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Pyrinadines B–G, new bis-pyridine alkaloids with an azoxy moiety from sponge *Cribrochalina* sp.

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Abstract—Six new cytotoxic bis-3-alkylpyridine alkaloids with an azoxy moiety, pyrinadines B–G (**1**–**6**), have been isolated from an Okinawan marine sponge *Cribrochalina* sp., and the structures were elucidated by spectroscopic data and chemical means.

1. Introduction

Marine sponges are a rich source of bioactive secondary metabolites with unprecedented skeletons. A number of 3-alkylpyridine alkaloids have been isolated from marine sponges of several genera.[1] Most of them possess a long aliphatic chain with a various nitrogen-containing terminus,[2] some of which have dimeric or polymeric structures of 3-alkylpyridine.[3] During our continuing search for bioactive substances from marine sponges,[4] we previously isolated cytotoxic pyridine alkaloids from sponges of the genera *Theonella*,[5] *Nyphates*,[6] and *Amphimedon*.[7] More recently, new bis-3-alkylpyridine alkaloids with an azoxy moiety, pyrinadines B–G (**1**–**6**), have been isolated together with pyrinadine A[8] (**7**) from an Okinawan marine sponge *Cribrochalina* sp. (SS-1115). Here, we describe the isolation and structure elucidation of pyrinadines B–G (**1**–**6**).

2. Results and discussion

The sponge *Cribrochalina* sp. (SS-1115) collected off Unten Port, Okinawa, was extracted with MeOH. EtOAc-soluble materials of the MeOH extract were subjected to a silica gel column ($CHCl_3$/MeOH) followed by an amino silica gel column (hexane/EtOAc) and then reversed-phase HPLC (J'sphere ODS-L80, CH_3CN/H_2O) to afford pyrinadines B (**1**, 0.0001%, wet weight), C (**2**, 0.0001%), D (**3**, 0.0003%), E (**4**, 0.0001%), F (**5**, 0.0001%), and G (**6**, 0.0001%) together with pyrinadine A[8] (**7**, 0.0003%) (Fig. 1).

Pyrinadine B (**1**) was revealed to have the molecular formula, $C_{36}H_{60}N_4O$, by HRESIMS [*m*/*z* 565.4839 $(M+H)^+$, Δ −0.6 mmu]. From the ^1H and ^{13}C NMR spectra of **1**, it was deduced that **1** was a congener of pyrinadine A (**7**). The characteristic band at 1505 cm^{-1} in the IR spectrum of **1** suggested the presence of an azoxy group. Aromatic proton signals [H-2, H-2′, H-6, and H-6′, δ_H 8.56 (4H); H-4 and H-4′, δ_H 7.49 (2H); H-5 and H-5′, δ_H 7.20 (2H)] in the ^1H NMR spectrum suggested that **1** possessed two 3-alkylpyridine rings. The ^{13}C NMR spectrum revealed five pairs of sp^2 carbon signals [C-2 and C-2′, δ_C 150.1 (2C, d); C-3 and C-3′, δ_C 138.0 (2C, s); C-4 and C-4′, δ_C 135.8 (2C, d); C-5 and C-5′, δ_C 123.2 (2C, d); C-6 and C-6′, δ_C 147.1 (2C, d)] due to the two pyridine rings. Thus, eight unsaturation numbers were accounted for. The ^{13}C NMR spectrum showed a pair of sp^3 carbon signals due to methylenes (C-19, δ_C 69.7; C-19′, δ_C 52.1) at relatively lower field as compared with those of methylenes in long alkyl chains (δ_C 26–30). The chemical shifts of C-19 and C-19′ indicated that these carbons were adjacent to azoxy moiety, which was the remaining part (N_2O) derived from the NMR data and molecular formula. The position of the oxygen atom in the azoxy moiety was elucidated to be on the C-19 on the basis of the ^1H and ^{13}C NMR chemical shifts of the C-19 and C-19′.[9] The geometry of the azoxy moiety was deduced to be *Z* from the UV absorption maximum (214 nm) of **1**, since those of the *Z*- and *E*-azoxy compounds have

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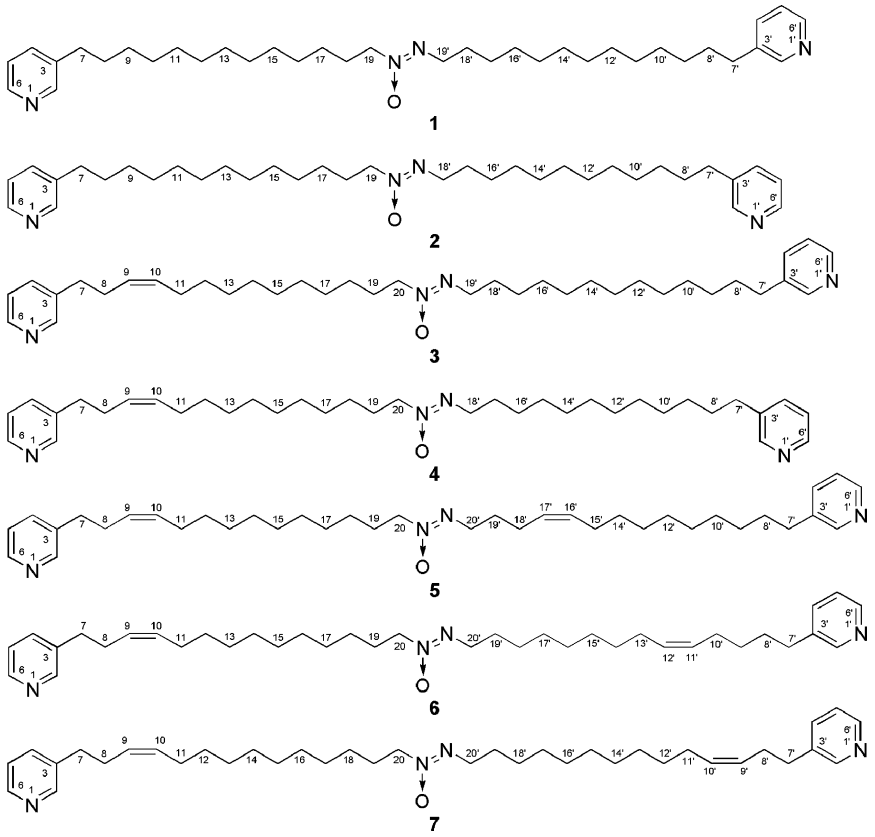

Figure 1. Structures of pyrinadines B–G (**1**–**6**) and A (**7**). The position of an oxygen atom in the azoxy group of **2**–**6** has not been determined.

been observed in the range of 220 ± 3 and 230 ± 3 nm, respectively.[10]

The ^{1}H–^{1}H COSY, HOHAHA, and HMBC spectra revealed the connectivity from two β-substituted pryidine rings to C-9 and C-9′ and from the azoxy moiety to C-17 and C-17′ (Fig. 2). Analysis of the ESI-MS/MS spectrum of **1** revealed connectivities from C-9 to C-17 and C-9′ to C-17′ (Fig. 3). Thus, the structure of pyrinadine B was concluded to be **1**.

The ^{1}H and ^{13}C NMR spectra of pyrinadines C–G (**2**–**6**) were almost identical with those of pyrinadines A[8] and B (**7** and **1**, respectively), suggesting that **2**–**6** were congeners of them. The spectral data of pyrinadine C (**2**) were almost identical with those of pyrinadine B (**1**) except for the molecular weight revealed from ESI-MS [*m*/*z* 551 $(M+H)^{+}$]. The molecular formula of **2**, $C_{35}H_{58}N_4O$, revealed by HRESIMS [*m*/*z* 551.4686 $(M+H)^{+}$, Δ −0.3 mmu] suggested that the carbon chain of **2** was short by one methylene unit as compared to that of **1**. Thus, the structure of pyrinadine C was elucidated to be **2**.

The molecular formulae of pyrinadines D (**3**), $C_{37}H_{60}N_4O$, and E (**4**) $C_{36}H_{58}N_4O$, were revealed from the HRESIMS. The ^{1}H and ^{13}C NMR spectra of **3** and **4** showed signals due to an olefin, suggesting that **3** and **4** possessed a disubstituted olefin in the carbon chain. *Z*-Geometry of the olefin at C-9 was assigned from the chemical shifts of C-8 and C-11.[11] Analysis of the ESI-MS/MS spectra of **3** and **4** revealed connectivities from two pyridine rings to azoxy moiety (Fig. 4). Thus, the structures of pyrinadines D and E were elucidated to be **3** and **4**, respectively.

The spectral data of pyrinadines F (**5**) and G (**6**) were similar to those of pyrinadine D (**3**). The HRESIMS revealed that molecular formulae of **5** and **6** were both $C_{38}H_{60}N_4O$. The ^{1}H and ^{13}C NMR spectra of **5** and **6** showed signals due to two olefins, suggesting that **5** and **6** possessed an additional disubstituted olefin in the carbon chain as compared to **3**. Geometry of olefins was assigned as all *Z* from the chemical shifts of allylic carbons.[11] Analysis of the ESI-MS/MS spectra of **5** and **6** revealed connectivities from two pyridine rings to azoxy moiety (Fig. 5). Thus, the structures of pyrinadines F and G were assigned as **5** and **6**, respectively.

Pyrinadines B–G (**1**–**6**) as well as A (**7**) are rare bis-pyridine alkaloids with an azoxy moiety from natural origins. Pyrinadines B–G (**1**–**6**) showed cytotoxicity against L1210 murine leukemia (IC_{50}, 13, 10, 10, 9, 7, and 7 μg/mL, respectively), while they did not show such activity against human epidermoid carcinoma KB cells in vitro (IC_{50} > 20 μg/mL).

3. Experimental

3.1. General

The IR and UV spectra were recorded on a JASCO FT/IR-5300 and a Shimadzu UV-1600PC spectropolarimeter. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker

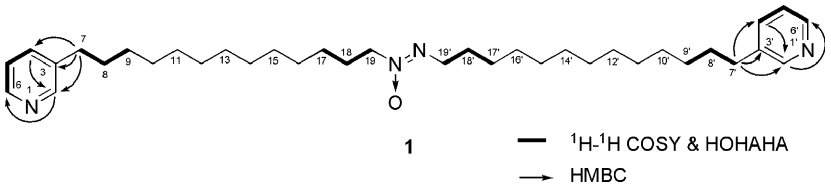

Figure 2. Selected 2D NMR correlations for pyrinadine B (**1**).

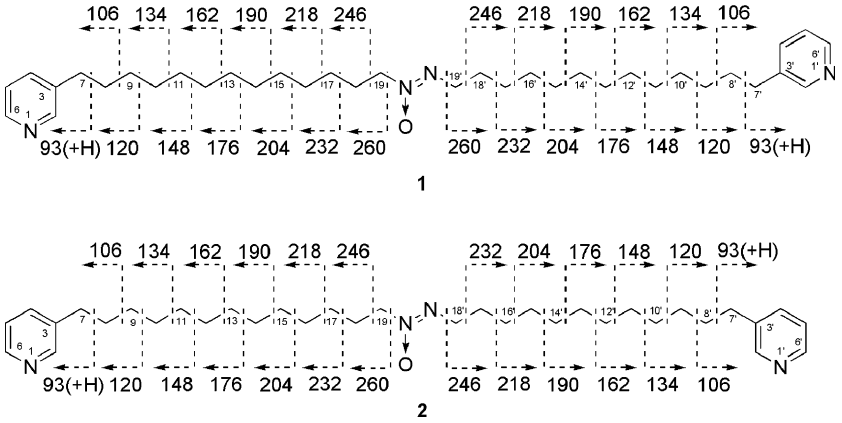

Figure 3. Fragmentation patterns of pyrinadine B (**1**) [parent ion; *m*/*z* 565 $(M+H)^+$] and pyrinadine C (**2**) [parent ion; *m*/*z* 551 $(M+H)^+$] in ESI-MS/MS. The position of an oxygen atom in the azoxy group of **2** has not been determined.

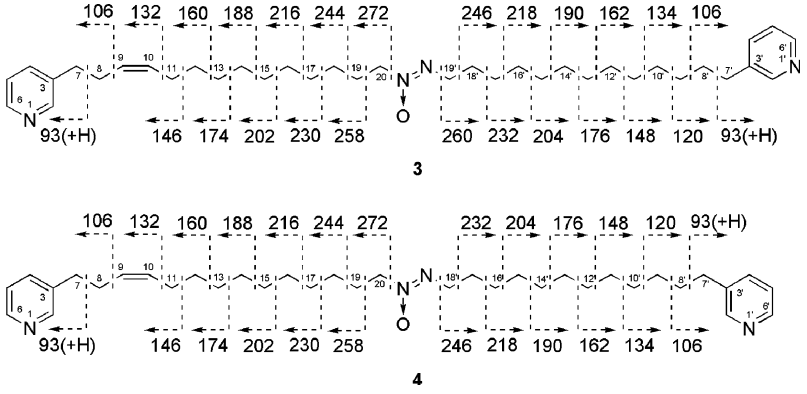

Figure 4. Fragmentation patterns of pyrinadine D (**3**) [parent ion; *m*/*z* 577 $(M+H)^+$] and pyrinadine E (**4**) [parent ion; *m*/*z* 563 $(M+H)^+$] in ESI-MS/MS. The position of an oxygen atom in the azoxy group of **3** and **4** has not been determined.

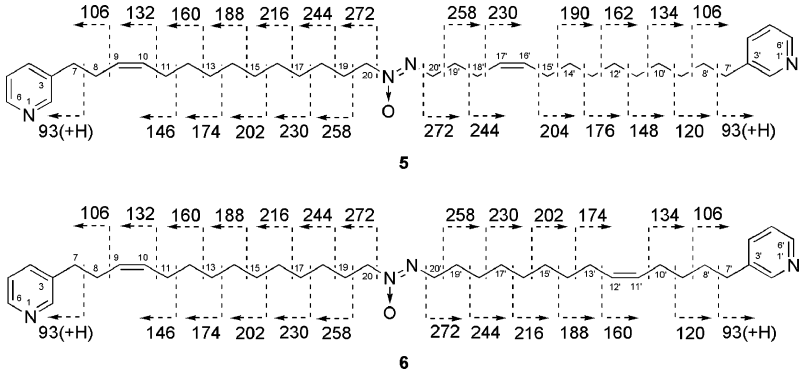

Figure 5. Fragmentation patterns of pyrinadine F (**5**) [parent ion; *m*/*z* 589 $(M+H)^+$] and pyrinadine G (**6**) [parent ion; *m*/*z* 589 $(M+H)^+$] in ESI-MS/MS. The position of an oxygen atom in the azoxy group of **5** and **6** has not been determined.

AMX-600 spectrometer using 2.5 mm micro cells (Shigemi Co., Ltd). The 7.26 and 77.0 ppm resonances of residual $CDCl_3$ were used as internal references for ^1H and ^{13}C NMR spectra, respectively. ESI mass spectra were obtained on a JEOL JMS-SX102A spectrometer.

3.2. Sponge description

The sponge *Cribrochalina* sp. (SS-1115; order Haplosclerida; family Niphatidae) was collected off Unten Port, Okinawa, and kept frozen until used. The genus of this sponge was identified by Dr. Jane Fromont. The color of this sponge was light to medium brown. Its surface was smooth with adherent membrane, interior dense, and compact mesohyl. The sponge was firm but very compressible and springy. One central large canal with smaller canals was draining into it. The sponge had dense close-meshed fiber skeleton. The primary fibers were 60 μm wide and centrally cored by small oxeas which form dense brushes at the surface. The secondary fibres were 30 μm wide and unispicular. The oxeas were smaller than 90× 5 μm. The voucher specimen was deposited at Graduate School of Pharmaceutical Sciences, Hokkaido University.

3.3. Extraction and isolation

The sponge (SS-1115, 0.27 kg, wet weight) was extracted with MeOH (1.0 L × 2), and the extract (26.11 g) was partitioned between hexane (3× 200 mL) and MeOH/H_2O (9:1, 200 mL), and subsequently MeOH/H_2O layer was extracted with EtOAc (3× 200 mL). The EtOAc-soluble materials (0.73 g) were subjected to a silica gel column ($CHCl_3$/MeOH, 10:0 to 0:10) to give pyridine alkaloid-containing fractions, which were separated by an amino silica gel column chromatography (hexane/EtOAc, 7:3) followed by reversed-phase HPLC (J'sphere ODS-L80, CH_3CN/H_2O) to afford pyrinadines B (**1**, 0.3 mg, t_R 36 min), C (**2**, 0.3 mg, t_R 32 min), D (**3**, 0.8 mg, t_R 33 min), E (**4**, 0.3 mg, t_R 24 min), F (**5**, 0.3 mg, t_R 22 min), G (**6**, 0.3 mg, t_R 21 min), and A (**7**, 0.8 mg, t_R 19 min).

3.4. Pyrinadine B (1)

Colorless oil; UV (MeOH) λ_{max} 214 (ε 11,700), 250 (4500), 257 (4700), 262 (4900), and 269 (3500) nm; IR (KBr) ν_{max} 2925, 2854, and 1505 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.56 (4H, m), 7.49 (2H, m), 7.20 (2H, m), 4.14 (2H, m), 3.39 (2H, m), 2.60 (4H, t, J = 7.7 Hz), 1.93 (2H, m), 1.68 (2H, m), 1.61 (4H, m), 1.0–1.4 (36H, m); ^{13}C NMR ($CDCl_3$) δ 150.1 (2C, d), 147.1 (2C, d), 138.0 (2C, s), 135.8 (2C, d), 123.2 (2C, d), 69.7 (t), 52.1 (t), 33.0 (2C, t), 31.1 (2C, t), 26–30 (20C, t); ESIMS (pos.) m/z 565 $(M+H)^+$; HRESIMS m/z 565.4839 $(M+H)^+$, Δ −0.6 mmu.

3.5. Pyrinadine C (2)

Colorless oil; UV (MeOH) λ_{max} 214 (ε 9200), 250 (4900), 256 (4700), 262 (4700), and 269 (3400) nm; IR (KBr) ν_{max} 2925, 2854, and 1505 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.45 (4H, m), 7.50 (2H, m), 7.22 (2H, m), 4.14 (2H, t, J = 7.3 Hz), 3.39 (2H, t, J = 7.3 Hz), 2.60 (4H, t, J = 7.1 Hz), 1.94 (2H, m), 1.69 (2H, m), 1.61 (4H, m), 1.0–1.4 (34H, m); ^{13}C NMR ($CDCl_3$) δ 149.8 (2C, d), 147.0 (2C, d), 137.8 (2C, s), 135.8 (2C, d), 123.3 (2C, d), 69.7 (t), 52.1 (t), 33.0 (2C, t), 31.1 (2C, t), 26–30 (19C, t); ESI-MS (pos.) m/z 551 $(M+H)^+$; HRESIMS m/z 551.4686 $(M+H)^+$, Δ −0.3 mmu.

3.6. Pyrinadine D (3)

Colorless oil; UV (MeOH) λ_{max} 213 (ε 9900), 251 (3600), 257 (4200), 262 (4600), and 269 (3400) nm; IR (KBr) ν_{max} 2925, 2853, and 1505 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.45 (4H, m), 7.50 (2H, m), 7.21 (2H, m), 5.39 (m), 5.36 (m), 4.14 (2H, m), 3.39 (2H, m), 2.66 (2H, t, J = 7.6 Hz), 2.60 (2H, t, J = 7.7 Hz), 2.35 (2H, dt, J = 7.6 and 7.5 Hz), 1.94 (2H, m), 1.93 (2H, m), 1.69 (2H, m), 1.61 (2H, m), 1.0–1.4 (32H, m); ^{13}C NMR ($CDCl_3$) δ 148.7 (d), 148.4 (d), 146.1 (d), 145.6 (d), 138.8 (s), 137.9 (d), 137.3 (d), 137.2 (d), 131.7 (d), 127.4 (d), 123.8 (d), 123.6 (d), 69.7 (t), 52.1 (t), 33.0 (2C, t), 31.0 (t), 28.6 (d), 27.2 (t), 26–30 (18C, t); ESI-MS (pos.) m/z 577 $(M+H)^+$; HRESIMS m/z 577.4840 $(M+H)^+$, Δ −0.5 mmu.

3.7. Pyrinadine E (4)

Colorless oil; UV (MeOH) λ_{max} 213 (ε 10,100), 250 (4100), 257 (4400), 262 (4600), and 269 (3300) nm; IR (KBr) ν_{max} 2925, 2853, and 1505 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.45 (4H, m), 7.51 (2H, m), 7.22 (2H, m), 5.40 (m), 5.36 (m), 4.14 (2H, t, J = 7.2 Hz), 3.39 (2H, t, J = 7.1 Hz), 2.67 (2H, t, J = 7.5 Hz), 2.61 (2H, t, J = 7.7 Hz), 2.36 (2H, dt, J = 7.5 Hz), 1.94 (2H, m), 1.93 (2H, m), 1.70 (2H, m), 1.61 (2H, m), 1.0–1.4 (30H, m); ^{13}C NMR ($CDCl_3$) δ 150.0 (d), 149.9 (d), 147.2 (d), 147.0 (d), 138.1 (2C, d), 136.0 (d), 135.9 (d), 131.5 (d), 127.7 (d), 123.3 (2C, d), 69.7 (t), 52.1 (t), 33.0 (t), 31.1 (t), 28.6 (t), 27.1 (2C, t), 26–30 (17C, t); ESI-MS (pos.) m/z 563 $(M+H)^+$; HRESIMS m/z 563.4692 $(M+H)^+$, Δ +0.4 mmu.

3.8. Pyrinadine F (5)

Colorless oil; UV (MeOH) λ_{max} 215 (ε 9300), 251 (3700), 257 (4400), 263 (4800), and 269 (3500) nm; IR (KBr) ν_{max} 2926, 2854, and 1505 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.52 (4H, m), 7.77 (2H, m), 7.46 (2H, m), 5.44 (m), 5.35 (2H, m), 5.33 (m), 4.14 (2H, m), 3.40 (2H, m), 2.78 (2H, m), 2.74 (2H, t, J = 7.5 Hz), 2.69 (2H, t, J = 7.6 Hz), 2.38 (2H, dt, J = 7.5 Hz), 2.01 (2H, m), 1.92 (2H, m), 1.89 (2H, m), 1.70 (2H, m), 1.65 (2H, m), 1.0–1.4 (26H, m); ^{13}C NMR ($CDCl_3$) δ 149.5 (2C, d), 146.9 (d), 146.9 (d), 146.0 (s), 137.0 (2C, d), 136.3 (2C, d), 131.5 (d), 130.3 (d), 129.3 (d), 127.6 (d), 69.7 (t), 52.1 (t), 33.1 (t), 33.0 (d), 31.1 (d), 28.7 (t), 27.2 (2C, t), 26–30 (17C, t); ESI-MS (pos.) m/z 589 $(M+H)^+$; HRESIMS m/z 589.4852 $(M+H)^+$, Δ +0.7 mmu.

3.9. Pyrinadine G (6)

Colorless oil; UV (MeOH) λ_{max} 213 (ε 10,300), 251 (4600), 257 (4600), 262 (4800), and 269 (3700) nm; IR

(KBr) ν_{max} 2920, 2854, and 1511 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.45 (4H, m), 7.52 (2H, m), 7.21 (2H, m), 5.43 (m), 5.36 (m), 5.35 (2H, m), 4.14 (2H, t, J = 7.2 Hz), 3.39 (2H, t, J = 7.0 Hz), 2.66 (2H, t, J = 7.6 Hz), 2.61 (2H, t, J = 7.6 Hz), 2.36 (2H, dt, J = 7.6 Hz), 2.10 (2H, m), 1.96 (2H, m), 1.94 (2H, m), 1.69 (2H, m), 1.62 (2H, m), 1.0–1.4 (26H, m); ^{13}C NMR ($CDCl_3$) δ 150.0 (2C, d), 147.3 (2C, d), 137.1 (2C, s), 136.1 (2C, d), 131.5 (d), 131.4 (d), 127.7 (2C, d), 123.3 (2C, d), 69.7 (t), 52.1 (t), 33.1 (t), 33.0 (t), 31.1 (t), 28.7(t), 27.7 (t), 27.2 (2C, t), 26–30 (15C, t); ESI-MS (pos.) m/z 589 $(M+H)^+$; HRESIMS m/z 589.4841 $(M+H)^+$, Δ −0.4 mmu.

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