Niphatesines E–H, New Pyridine Alkaloids from the Okinawan Marine Sponge *Niphates* sp.

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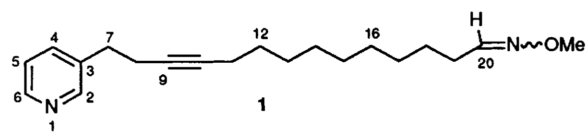
Four new mono-3-alkyl-substituted pyridine alkaloids, niphatesines E–H (**1**–**4**), possessing oxime methyl ether or methoxyamine functionality have been isolated from the Okinawan marine sponge *Niphates* sp. and the structures elucidated by spectral and chemical means. These pyridine alkaloids exhibited cytotoxic and antimicrobial activities.

During our continuing studies on bioactive substances from Okinawan marine organisms,[1] we previously described the isolation and structure elucidation of niphatesines A–D, monosubstituted pyridine alkaloids possessing antileukaemic activity from an extract of the Okinawan sponge *Niphates* sp.[2] Further investigation of the constituents of this sponge has now led to the isolation of four new pyridine alkaloids, niphatesines E–H (**1**–**4**), with cytotoxic and antimicrobial activities. Here we describe the isolation and structure elucidation of these new alkaloids **1**–**4**.

The sponge *Niphates* sp. was collected off the Kerama Islands, Okinawa, and kept frozen until used. The methanol extract was partitioned between ethyl acetate and water. The ethyl acetate-soluble fraction was subjected to repeated silica gel flash column chromatography and elution with chloroform–methanol and hexane–ethyl acetate, followed by reversed-phase HPLC to give niphatesines E (**1**, 0.0005% yield, wet weight), F (**2**, 0.001%), G (**3**, 0.001%), and H (**4**, 0.002%) together with the known ikimine A (**5**, 0.002%).[3]

The molecular formula of niphatesine E (**1**) was determined to be $C_{20}H_{30}N_2O$ by high-resolution fast-atom bombardment mass spectrometry (HR-FAB-MS) data [m/z 315.2430 $(M + H)^+$, Δ −0.6 mmu]. The UV absorption maximum at 262 nm implied the presence of the same pyridine chromophore as that of niphatesines A–D.[2] The ^1H and ^{13}C NMR signals for the aromatic region [δ_H 8.84 (br s, 2-H), 8.79 (d, J 5.9 Hz, 6-H), 8.29 (d, J 8.1 Hz, 4-H) and 7.83 (dd, J 8.1 and 5.9 Hz, 5-H); δ_C 144.4 (d, C-4), 142.7 (s, C-3), 140.7 (d, C-2), 140.5 (d, C-6) and 125.9 (d, C-5)] also suggested the presence of a mono-3-alkyl-substituted pyridine ring on the basis of comparison with spectral data of niphatesines[2] and theonelladins.[4] The ^{13}C NMR signals at δ_C 83.9 (s) and 76.0 (s; overlapped with the signal of $CDCl_3$) were indicative of the presence of a disubstituted alkyne moiety. Six degrees out of the seven unsaturation numbers were accounted for by the pyridine ring and alkyne group. In the ^1H NMR spectrum of compound **1** a methoxy and an olefinic proton were observed as split signals in the ratio 1.4:1 (δ_H 3.81 and 3.87, each singlet; δ_H 7.36 and 6.66, each triplet, J 6.5 Hz, respectively). The ^{13}C signals at δ_C 152.1 and 151.1 (C-20) and δ_C 61.6 and 6.1.2 (OMe) also appeared as split signals in ratios ~1.4:1. These ^1H and ^{13}C signals were assignable to an oxime methyl ether (−CH=NOMe) present at the terminus of the alkyl chain, existing as a mixture of *E* and *Z* isomers. The major isomer was assigned as *E* on the basis of the ^1H chemical shifts (δ_H 7.36 and 6.66, 1.4:1, 20-H), since the proton attached to the oxime carbon of the *E*-isomer usually resonates at lower field than does that of the *Z*-isomer.[5] The presence of the oxime methyl ether group was further confirmed by the intense fragment ion peaks observed at m/z 283 $(M - OCH_3)^+$ and 256 $(M - CH{=}NOMe)^+$ in the electron-impact mass spectrum (EIMS) of compound **1** as well as the IR absorption band of compound **1** at 1680 cm^{-1}. The remaining one unsaturation was thus characterized. The position of the alkyne group of compound **1** was deduced to be at C-9 and C-10 from the EIMS fragmentation pattern (Fig. 1), which was virtually the same pattern as that of niphatesine A.[2] The structure of niphatesine E was, therefore, concluded to be **1**.



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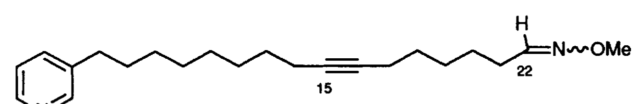

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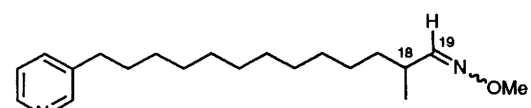

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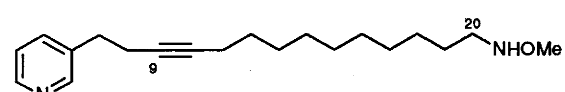

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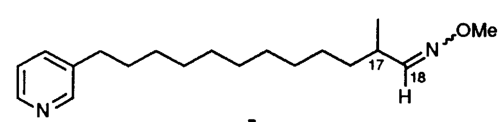

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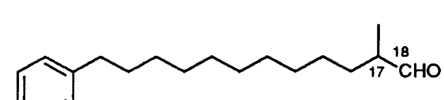

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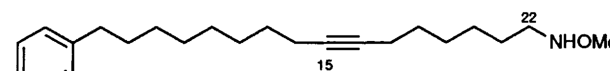

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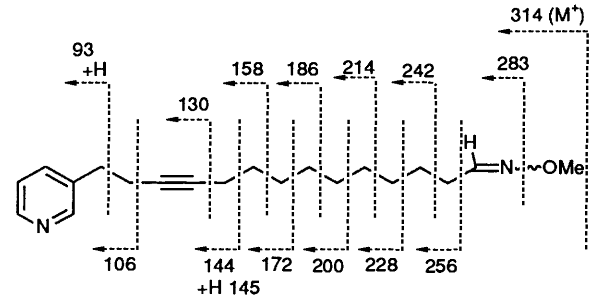

1

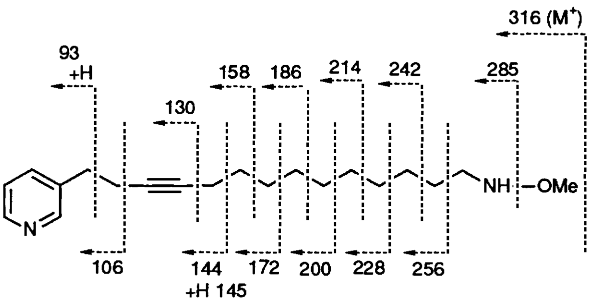

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Fig. 1 The EIMS fragment ions (*m*/*z* of niphatesines E **1** and H **4**

Niphatesine F (**2**) was shown to have the molecular formula $C_{22}H_{34}N_2O$ by HR-FAB-MS data [*m*/*z* 343.2750 $(M + H)^+$, Δ +0.1 mmu]. The spectral data of niphatesine F (**2**) resembled closely those of niphatesine E (**1**) except for the molecular weight, suggesting that compound **2** contained two more methylene units (28 amu; $CH_2 \times 2$) in the side-chain compared with compound **1**. A methoxyimino group was also revealed to be present at the terminus of the side-chain by ^1H and ^{13}C NMR spectroscopy of a mixture of *E* and *Z* isomers in the ratio 1.7:1 (δ_H 3.81 and 3.86, each singlet, OMe; δ_H 7.36 and 6.66, each triplet, *J* 6.2 Hz, 22-H; δ_C 61.5 and 61.2, OMe; δ_C 151.9 and 150.9, C-22, respectively), the major one again being the *E*-isomer. The position of the alkyne group was clearly revealed to be at C-15 and C-16 on the basis of a comparison of the EIMS fragmentation pattern with that of niphatesine B[2] [*m*/*z* 228 (fission of C-17/C-18 bond) and 176 (fission of C-13/C-15 bond)]. Thus the structure of niphatesine F was assigned to be **2**.

Niphatesine G (**3**) was also a mono-3-alkyl-substituted pyridine alkaloid with an alkyl side-chain terminated by an oxime methyl ether group. The molecular formula of compound **3** was established to be $C_{20}H_{34}N_2O$ by HR-FAB-MS data [**3**: *m*/*z* 319.2743 $(M + H)^+$, Δ −0.6 mmu]. The ^1H NMR spectrum of compound **3** was quite similar to that of ikimine A (**5**, 2.7:1 mixture of *E*/*Z* isomers), which was also isolated from this sponge. Ikimine A (**5**) was previously isolated from an unidentified Micronesian sponge.[3] The ^1H NMR spectrum of compound **3** revealed signals due to a secondary methyl group (δ_H 1.05, 3 H, d, *J* 6.6 Hz) as well as the proton attached to the oxime carbon, observed as a doublet (δ_H 7.20 and 6.44, *J* 7.7 Hz, 19-H; *E*:*Z* 3.2:1). The difference in the spectral properties of compounds **3** and **5** was found only in the molecular weight [HR-FAB-MS of **5**: *m*/*z* 305.2585 $(M + H)^+$, $C_{19}H_{33}N_2O$, Δ −0.8 mmu]. Compound **3** was, therefore, shown to possess one more CH_2 unit (14 amu) between the 3-position of the pyridine ring and the terminal methoxy imino group than has compound **5**. The presence of the oxime methyl ether group in compound **5** was confirmed by the following chemical tests: on treatment with 3 mol dm^{-3} hydrochloric acid in methanol under reflux for 20 h, compound **5** was converted into the corresponding aldehyde [**6**, *m*/*z* 275 (M^+) and 246 $(M - CHO)^+$]. The ^1H NMR spectrum of the aldehyde **6** no longer exhibited the signal due to a methoxy group, but instead the signal due to an aldehyde group was observed at δ_H 9.61 as a doublet (*J* 1.9 Hz).

The molecular formula of niphatesine H (**4**), $C_{20}H_{32}N_2O$, established by HR-FAB-MS [*m*/*z* 317.2600 $(M + H)^+$, Δ +0.7 mmu], implied that niphatesine H (**4**) possesses two more hydrogen atoms than does niphatesine E (**1**). The UV absorption of compound **4** (λ_{max} 262 nm) was similar to that of compound **1** while the IR spectrum of compound **4** did not show the absorption band at 1680 cm^{-1}, thus suggesting the presence of a pyridine chromophore and the absence of the oxime group in compound **4**. The ^1H and ^{13}C NMR spectra of compound **4** revealed signals due to a methoxy [δ_H 3.93 (3 H, s); δ_C 61.4 (q)], a disubstituted alkyne [δ_C 84.2 (s) and 76.0 (s; overlapped with the signal of $CDCl_3$)], and a heteroatom-bearing sp^3 methylene [δ_H 3.26 (2 H, br s); δ_C 49.4 (t)] unit. These spectral features were parallel to those of niphatyne A (**7**),[6] previously isolated from a Fijian sponge (*Niphates* sp.), and compound **4** was, therefore, inferred to be a 3-alkylpyridine alkaloid with a methoxy amino group at the terminus of the alkyl chain. The position of the alkyne functionality of compound **4** was deduced to be at C-9 and C-10 on the basis of comparison of the EIMS data with those of niphatesine E (**1**) as shown in Fig. 1. Thus niphatesine H (**4**) was shown to be the 20,*N*-dihydro form of niphatesine E (**1**). The oxime bond of niphatesine E (**1**) was veritably reduced by treatment with sodium cyanoborohydride to afford niphatesine H (**4**) almost quantitatively. By the same procedure niphatesine F (**2**) was converted into known niphatyne A (**7**),[6] which was also isolated from this sponge, to verify the structure of compound **2**.

Niphatesines E–H (**1–4**) and compound **5** exhibited cytotoxic activity against murine lymphoma L1210 cells and human epidermoid carcinoma KB cells *in vitro*. The inhibitory activity (%) at 10 µg/cm^3 against L1210 and KB cells, respectively, were as follows: **1**: 27.0% and 22.7%, **2**: 41.2% and 19.4%, **3**: >50% (IC_{50} 7.9 µg/cm^3) and 16.8%, **4**: >50% (IC_{50} 1.9 µg/cm^3) and >50% (IC_{50} 6.0 µg/cm^3), and **5**: >50% (IC_{50} 5.4 µg/cm^3) and 26.6%. Compounds **1–5** also showed antimicrobial activity against some fungi and gram-positive bacteria as shown in Table 1.

Experimental

The IR and UV spectra were recorded on a JASCO A-102 and Shimadzu UV-220 spectrophotometer, respectively. ^1H and ^{13}C NMR spectra were recorded on a JEOL GX-270 spectrometer. *J*-Values are given in Hz. FAB mass spectra were obtained on a JEOL HX-110 spectrometer using 2-nitrobenzyl alcohol as matrix. EI mass spectra (EIMS) were recorded on a JEOL DX-303 spectrometer. Wako C-300 silica gel (Wako Pure Chemical) was used for glass column chromatography, and TLC was carried out on Merck silica gel GF254.

Isolation.—The sponge *Niphates* sp. (0.52 kg wet weight) collected by SCUBA off the Kerama Islands, Okinawa, was kept frozen until used. The methanol extract of the sponge was evaporated under reduced pressure and the residue (100 g) was dissolved in a mixed solvent of ethyl acetate (600 cm^3) and water (600 cm^3) and was then partitioned between ethyl acetate (600 $cm^3 \times 3$) and water (600 cm^3). The ethyl acetate-soluble material (8.43 g) was partly (3.85 g) subjected to silica gel flash column chromatography with gradient elution of methanol in chloroform (0–5%) to give an active fraction (1.21 g), which was further purified by silica gel flash column chromatography and elution with gradually increasing amounts of ethyl acetate in hexane (0–100%). The fraction (89.8 mg) eluted with 20% ethyl acetate in hexane was further purified by HPLC [YMC-Pack AM-324 ODS, Yamamura Chemical; 10 × 250 mm; eluent,

Table 1 Antimicrobial activity of compounds **1–5**

	MIC-values (μg/cm^3)[a]				
	1	2	3	4	5
Candida albicans	266	266	*b*	33	266
Cryptococcus neoformans	133	133	133	16.5	133
Paecilomyces variotii				33	
Aspergillus niger				133	133
Staphylococcus aureus	16.5	16.5	8.2	16.5	33
Sarcina lutea	133	133	133	33	66
Bacillus subtilis	266	266	266	133	133
Escherichia coli					

[a] Minimum inhibitory concentration. [b] No value here and elsewhere denotes 'inactive at 266 μg/cm^3'.

acetonitrile–water–trifluoroacetic acid (50:50:0.1)] to yield niphatesines E (**1**, 2.7 mg; t_R 20.0 min), F (**2**, 5.4 mg; t_R 32.8 min), G (**3**, 5.4 mg; t_R 44.0 min) and compound **5** (8.5 mg; t_R 28.0 min). The fraction (225.0 mg) from the second silica gel column eluted with 50% ethyl acetate in hexane was separated by HPLC [YMC-Pack AM-324 ODS; 10 × 250 mm; eluent, acetonitrile–water–trifluoroacetic acid (30:70:0.1)] to give niphatesine H (**4**, 9.5 mg; t_R 15.6 min) as well as the known compound niphatyne A (**7**, 16.5 mg; t_R 35.6 min).[6]

Niphatesine E **1**.—An oil: λ_{max}(MeOH)/nm 262 (ε 1900); ν_{max}(KBr)/cm^{-1} 2910, 2850, 1680, 1200 and 1140; δ_H(CDCl$_3$) 8.84 (1 H, s, 2-H), 8.79 (1 H, d, *J* 5.9, 6-H), 8.29 (1 H, d, *J* 8.1, 4-H), 7.83 (1 H, dd, *J* 5.9 and 8.1, 5-H), 7.36 and 6.66 (1 H, t, *J* 6.5, 20-H; 1.4:1 ratio), 3.87 and 3.81 (3 H, s, OMe; 1:1.4 ratio), 2.99 (2 H, t, *J* 6.6, 7-H$_2$), 2.58 (2 H, t, *J* 6.6, 8-H$_2$), 2.06–2.18 (4 H, m, 11- and 19-H$_2$) and 1.20–1.43 (14 H, m, 12–18 H$_2$); δ_C(CDCl$_3$) 152.1 and 151.1 (d, C-20; 1.4:1 ratio), 144.4 (d, C-4), 142.7 (s, C-3), 140.7 (d, C-2), 140.5 (d, C-6), 125.9 (d, C-5), 83.9 (s, C-10), 76.0 (s, C-9), 61.6 and 61.2 (q, OMe; 1.4:1 ratio), 32.1 (t), 29.7 (t), 29.3 (t), 29.2 (t), 29.0 (t), 26.8 (t), 26.2 (t), 25.6 (t), 20.0 (t) and 18.6 (t); EIMS *m/z* 314 (5, M$^+$), 283 (80), 256 (10), 242 (35), 228 (11), 214 (12), 200 (16), 186 (20), 172 (18), 158 (37), 145 (30), 144 (25), 130 (13), 118 (12), 106 (28) and 93 (100) [HR-FAB-MS *m/z*, 315.2430. Calc. for $C_{20}H_{31}N_2O$: (M + H), 315.2436].

Niphatesine F **2**.—An oil; λ_{max}(MeOH)/nm 263 (ε 3100); ν_{max}(KBr)/cm^{-1} 2920, 2850, 1680, 1460 and 1200; δ_H(CDCl$_3$) 8.73–8.75 (2 H, br s, 2- and 6-H), 8.21 (1 H, d, *J* 8.4, 4-H), 7.81–7.86 (1 H, m, 5-H), 7.36 and 6.66 (1 H, t, *J* 6.2, 22-H; 1.7:1 ratio), 3.86 and 3.81 (3 H, s, OMe; 1:1.7 ratio), 2.94 (2 H, m, 21-H$_2$), 2.83 (2 H, t, *J* 7.7, 7-H$_2$), 2.10–2.17 (4 H, m, 14- and 17-H$_2$), 1.67–1.70 (2 H, m, 8-H$_2$) and 1.25–1.51 (16 H, br s, 9–13- and 18–20-H$_2$); δ_C(CDCl$_3$) 151.9 and 150.9 (d, C-22; 1.7:1 ratio), 144.7 (d, C-4), 142.9 (s, C-3), 141.7 (d, C-2), 139.5 (d, C-6), 126.3 (d, C-5), 80.3 and 79.9 (s, C-15 and -16), 61.5 and 61.2 (q, OMe; 1.7:1 ratio), 32.9 (t), 30.5 (t), 29.4 (t), 29.1 (t), 29.0 (t), 28.9 (t), 28.8 (t), 28.7 (t), 28.3 (t) and 18.7 (t); EIMS *m/z* 342 (26, M$^+$), 311 (28), 284 (17), 256 (10), 242 (34), 228 (21), 176 (28), 162 (20), 148 (26), 134 (28), 120 (32), 106 (17) and 93 (100) [HR-FAB-MS *m/z*, 343.2750. Calc. for $C_{22}H_{35}N_2O$: (M + H), 343.2749].

Niphatesine G **3**.—An oil; λ_{max}(MeOH)/nm 263 (ε 1500); ν_{max}(KBr)/cm^{-1} 2920, 2850, 1670, 1200, 1140 and 720; δ_H(CDCl$_3$) 8.70–8.76 (2 H, br s, 2- and 6-H), 8.17 (1 H, d, *J* 8.1, 4-H), 7.77–7.81 (1 H, m, 5-H), 7.20 and 6.44 (1 H, d, *J* 7.7, 19-H; 3.2:1 ratio), 3.84 and 3.81 (3 H, s, OMe; 3.2:1 ratio), 2.82 (2 H, t, *J* 7.7, 7-H$_2$), 2.33 (1 H, m, 18-H), 1.67–1.69 (2 H, m, 8-H$_2$), 1.25–1.32 (18 H, br s, 9–17-H$_2$) and 1.05 (3 H, d, *J* 6.6, 18-Me); EIMS *m/z* 318 (6, M$^+$), 287 (100), 260 (111), 232 (38), 218 (7), 204 (10), 190 (9), 176 (8), 162 (9), 148 (11), 134 (8), 120 (18), 106 (45) and 93 (40) [HR-FAB-MS *m/z*, 319.2743. Calc. for $C_{20}H_{35}N_2O$: (M + H), 319.2749].

Niphatesine H **4**.—An oil; λ_{max}(MeOH)/nm 262 (ε 2500); ν_{max}(KBr)/cm^{-1} 2920, 2850, 1200, 1130 and 720; δ_H(CDCl$_3$) 8.78–8.80 (2 H, br s, 2- and 6-H), 8.23 (1 H, d, *J* 7.7, 4-H), 7.86–7.89 (1 H, m, 5-H), 3.93 (3 H, s, OMe), 3.26 (2 H, br s, 20-H$_2$), 2.98 (2 H, t, *J* 6.6, 7-H$_2$), 2.59 (2 H, t, *J* 6.0), 2.08 (2 H, m), 1.73 (2 H, br s) and 1.16–1.29 (14 H, br s); δ_C(CDCl$_3$) 145.2 (d, C-4), 142.0 (s, C-3), 140.8 (d, C-2), 140.5 (d, C-6), 126.4 (d, C-5), 84.2 (s, C-10), 76.0 (s, C-9), 61.4 (q, OMe), 49.4 (t, C-20), 31.9 (t), 29.0 (t), 28.8 (t), 28.7 (t), 28.7 (t), 28.7 (t), 28.4 (t), 27.8 (t), 26.1 (t), 23.6 (t), 19.8 (t) and 18.4 (t); EIMS *m/z* 316 (5, M$^+$), 285 (95, M$^+$ − OCH$_3$), 256 (18), 242 (12), 228 (15), 214 (20), 200 (21), 186 (22), 172 (18), 158 (45), 145 (30), 144 (20), 130 (10), 106 (25) and 93 (100) [HR-FAB-MS *m/z*, 317.2600. Calc. for $C_{20}H_{33}N_2O$: (M + H), 317.2593].

Compound **5**.—An oil; λ_{max}(MeOH)/nm 263 (ε 3300); ν_{max}(KBr)/cm^{-1} 2920, 2850, 1680, 1470 and 1200; δ_H(CDCl$_3$) 8.67–8.86 (2 H, br s, 2- and 6-H), 8.21 (1 H, d, *J* 6.2, 4-H), 7.82–7.84 (1 H, m, 5-H), 7.20 and 6.46 (1 H, d, *J* 8, 18-H; 2.7:1 ratio), 3.84 and 3.80 (3 H, s, OMe; 1:2.7 ratio), 2.82 (2 H, t, *J* 5.1, 7-H$_2$), 2.30–2.35 (1 H, m, 17-H), 1.69 (2 H, m, 8-2H), 1.25–1.32 (16 H, br s, 9–16 H$_2$) and 1.04 (3 H, d, *J* 6.6, 17-Me); δ_C(CDCl$_3$) 157.1 and 155.7 (d, C-18; 2.7:1 ratio), 145.0 (d, C-4), 143.2 (s, C-3), 141.6 (d, C-2), 139.5 (d, C-6), 126.4 (d, C-5), 61.5 and 61.1 (q, OMe; 2.7:1 ratio), 34.8 (t), 34.3 (d, C-17), 32.9 (t), 30.5 (t), 29.5 (t), 29.4 (t), 29.3 (t), 29.1 (t), 29.0 (t), 27.2 (t), 27.0 (t) and 18.2 and 17.6 (q, 17-Me; 2.7:1 ratio); EIMS *m/z* 304 (21, M$^+$), 273 (92), 246 (12), 232 (8), 218 (20), 204 (15), 190 (16), 176 (14), 162 (18), 148 (20), 134 (15), 120 (25), 106 (95) and 93 (100) [HR-FAB-MS *m/z*, 305.2584. Calc. for $C_{19}H_{33}N_2O$: (M + H), 305.2593].

Hydrolysis of Compound **5**.—Compound **5** (1.5 mg) was treated with 3 mol dm^{-3} HCl–MeOH (1:1; 5 cm^3) under reflux for 20 h. After neutralization with aq. 3 mol dm^{-3} KOH, the reaction mixture was extracted with dichloromethane (5 cm^3 × 3). The organic layer was partially evaporated, passed through an SEP-PAK cartridge C_{18} (Waters Associates), and eluted with acetonitrile–water (1:1) to afford the aldehyde **6** (1.4 mg); δ_H 9.61 (d, *J* 1.9), 8.45 (2 H, br s, 2- and 6-H), 7.52 (1 H, d, *J* 7.9, 4-H) and 7.21 (1 H, m, 5-H); EIMS *m/z* 275 (11, M$^+$), 246 (29, M$^+$ − CHO), 218 (42), 204 (35), 190 (17), 176 (15), 162 (18), 148 (13), 134 (15), 120 (25), 106 (100) and 93 (85).

Reduction of Niphatesines E (**1**) *and F* (**2**).—To a solution of niphatesine E (**1**, 2.0 mg) in methanol (5 cm^3) was added sodium cyanoborohydride (1.5 mg) and the pH-value of this solution was adjusted to 2 by addition of aq. 3 mol dm^{-3} HCl. After the mixture had been stirred at room temperature for 6 h, the solvent was removed under reduced pressure and the residue was dissolved in ethyl acetate and washed with water. Evaporation of the organic layer afforded niphatesine H (**4**, 2.0 mg), which was identified by comparison of TLC, ^{1}H NMR and EIMS data. Under the same conditions niphatesine F (**2**, 4.3 mg) was treated with $NaBH_3CN$ (3.6 mg) to give niphatyne A (**7**, 4.1 mg).

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