New Sesquiterpene Formamides from the Marine Sponge *Axinyssa* sp.

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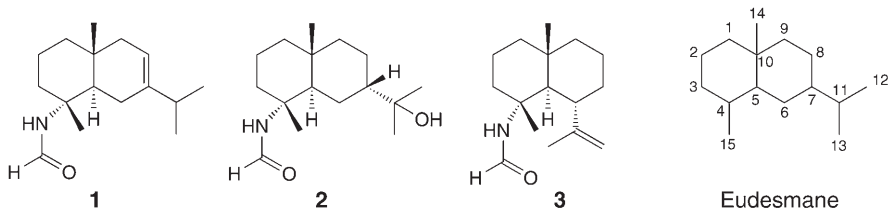
Chemical investigation of an extract of the marine sponge *Axinyssa* sp., collected from the South China Sea, led to the isolation and identification of two new sesquiterpene formamides, (4*R**,5*R**,10*R**)-4-formamidoeudesm-7-ene (**1**) and (4*R**,5*R**,7*S**,10*R**)-4-formamidoeudesman-11-ol (**2**), together with the known 4α-formamidogorgon-11-ene (**3**). Their structures were elucidated by spectroscopic methods, including 1D- and 2D-NMR spectroscopy, high-resolution ESI-TOF mass spectrometry, as well as X-ray single-crystal diffraction experiments. Possible biosynthetic pathways for **1**–**3** were also proposed. The human nasopharyngeal cancer cell line CNE-2, human cervical cancer cell line HeLa, and human normal liver cell line L02 were used to examine the cytotoxic activities of **1**–**3** *in vitro*. As the results, **1** showed significant cytotoxic activity against CNE-2, HeLa, and L02 cell lines with the IC_{50} values of 13.8, 7.5, and 38.0 μg/ml, resp.

Introduction. – In the last decades, the sponges of the genus *Axinyssa* (order Halichondria) have been reported to contain a wide variety of the nitrogenous sesquiterpene metabolites, including isocyanides, isothiocyanates, thiocyanates, and formamides. Their carbon skeletons included monocyclic bisabolene [1], bicyclic eudesmane [2], cadinene [3], gorgonane [2][4], tricyclic pupukaenane [5–8], cubebane [5], and aromadendrane [5]. Evaluation of biological activities of these N-containing sesquiterpenes has rendered some of them potent in cytotoxic [7], antimicrobial [3], anthelmintic [9], antimalarial [6], and antifouling [10] assays.

Specimens of the sponge *Axinyssa* sp. were collected by hand using Scuba from Hainan Island in the South China Sea. In view of the prior chemical literatures on *Axinyssa*, we expected to isolate some new nitrogenous sesquiterpenes. The ^{1}H-NMR spectrum of the semipure MeOH extract, obtained after partitioning with AcOEt and H_2O, showed many characteristic formamide group signals around δ(H) 5 and 8 ppm, representing resonances of the NH and HCO groups, respectively. Alternatively, the ^{13}C-NMR spectrum showed a series of resonances around δ(C) 160 ppm. Therefore, the crude extract was assumed to be rich in sesquiterpene formamides. The AcOEt-soluble extracts of the sponge crude extract were subjected to a standard separation procedure that involved flash chromatography on silica gel, followed by repeated HPLC to obtain pure compounds **1**–**3**. Here, we describe the structure elucidation and cytotoxic activity of these compounds.



Results and Discussion. – 1. *Structure Elucidation.* Compound **1**, a white solid, was isolated as an epimeric mixture of the (*Z*)- and (*E*)-isomers with 3:2 ratio. The molecular formula $C_{16}H_{27}NO$ was established by analysis of the HR-ESI-TOF-MS (*m/z* 250.2158 ($[M+H]^+$)). The presence of two sets of signals in the ^{1}H- and ^{13}C-NMR spectra was due to restricted rotation around the NH–CO bond, forming the (*Z*)- and (*E*)-isomer. This characteristic phenomenon was also observed in other sesquiterpene formamides [2][3][11]. By careful analysis of the ^{1}H- and ^{13}C-NMR, DEPT, and HMQC spectra, the sixteen ^{13}C signals were assigned to contained four Me, five CH_2, and four CH groups, and three quaternary C-atoms. In the IR spectrum, the prominent bands at 3282, 2839, 2753, and 1661 cm^{-1} were assigned to the formamide functionality, which was supported by the NMR data (*Table* in *Exper. Part*) for CHO (major (*Z*)-isomer: δ(C) 160.6, δ(H) 8.03 (*d*, *J* = 2.0); minor (*E*)-isomer: δ(C) 163.7, δ(H) 8.25 (*d*, *J* = 12.5)) and NH (major (*Z*)-isomer: δ(H) 5.40 (*d*, *J* = 2.0); minor (*E*)-isomer: δ(H) 5.82 (*d*, *J* = 12.5)). Another functional group, a trisubstituted C=C bond, was identified by ^{13}C-NMR spectrum (major (*Z*)-isomer: δ(C) 134.0 (*s*); 128.1 (*d*); minor (*E*)-isomer: δ(C) 140.5 (*s*), 127.9 (*d*)). Thus, **1** must be bicyclic to account for the four degrees of unsaturation required by the molecular formula.

In the ^{1}H-NMR spectrum, the major (*Z*)-isomer of **1** displayed *singlet* signals of two Me groups at δ(H) 1.46 and 1.51, *doublet* signals for two Me groups at δ(H) 0.97 (*d*, *J* = 7.0) and 0.87 (*d*, *J* = 7.0). The latter two Me groups were connected to the CH group (δ(C) 35.0, δ(H) 2.12 (*sept.*, *J* = 7.0)), forming an i-Pr moiety. Further NMR studies including HMQC, HMBC, and ^{1}H,^{1}H-COSY data allowed to assemble the structure of **1** as shown in *Fig. 1*. The correlations of NH/C(4) and H–C(15)/C(4) in the HMBC spectrum confirmed the formamide and Me groups were at C(4). The cross-peaks of H–C(5)/H_{ax}–C(6), H–C(5)/H_{eq}–C(6), H–C(8)/H_{ax}–C(9), and H–C(8)/H_{eq}–C(9) in ^{1}H,^{1}H-COSY, as well as the HMBC correlations H–C(11)/C(7), H–C(12)/C(7), H–C(13)/C(7), H–C(11)/C(8), H–C(5)/C(7), and H–C(11)/C(6) revealed that the i-Pr group was connected to the olefinic quaternary C-atom C(7). Based on this analysis, **1** was determined to have an eudesmane (=decahydro-4a,8-dimethyl-2-(1-methylethyl)naphthalene) skeleton.

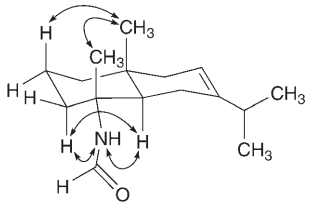


Fig. 1. *Selected ROESY correlations of* **1**

The relative configuration about the bicyclic ring system was determined by ROESY correlations. The correlations H–C(14)/H_{eq}–C(1), H–C(14)/H_{ax}–C(2), and H–C(14)/H–C(15) suggested Me(14) and Me(15) groups in the axial position. Likewise, the axial position for H–C(5) was established based on the presence of the correlations H–C(5)/H_{ax}–C(3) and H–C(5)/NH. Thus, **1** was established as (4*R**,5*R**,10*R**)-4-formamidoeudesm-7-ene. By comparison with the NMR data of previously reported sesquiterpene formamides, we found that (4*R*,5*R*,7*S*,10*R*)-4-formamidoeudesm-11-ene (**4**) [2], which had been isolated from Caribbean sponge *Axinyssa ambrosia*, had the same skeleton. The major difference found was the position of the C=C bond located between C(7) and C(8) in **1**, between C(11) and C(12) in **4**.

Compound **2** was isolated as colorless oil as the mixture of (*Z*)- and (*E*)-isomers in 3:1 ratio. The molecular formula $C_{16}H_{29}NO_2$ was assigned on the basis of HR-ESI-TOF-MS data (*m*/*z* 250.2254 ($[M+H-H_2O]^+$)). The 3321-cm^{-1} band and a prominent band at 1665 cm^{-1} in the IR spectrum, and the CH resonance at δ(C) 159.7/160.5, which was connected to the broad *singlet* at δ(H) 8.13/8.24 in the HMQC spectrum, were assigned to a formamide group. The broad band at 3549 cm^{-1} in the IR spectrum and the broad *singlet* at δ(H) 4.18 (1 H) indicated the presence of a OH group. The ^{13}C-NMR (*Table* in *Exper. Part*) spectrum of **2** showed signals for four Me, six CH_2, three CH groups, and three quaternary C-atoms. Three degrees of unsaturation were required by this molecular formula. The absence of a C=C bond indicated that **2** must, therefore, be bicyclic. Of the major (*Z*)-isomer, the ^{1}H-NMR spectrum recorded in $CDCl_3$ contained four Me singlets at δ(H) 0.96, 1.23, 1.30, and 1.52. In the HMBC spectrum, the latter two methyl signals showed correlations with the quaternary C-atom at δ(C) 61.5, which beared a OH group. Accordingly, the partial structure –C(OH)Me_2 was assembled. Further interpretation of the HMBC and ^{1}H,^{1}H-COSY spectra showed that this fragment was located at C(7), forming the same eudesmane skeleton.

The relative configuration of the stereogenic centers in **2** was determined in the same manner as described for **1**. The ROESY correlations H–C(5)/H–C(12) and H–C(5)/H–C(13) indicated that H–C(7) was equatorial. By interpretation of the ROESY spectrum, the relative configurations of C(4), C(5), and C(10) in **2** were all consistent with those of **1**. Therefore, **2** was identified as (4*R**,5*R**,7*S**,10*R**)-4-formamidoeudesman-11-ol.

Compound **3** was identified as 4α-formamidogorgon-11-ene, by comparing its ^{13}C-NMR data with the reported values [10]. However, the detailed ^{1}H- and ^{13}C-NMR data (*Table* in *Exper. Part*) for the (*E*)- and (*Z*)-isomers of **3** are reported herein for the first time. The crystal structure and the X-ray diffraction data for this compound at room temperature were also reported (see *Fig. 2*)[1]).

2. *Biosynthetic Aspects and Biological Properties.* Though eudesmane-type sesquiterpenoids are of universal occurrence in nature and exhibit a wide range of biological

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[1]) Crystal data: crystallized from AcOEt, $C_{16}H_{27}NO$, M_r 249.21, monoclinic system, space group *P*2(1), $a = 9.33940(10)$, $b = 12.68670(10)$, $c = 13.33430(10)$ Å, $V = 1507.90(2)$ Å^3, $Z = 5$, $d = 1.094$ g/cm^3. Crystal size $0.40 \times 0.10 \times 0.10$ mm. CCDC-662116 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge *via* www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting the *Cambridge Crystallographic Data Centre*, 12, Union Road, Cambridge CB21EZ, UK; fax: +44 1223 336033.

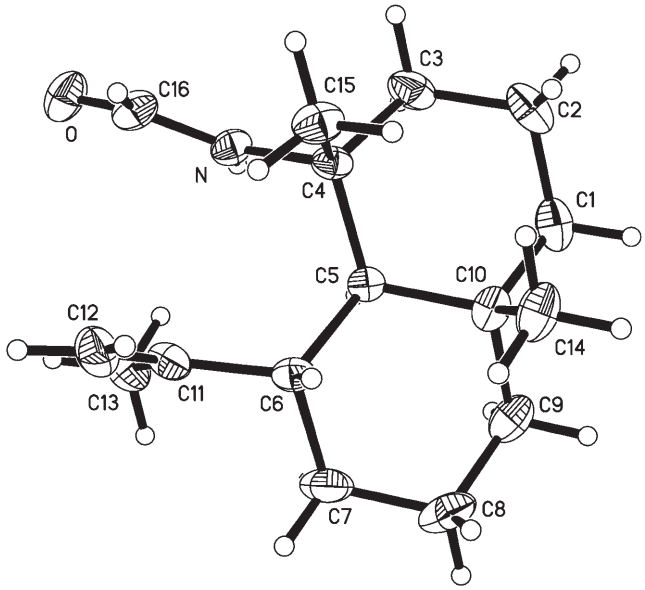


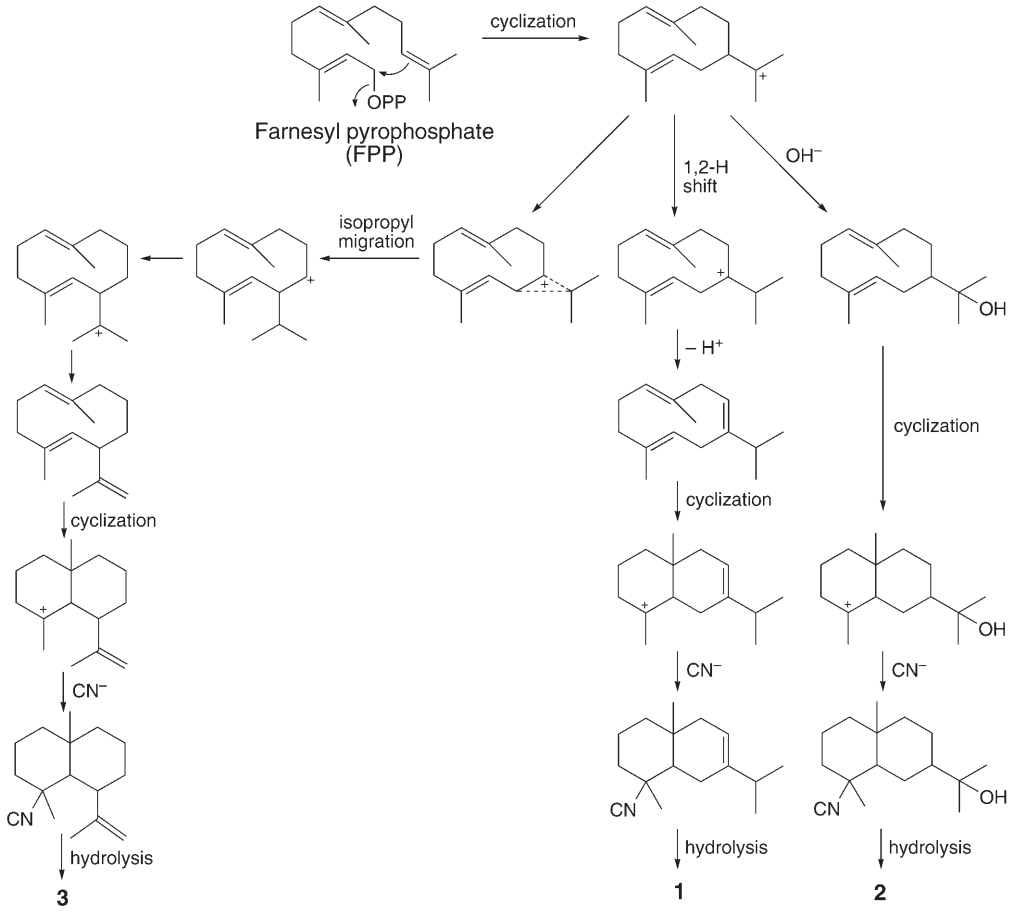
Fig. 2. *X-Ray crystal structure of* **3**

activities [12], N-containing sesquiterpenoids, including isocyanides, isothiocyanates, and formamides, are rather rare, and most of them were found in sponges of the order Halichondria [13]. The presence of the unusual sesquiterpenoid isocyanides generally accompanied by the corresponding isothiocyanates and formamide derivatives was considered as an evidence of the strict biogenetic relationship between these three classes of compounds. Previous experimental results obtained by *Simpson* and *Garson* revealed inorganic cyanide was a precursor of both the isocyanide and isothiocyanate moieties of these sesquiterpenoids. Further, the isocyanides could be degraded by acid hydrolysis to the formamides [14]. With these results in mind, we propose the biosynthetic pathways for **1**, **2**, and **3** in the *Scheme*. The eudesmane skeleton was shown to be the product of two cyclizations of farnesyl pyrophosphate (FPP). As for **3**, a nonisoprenoid sesquiterpene skeleton, we postulated that the gorgonene skeleton was derived from a rearrangement of the cationic intermediate, which leads to the germacrance skeleton resulting in a misplaced isopropenyl group.

The human nasopharyngeal cancer cell line CNE-2, human cervical cancer cell line HeLa, and human normal liver cell line L02 were used to examine the cytotoxic activities of **1**–**3** *in vitro*. Compound **1** showed significant cytotoxic activities against CNE-2, HeLa, and L02 cell lines with the IC_{50} values of 13.8, 7.5, and 38.0 μg/ml, respectively. In contrast, **2** and **3** were apparently inactive in this assay (IC_{50} values > 100 μg/ml).

This work was supported by the *National Natural Science Foundation of China* (No. 20502036 and 20602044) and the *Natural Science Foundation of Guangdong Province* (No. 05300667). We would like to thank the staff of *Administration of Hainan Sanya National Coral Reef Reserve*, who gave the permission for us to collect the sample.

Scheme. *Proposed Biosynthetic Pathway for* **1**, **2**, *and* **3**



Experimental Part

General. HPLC: *Phenomenex C18* reverse-phase (RP) column (250 × 10 mm), with a single-wavelength (230 nm) UV detector and an ELS detector. Optical rotations: *Jasco DPI-370* digital polarimeter. IR Spectra: *Perkin-Elmer 337* spectrophotometer, in CH_2Cl_2 soln.; in cm^{-1}. ^1H- and ^{13}C-NMR spectra: *Varian Inova-500* spectrometer, at 500/125 MHz, resp.; in $CDCl_3$ or $(D_6)DMSO$ soln.; δ in ppm rel. to Me_4Si, J in Hz. HR-ESI-TOF-MS: *Mariner 5200* mass spectrometer; in m/z.

Sponge Material. The marine sponge *Axinyssa* sp. was collected in July 2007 by scuba diving at a depth of 10–15 m along the coast of Hainan Island in the South China Sea. A voucher specimen (No. 2007 H12) was deposited at the Research Center of Organic Natural Products, Sun Yat-sen University, Guangzhou, P. R. China.

Extraction and Isolation. Axinyssa sp. (110 g, dry weight) was soaked in MeOH three times (3 × 1000 ml), and the MeOH soln. was evaporated under reduced pressure to give the MeOH extract (9.2 g). The MeOH extract was further partitioned with AcOEt and H_2O, and the org. layer was concentrated under reduced pressure to give the AcOEt extract (5.7 g). The AcOEt extract was

Table. *^{1}H- and ^{13}C-NMR Data of* **1**, **2**, *and* **3**. At 500 and 125 MHz, resp., in $CDCl_3$; δ in ppm, J in Hz. Values after the bias (/) are for the minor isomer.

Position	**1** ((*Z*)/(*E*) 3 : 2)		**2** ((*Z*)/(*E*) 3 : 1)		**3** ((*E*)/(*Z*) 5 : 2)	
	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)
H_{ax}–C(1)	1.77–1.83 (*m*)/1.75–1.81 (*m*)	39.9/41.2 (*t*)	1.01 (*ddd*, *J* = 13.0, 13.0, 3.0)/ 1.08 (*ddd*, *J* = 13.0, 13.0, 4.0)	43.7/43.9 (*t*)	1.13 (*ddd*, *J* = 13.0, 13.0, 3.5)	41.6/41.2 (*t*)
H_{eq}–C(1)	1.94–1.99 (*m*)/1.81–1.86 (*m*)		1.44 (*ddd*, *J* = 13.0, 3.0, 3.0)/ 1.48 (*ddd*, *J* = 13.0, 3.0, 3.0)		1.37 (*ddd*, *J* = 13.0, 5.0, 3.0)	
H_{ax}–C(2)	1.40–1.45 (*m*)	27.9/28.0 (*t*)	1.39–1.45 (*m*)/1.41–1.46 (*m*)	21.36/21.39 (*t*)	1.46–1.50 (*m*)	18.7/18.8 (*t*)
H_{eq}–C(2)	1.64–1.69 (*m*)		1.56–1.62 (*m*)/1.61–1.66 (*m*)		1.64–1.68 (*m*)	
H_{ax}–C(3)	1.39–1.43 (*m*)/1.40–1.45 (*m*)	34.4/34.5 (*t*)	1.06 (*ddd*, *J* = 13.0, 13.0, 3.0)/ 1.11 (*ddd*, *J* = 13.0, 13.0, 3.5)	40.7/41.0 (*t*)	1.56–1.60 (*m*)	42.8 (*t*)
H_{eq}–C(3)	1.79–1.84 (*m*)/1.82–1.86 (*m*)		1.52 (*ddd*, *J* = 13.0, 3.0, 3.0)/ 1.55 (*ddd*, *J* = 13.0, 3.0, 3.0)		1.68 (*ddd*, *J* = 11.0, 3.0, 3.0)	
C(4)	–	61.3/60.4 (*s*)	–	64.5/63.1 (*s*)	–	57.1/58.6 (*s*)
H–C(5)	1.583 (*dd*, *J* = 6.5, 6.5)/1.576 (*dd*, *J* = 6.5, 6.5)	37.182/37.180 (*d*)	1.13 (*dd*, *J* = 13.0, 3.0)/ 1.11 (*dd*, *J* = 13.0, 3.0)	55.8/55.7 (*d*)	1.32 (*d*, *J* = 11.0)	55.8 (*d*)
H_{ax}–C(6)	1.50–1.54 (*m*)/1.64–1.68 (*m*)	23.01/22.98 (*t*)	1.15–1.19 (*m*)/1.16–1.21 (*m*)	26.1/26.6 (*t*)	2.47 (*ddd*, *J* = 11.0, 11.0, 3.5)	44.5 (*d*)
H_{eq}–C(6)	1.82–1.87 (*m*)/1.90–1.95 (*m*)		1.70–1.74 (*m*)/1.72–1.76 (*m*)			
C(7) or H_{ax}–C(7)	–	140.0/140.5 (*s*)	–	43.6/43. 3 (*d*)	1.42–1.46 (*m*)	34.1 (*t*)
H_{eq}–C(7)	–		1.42–1.47 (*m*)/1.50–1.55 (*m*)		1.51–1.55 (*m*)	
H–C(8) or H_{ax}–C(8)	5.22 (br. *s*)/5.21 (br. *s*)	128.1/127. 9 (*d*)	1.28 –1.31 (*m*)/1.25–1.29 (*m*)	20.4/20.3 (*t*)	1.41–1.46 (*m*)/ 1.40–1.45 (*m*)	21.2/21.0 (*t*)
H_{eq}–C(8)	–		1.71–1.75 (*m*)/1.63–1.68 (*m*)		1.54–1.60 (*m*)/ 1.45–1.51 (*m*)	
H_{ax}–C(9)	1.75 (*d*, *J* = 14.5)/1.74 (*d*, *J* = 14.0)	54.2/55.6 (*t*)	1.49 –1.53 (*m*)/1.40–1.44 (*m*)	40.3/39.5 (*t*)	1.15 (*ddd*, *J* = 13.0, 13.0, 3.5)	45.4/45.1 (*t*)
H_{eq}–C(9)	2.03 (*d*, *J* = 14.5)/1.83 (*d*, *J* = 14.0)		2.08 (*ddd*, *J* = 13.0, 3.0, 3.0)/ 2.72 (*ddd*, *J* = 13.0, 3.0, 3.0)		1.28–1.33 (*m*)	
C(10)	–	46.2 (*s*)	–	33.6/33.8 (*s*)	–	35.9 (*s*)

Table (cont.)

Position	**1** ((*Z*)/(*E*) 3 : 2)		**2** ((*Z*)/(*E*) 3 : 1)		**3** ((*E*)/(*Z*) 5 : 2)	
	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)
H–C(11) or C(11)	2.12 (*sept.*, $J = 7.0$)/2.13 (*sept.*, $J = 7.0$)	35.0 (*d*)	–	61.5/62.7 (*s*)	–	150.4 (*s*)
Me(12) or H_a–C(12)	0.97 (*d*, $J = 7.0$)/0.98 (*d*, $J = 7.0$)	21.7/21.6 (*q*)	1.52 (*s*)/1.40 (*s*)	27.2/31.5 (*q*)	4.74 (br. *s*)/ 4.69 (br. *s*)	111.1/112.4 (*t*)
H_b–C(12)	–		–		4.86 (*d*, $J = 2.0$)/ 4.82 (br. *s*)	
Me(13)	0.87 (*d*, $J = 7.0$)	21.6/15.5 (*q*)	1.30 (*s*)/1.26 (*s*)	21.1/18.7 (*q*)	1.62 (*s*)	18.6 (*q*)
Me(14)	1.51 (*s*)	27.2 (*q*)	0.96 (*s*)/0.97 (*s*)	18.4/18.9 (*q*)	1.01 (*s*)/0.98 (*s*)	20.8/20.6 (*q*)
Me(15)	1.46 (*s*)	30.4 (*q*)	1.23 (*s*)/1.18 (*s*)	24.6/26.2 (*q*)	1.31 (*s*)	20.43/20.48 (*q*)
C(16)HO	8.03 (*d*, $J = 2.0$)/8.25 (*d*, $J = 12.5$)	160.6/163.7 (*d*)	8.13 (*s*)/8.24 (br. *s*)	159.7/160.5 (*d*)	8.12 (*d*, $J = 12.5$)/ 7.87 (*d*, $J = 2.5$)	161.7/160.2 (*d*)
NH	5.40 (*d*, $J = 2.0$)/5.82 (*d*, $J = 12.5$)		4.21 (br. *s*)		5.74 (*d*, $J = 12.5$)/ 5.01 (*d*, $J = 2.5$)	
OH	–		4.21 (br. *s*)		–	

fractionated by a silica-gel flash chromatography (FC) with hexane/AcOEt/MeOH stepwise gradient to afford 21 *Fractions. Fr. 6* and *7* (210 mg in total) were further fractionated by *prep*. RP-HPLC with a gradient of 20:80 up to 0:100 H_2O/MeCN to afford **2** (16 mg) and **3** (38 mg). *Fr. 10* (61 mg) was further purified by repeated RP-HPLC (H_2O/MeOH 60:40–0:100) to yield **1** (8 mg).

*(4*R**,5*R**,10*R**)-4-Formamidoeudesm-7-ene* (**1**). White solid. M.p. 61°. $[\alpha]_D^{20} = +46.7$ ($c = 0.15$, MeOH). IR (CH_2Cl_2): 3282, 3047, 2959, 2932, 2870, 2839, 2753, 1661, 1536, 1464, 1444, 1384, 1360, 1327, 1269, 1200, 1150, 1014, 971, 863, 731. ^{1}H- and ^{13}C-NMR: see the *Table*. HR-ESI-TOF-MS: 250.2158 ($[M+H]^+$, $C_{16}H_{28}NO^+$; calc. 250.2165).

*(4*R**,5*R**,7*S**,10*R**)-4-Formamidoeudesman-11-ol* (**2**). Colorless oil. $[\alpha]_D^{20} = +57.3$ ($c = 0.15$, MeOH). IR (CH_2Cl_2): 3549, 3321, 2998, 2965, 2931, 2862, 2848, 1665, 1458, 1442, 1379, 1365, 1358, 1315, 1289, 1246, 1161, 1110, 1016, 764, 750. ^{1}H- and ^{13}C-NMR: see the *Table*. HR-ESI-TOF-MS: 250.2254 ($[M+H-H_2O]^+$, $C_{16}H_{28}NO^+$; calc. 250.2165).

4α-Formamidogorgon-11-ene (**3**). Colorless solid. M.p. 106°. $[\alpha]_D^{20} = -117.5$ ($c = 0.20$, MeOH). IR (CH_2Cl_2): 3203, 3063, 3020, 2976, 2928, 2868, 2851, 1681, 1640, 1538, 1500, 1459, 1443, 1384, 1375, 1337, 1326, 1307, 1265, 1191, 1104, 1050. ^{1}H- and ^{13}C-NMR ($CDCl_3$): see the *Table*. ^{13}C-NMR ((D_6)DMSO, 125 MHz): 43.5/41.1 (C(1)); 18.1/18.4 (C(2)); 45.2/44.8 (C(3)); 55.8/56.6 (C(4)); 52.7 (C(5)); 43.8/44.0 (C(6)); 33.8/33.5 (C(7)); 20.9/20.7 (C(8)); 43.5 (C(9)); 35.1/35.3 (C(10)); 149.4/149.5 (C(11)); 111.0/111.3 (C(12)); 17.8/17.4 (C(13)); 21.2 (C(14)); 20.3/20.1 (C(15)); 161.6/160.4 (C(17)). HR-ESI-TOF-MS: 250.2211 ($[M+H]^+$, $C_{16}H_{28}NO^+$; calc. 250.2165).

X-Ray Crystallography. Crystals of **3** were obtained by recrystallization from AcOEt soln. X-Ray diffraction data were collected on a *Oxford Diffraction Xcalibur Nova* X-ray single-crystal diffractometer with CuK_α radiation ($\lambda = 1.54178$ Å) at r.t. The data were processed using CrysAlis. The structure was solved by direct method. The H-atoms were added in ideal positions and refined as riding models. The structure was refined using full-matrix least-squares based on F^2 with the program SHELXL.

Cytotoxicity Assay. The *in vitro* cytotoxicities of **1**, **2**, and **3** were determined by means of the colorimetric MTT (= 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay. The human nasopharyngeal cancer cell line CNE-2, human cervical cancer cell line HeLa, and human normal liver cell line L02 were seeded in 96-well plates at a density of 3×10^7 cells/l, and the compounds were added at various concentrations (0.125–25 mg/l). After 48 h, MTT was added to the culture medium at a final concentration of 0.5 mg/ml, and the plates were incubated for 4 h at 37°. The insoluble formazan product was then precipitated by centrifugation, and the supernatant was removed. The formazan crystals were dissolved in DMSO (150 µl) with gentle shaking at r.t. The UV/VIS absorbance at 570 nm was recorded with a *Bio-tekELx800* (*Bio-tek*, USA) microplate reader, and the data were analyzed in the usual way.

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Received October 9, 2007