C-9–C-10 by ^{1}H–^{1}H correlation spectroscopy (COSY) cross-peaks for H_2-7/H_2-8 and H_2-8/H-9

* To whom correspondence should be addressed. e-mail: jkobay@pharm.hokudai.ac.jp

and EI-MS fragment ion peaks at *m*/*z* 106 and 132. The *Z*-geometry of the double bond was deduced from the ^{13}C chemical shifts for the allylic methylene carbons (C-8, δ_C 28.8; C-11, δ_C 27.1).[20] Thus compound **5** was assigned as a 3-alkyl (C_{14}) pyridine with *E* and *Z*-forms (3 : 2) at the oxime terminus.

The molecular formula, $C_{19}H_{30}N_2O$, of compound **6** was established by HR-EI-MS (*m*/*z* 302.2362 $[M]^+$, Δ +0.4 mmu). ^1H-NMR data revealed a 3-alkyl pyridine moiety, a disubstituted olefin, and an oxime terminus consisting of a 3 : 2 mixture of *E*- and *Z*-forms. The position of the olefin was inferred as C-15–C-16 by EI-MS fragment ion peaks at *m*/*z* 190 and 216 (Fig. 2). This was also supported by EI-MS fragment ions at *m*/*z* 190, 205, and 220 observed for the reduction product (**9**) of **6** with D_2. The carbon chemical shifts of C-14 and C-17 (δ_C 29.2 and 30.5, respectively) of **6** were indicative of 15*Z*-geometry.[20] Thus compound **6** was elucidated to be a $\Delta^{15(16)}$ analogue of **5**.

Compounds **7** and **8** were revealed to possess the molecular formulae, $C_{18}H_{30}N_2O$ and $C_{17}H_{28}N_2O$, respectively, by

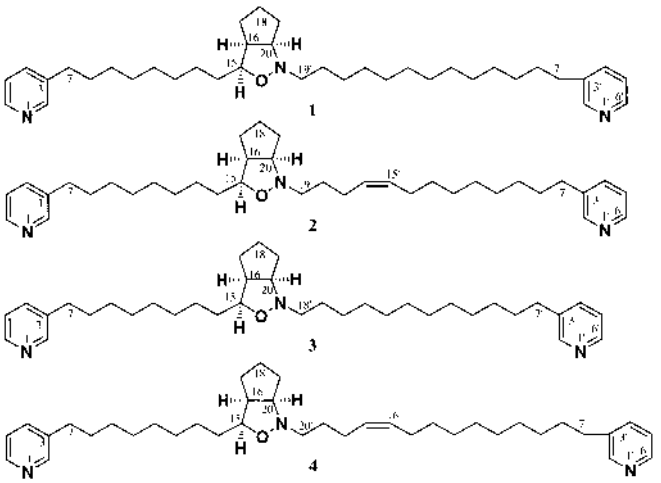

Chart 1. Structures of **1**—**4**

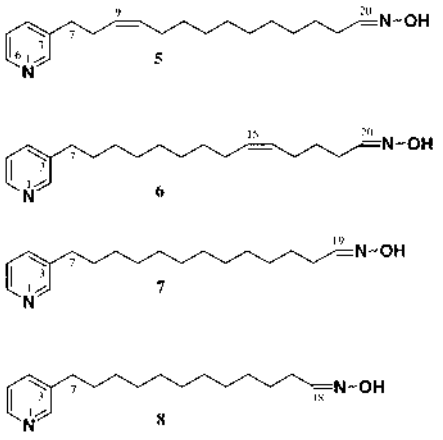

Chart 2. Structures of **5**—**8**

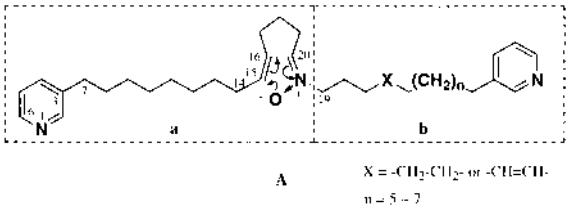

Chart 3. Structure of A

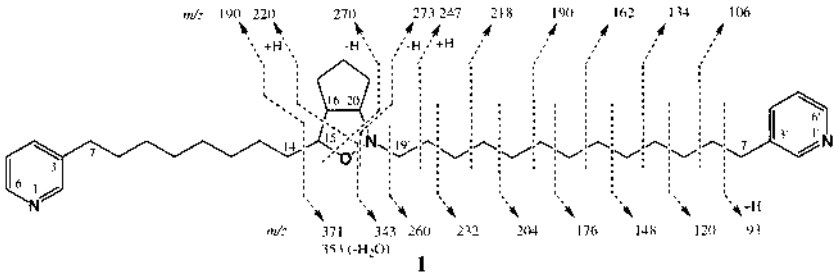

Fig. 1. Fragmentation Pattern of Pyrinodemin B (**1**) in EI-MS (Parent Ion; *m*/*z* 561 $[M]^+$)

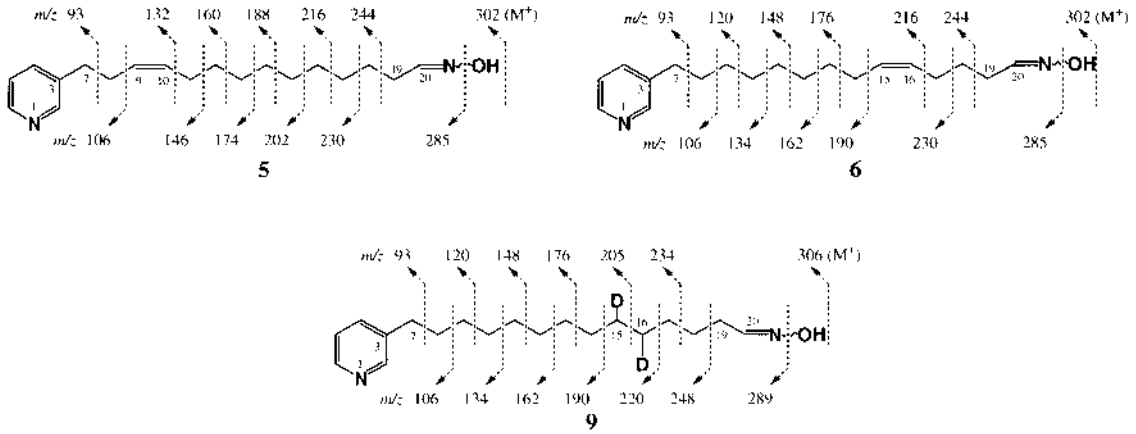

Fig. 2. Fragmentation Patterns of Compounds **5** and **6** and Reduction Product (**9**) of **6** in EI-MS

Table 1. Antimicrobial Activity of Pyrinodemin A (**4**) and Compounds **5**—**8**

Test organisms	MIC (μg/ml)				
	4	**5**	**6**	**7**	**8**
Candida albicans ATCC 90028	>33	>33	16	16	33
Cryptococcus neoformans ATCC 900112	33	33	16	16	16
Aspergillus niger ATCC 40406	33	>33	8	4	8
Paecilomyces variotii YM-1	33	33	16	16	16
Trichophyton mentagrophytes ATCC 40769	>33	>33	>33	>33	16
Staphyloccus aureus 209P	>33	8	4	2	16
Micrococcus luteus IFM 2066	>33	16	16	8	16
Bacillus subtilis PCI 189	>33	33	>33	8	16
Corynebacterium xerosis IFM 2057	>33	33	4	4	8
Escherichia coli NIJ JC2	>33	>33	>33	>33	>33

Mueller Hinton broth and Sabouraud dextrose broth were used for bacteria and fungi, respectively.

HR-EI-MS. ^{1}H-NMR data of **7** and **8** indicated that both have a 3-alkyl pyridine moiety and an oxime terminus consisting of a 3 : 2 mixture of *E*- and *Z*-forms. The ^{1}H-NMR spectra of compounds **7** and **8** showed that both had no internal double bond. Analyses of EI-MS fragmentation patterns of **7** and **8** implied the presence of the saturated linear C_{12} and C_{11} alkyl chains, respectively, between the pyridine ring and the oxime group. Compounds **7** and **8** were thus identified as saturated analogues of compound **5** (or **6**), respectively.

Pyrinodemins B—D (**1**—**3**) are unique *bis*-3-alkylpyridine alkaloids with a *cis*-cyclopent[*c*]isoxazolidine moiety like pyrinodemin A[17] (**4**). Biosynthetically, the cyclopent[*c*]isoxazolidine moiety in pyrinodemins may be generated from a linear *bis*-pyridine such as A through 1,3-dipolar cycloaddition. Furthermore, the structure of unit **a** in A corresponds to that of compound **6**, while the structure of unit **b** in A corresponds to that of **6**—**8**, or their homologues without the N–OH unit. Pyrinodemins B—D (**1**—**3**) exhibited potent cytotoxicity against murine leukemia L1210 (IC_{50}, 0.07, 0.06, and 0.08 μg/ml, respectively) and KB epidermoid carcinoma cells (IC_{50}, 0.5 μg/ml, each) *in vitro*, comparable to pyrinodemin A[17] (**4**), whereas compounds **5**—**8** did not show cytotoxicity (IC_{50}>10 μg/ml). On the other hand, compounds **5**—**8** exhibited antimicrobial activity against some fungi and Gram-positive bacteria (Table 1), and pyrinodemin A (**4**) showed relatively weak antifungal activity.

Experimental

IR and UV spectra were recorded on JASCO FT/IR-5300 and JASCO Ubest-35 spectrophotometers, respectively. ^{1}H- and ^{13}C-NMR spectra were recorded on Bruker AMX-600 and ARX-500 spectrometers, respectively. EI-MS were obtained on a JEOL DX-303 spectrometer at 70 eV, and positive ion FAB-MS were measured on a JEOL HX-110 spectrometer using glycerol as a matrix.

Extraction and Isolation The sponge *Amphimedon* sp. (1.0 kg, wet weight) collected off Okinawa Island was extracted with MeOH (1 l×2). The methanolic extract (71 g) was partitioned between EtOAc (400 ml×3) and 1 M NaCl solution. Parts (1.5 g) of the EtOAc soluble materials (5.11 g) were subjected to a silica gel column ($CHCl_3$–MeOH, 100 : 0→95 : 5). The fraction eluting with 98% $CHCl_3$–MeOH was subjected to silica gel column chromatography (hexane–EtOAc, 1 : 2) and reversed-phase HPLC (Luna Phenyl-hexyl, 5 μm, Phenomenex®, 4.6×250 mm; eluent: MeOH–H_2O, 91 : 9; flow rate: 1 ml/min; UV detection at 264 nm) to afford pyrinodemins B (**1**, 0.00009%, wet weight, t_R 28 min), C (**2**, 0.00005%, t_R 26 min), and D (**3**, 0.00004%, t_R 24 min) together with pyrinodemin A (**4**, 0.00011%, t_R 31 min). The fraction eluting with 95% $CHCl_3$–MeOH was separated on a silica gel column (hexane–EtOAc, 1 : 1) and then reversed-phase HPLC (Luna Phenyl-hexyl; eluent: CH_3CN–H_2O, 45 : 55; flow rate: 1 ml/min; UV detection at 264 nm) to yield compounds **5** (0.0005%, t_R 42 min), **6** (0.0004%, t_R 38 min), **7** (0.0004%, t_R 35 min), and **8** (0.0003%, t_R 28 min).

Pyrinodemin B (**1**): UV λ_{max} (MeOH) nm (ε): 264 (6000). IR (neat) cm^{-1}: 1575. ^{1}H-NMR ($CDCl_3$) δ: 1.2—1.3 (24H), 1.33 (4H, m, H_2-9 and H_2-9′), 1.40 (1H, m, H-17), 1.42 (1H, m, H-18), 1.44 (1H, m, H-14), 1.50 (2H, m, H_2-18′), 1.55 (1H, m, H-14), 1.60 (4H, m, H_2-8 and H_2-8′), 1.64 (1H, m, H-17), 1.65 (1H, m, H-19), 1.66 (1H, m, H-18), 1.76 (1H, m, H-19), 2.59 (1H, m, H-19′), 2.60 (4H, t, J=7.6 Hz, H_2-7 and H_2-7′), 2.83 (1H, m, H-16), 2.84 (1H, m, H-19′), 3.46 (1H, m, H-20), 4.05 (1H, m, H-15), 7.19 (2H, br t, J=7.0 Hz, H-5 and H-5′), 7.48 (2H, d, J=7.5 Hz, H-4 and H-4′), 8.42 (2H, br s, H-2 and H-2′), 8.44 (2H, m, H-6 and H-6′). ^{13}C-NMR ($CDCl_3$) δ: 26.3 (t), 26.5 (t), 27.9 (t), 28.9 (2C, t), 29.2 (2C, t), 29.4—29.8 (12C, t), 31.2 (2C, t), 33.0 (2C, t), 33.6 (t), 49.3 (d), 57.3 (t), 72.2 (d), 77.2 (d), 122.5 (2C, d), 135.4 (2C, d), 137.5 (2C, s), 146.5 (2C, d), 150.0 (2C, d). FAB-MS *m/z*: 562 $[M+H]^+$. HR-FAB-MS *m/z*: 562.4758 (Calcd for $C_{37}H_{60}N_3O$ $[M+H]^+$: 562.4736).

Pyrinodemin C (**2**): UV λ_{max} (MeOH) nm (ε): 263 (5800). IR (neat) cm^{-1}: 1575. ^{1}H-NMR ($CDCl_3$) δ: 1.2—1.3 (16H), 1.33 (4H, m), 1.40 (1H, m), 1.42 (1H, m), 1.44 (1H, m), 1.55 (1H, m), 1.58 (2H, m), 1.60 (4H, m), 1.64 (1H, m), 1.65 (1H, m), 1.66 (1H, m), 1.76 (1H, m), 2.03 (4H, m), 2.59 (1H, m), 2.60 (4H, t, J=7.6 Hz), 2.82 (1H, m), 2.84 (1H, m), 3.46 (1H, m), 4.05 (1H, m), 5.34 (2H, m), 7.19 (2H, br t, J=7.0 Hz), 7.48 (2H, d, J=7.5 Hz), 8.42 (2H, br s), 8.44 (2H, m). EI-MS *m/z* (rel. int. %): 93 (100), 106 (98), 120 (11), 134 (7), 148 (8), 162 (12), 176 (18), 190 (8), 217 (5), 220 (26), 230 (4), 270 (5), 271 (18), 301 (9), 315 (5), 339 (2), 351 (36), 369 (5), 541 (2), 559 ($[M]^+$, 2). FAB-MS *m/z*: 560 $[M+H]^+$. HR-FAB-MS *m/z*: 560.4558 (Calcd for $C_{37}H_{58}N_3O$ $[M+H]^+$: 560.4579).

Pyrinodemin D (**3**): UV λ_{max} (MeOH) nm (ε): 264 (6200). IR (neat) cm^{-1}: 1575. ^{1}H-NMR ($CDCl_3$) δ: 1.2—1.3 (22H), 1.33 (4H, m), 1.40 (1H, m), 1.42 (1H, m), 1.44 (1H, m), 1.50 (2H, m), 1.55 (1H, m), 1.60 (4H, m), 1.64 (1H, m), 1.65 (1H, m), 1.66 (1H, m), 1.76 (1H, m), 2.59 (1H, m), 2.60 (4H, t, J=7.6 Hz), 2.83 (1H, m), 2.84 (1H, m), 3.46 (1H, m), 4.05 (1H, m), 7.19 (2H, brt, J=7.0 Hz), 7.48 (2H, d, J=7.5 Hz), 8.42 (2H, brs), 8.44 (2H, m). EI-MS *m/z* (rel. int. %): 93 (100), 106 (93), 120 (11), 134 (7), 148 (8), 162 (12), 176 (18), 190 (10), 204 (6), 218 (12), 220 (26), 233 (8), 259 (20), 270 (4), 301 (10), 315 (6), 329 (4), 339 (43), 357 (3), 529 (2) 547 ($[M]^+$, 1). FAB-MS *m/z*: 548 $[M+H]^+$. HR-FAB-MS *m/z*: 548.4559 (Calcd for $C_{36}H_{58}N_3O$ $[M+H]^+$: 548.4580).

Compound **5**: UV λ_{max} (MeOH) nm (ε): 264 (3300). IR (neat) cm^{-1}: 3200, 2925, 1575. ^{1}H-NMR ($CDCl_3$) δ: 1.2—1.3 (12H), 1.45 (2H, m, H_2-18), 1.92 (2H, m, H_2-11), 2.19 (1.2H, m, H_2-19), 2.36 (2.8H, m, H_2-8 and H_2-19), 2.66 (2H, t, J=7.6 Hz, H_2-7), 5.37 (2H, m, H-9 and H-10), 6.71 (0.4H, t, J=5.1 Hz, H-20), 7.21 (1H, t, J=5.6 Hz, H-5), 7.43 (0.6H, t, J=6.0 Hz, H-20), 7.49 (1H, d, J=5.6 Hz, H-4), 8.47 (2H, m, H-2 and H-6). ^{13}C-NMR ($CDCl_3$) δ: 24.4 (0.4C, t, C-19), 27.1 (t, C-11), 28.8 (t, C-8), 28.9 (t), 29.1 (0.6C, t, C-19), 29.4—29.8 (5C, t), 33.1 (t, C-7), 123.2 (d, C-5), 127.7 (d, C-9), 131.5 (d, C-10), 136.1 (d, C-4), 137.5 (s, C-3), 147.2 (d, C-2), 149.9 (d, C-6), 152.3 (0.6C, d, C-20), 152.8 (0.4C, d, C-20). HR-EI-MS *m/z*: 302.2352 (Calcd for $C_{19}H_{30}N_2O$ $[M]^+$: 302.2358).

Compound **6**: UV λ_{max} (MeOH) nm (ε): 264 (3200). IR (neat) cm^{-1}: 3200, 2925, 1575. ^{1}H-NMR ($CDCl_3$) δ: 1.2—1.3 (10H), 1.45 (2H, m), 1.55 (2H, m), 2.02 (2H, m), 2.10 (2H, m), 2.19 (1.2H, m), 2.38 (0.8H, m), 2.65 (2H, t, J=7.6 Hz), 5.37 (1H, m), 5.40 (1H, m), 6.71 (0.4H, t, J=5.1 Hz), 7.21 (1H, t, J=5.6 Hz), 7.43 (0.6H, t, J=6.0 Hz), 7.49 (1H, d, J=5.6 Hz), 8.47 (2H, m). HR-EI-MS *m/z*: 302.2362 (Calcd for $C_{19}H_{30}N_2O$ $[M]^+$: 302.2358).

Compound **7**: UV λ_{max} (MeOH) nm (ε): 264 (3100). IR (neat) cm^{-1}: 3200, 2925, 1575. ^{1}H-NMR ($CDCl_3$) δ: 1.2—1.3 (16H), 1.45 (2H, m), 1.55 (2H, m), 2.19 (1.2H, m), 2.38 (0.8H, m), 2.65 (2H, t, J=7.6 Hz), 6.71 (0.4H, t, J=5.1 Hz), 7.21 (1H, t, J=5.6 Hz), 7.43 (0.6H, t, J=6.0 Hz), 7.49 (1H, d, J=5.6 Hz), 8.47 (2H, m). EI-MS *m*/*z* (rel. int. %): 93 (100), 106 (93), 120 (28), 134 (12), 148 (18), 162 (29), 176 (33), 190 (16), 204 (22), 218 (24), 232 (78), 273 (9), 290 ([M]$^+$, 4). HR-EI-MS *m*/*z*: 290.2337 (Calcd for $C_{18}H_{30}N_2O$ [M]$^+$: 290.2358).

Compound **8**: UV λ_{max} (MeOH) nm (ε): 264 (3200). IR (neat) cm^{-1}: 3200, 2925, 1575. ^{1}H-NMR ($CDCl_3$) δ: 1.2—1.3 (14H), 1.45 (2H, m), 1.55 (2H, m), 2.19 (1.2H, m, H_2-17), 2.38 (0.8H, m), 2.65 (2H, t, J=7.6 Hz), 6.71 (0.4H, t, J=5.1 Hz), 7.21 (1H, t, J=5.6 Hz), 7.43 (0.6H, t, J=6.0 Hz), 7.49 (1H, d, J=5.6 Hz), 8.47 (2H, m). EI-MS *m*/*z* (rel. int. %): 93 (100), 106 (86), 120 (20), 134 (9), 148 (14), 162 (13), 176 (16), 190 (10), 204 (18), 218 (74), 259 (4), 276 ([M]$^+$, 2). HR-EI-MS *m*/*z*: 276.2185 (Calcd for $C_{17}H_{28}N_2O$ [M]$^+$: 276.2202).

Reduction of Compound 6 To a solution of compound **6** (0.1 mg) in MeOH-d_4 (70 μl) was added 5% palladium on activated carbon (10 μg), and the mixture was stirred at room temperature for 1 h under a deuterium atmosphere. After filtration of the catalyst, the filtrate was evaporated *in vacuo* to afford compound **9** (0.08 mg): HR-EI-MS *m*/*z*: 306.2630 (Calcd for $C_{19}H_{30}D_2N_2O$ [M]$^+$: 306.2638).

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