MULUNDOCANDIN, A NEW LIPOPEPTIDE ANTIBIOTIC

II. STRUCTURE ELUCIDATION

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(Received for publication September 4, 1986)

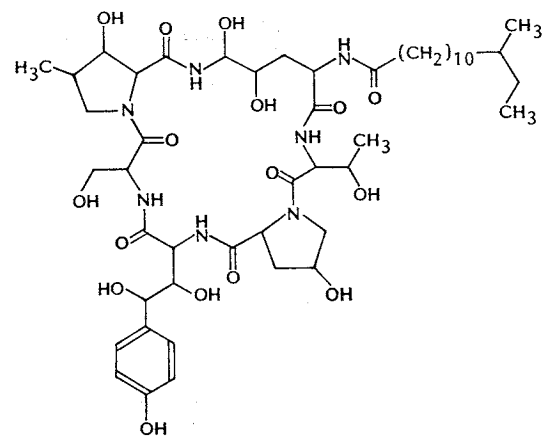
The structure of a new antifungal antibiotic, mulundocandin, $C_{48}H_{77}N_7O_{16}$, was elucidated by high field NMR experiments *e.g.*, homo- and heteronuclear correlation spectra, distortionless enhancement by polarization transfer (DEPT) spectra as well as nuclear Overhauser effect. The compound is a lipopeptide antibiotic belonging to the echinocandin class.

A new lipopeptide antifungal antibiotic, named mulundocandin, was isolated from the culture filtrate of *Aspergillus sydowi* (Bainier and Sartory) Thom and Church var. nov. *mulundensis* Roy[1]. The antibiotic is a new member of the echinocandin type of antibiotics which are neutral cyclic peptides with a fatty acid side chain[2]. In this paper we report the structure elucidation of mulundocandin.

The antibiotic, present both in the culture filtrate and mycelium, was isolated from the ethyl acetate extract of the culture filtrate by silica gel column chromatography and droplet counter-current chromatography. Mulundocandin (**1**) was obtained as a colorless amorphous powder, mp 225°C; $[\alpha]_D^{25}$ −42.77° (*c* 1.6, MeOH); elemental analysis C 55.70, H 7.82, N 11.29%.

The molecular weight of the compound was deduced to be *m/z* 1,007 from the fast atom bombardment MS (FAB-MS) of the cluster ions of mulundocandin with lithium and sodium. Thus in the presence of lithium iodide in 3-nitrobenzyl alcohol matrix the $[M+Li]^+$ peak appeared at *m/z* 1,014 and in the presence of sodium acetate in the same matrix the $[M+Na]^+$ peak was observed at *m/z* 1,030. Without any additive to promote clustering the compound did not exhibit the $[M+H]^+$ peak as the most abundant peak due to extensive loss of H_2O—the most intense peak was observed at *m/z* 990 and in addition, an $[M+Na]^+$ cluster ion of variable intensity appeared.

The electron impact MS (EI-MS) of the trimethylsilylated mulundocandin gave a cluster of peaks at nominal mass of 1,799. This corresponds to trimethylsilylation at 11 sites. The intensity of the different signals of the isotopic cluster of the molecular ion matched with the intensity pattern of the isotopic cluster calculated for $C_{61}H_{165}N_7O_{16}Si_{11}$. The molecular formula of mulundocandin is then $C_{48}H_{77}N_7O_{16}$. The UV



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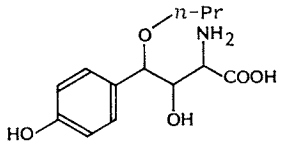
spectrum of mulundocandin[1] (λ_{max} 223 (sh), 275, 282 (sh) nm in methanol; λ_{max} 245, 290 nm in alkaline methanol) is similar to the UV spectrum of the echinocandin type of antibiotics[2]. Mulundocandin (**1**) gave no coloration with ninhydrin. However upon acid hydrolysis, the hydrolysate showed intense violet coloration with ninhydrin indicating the presence of amino acids. The acid hydrolysate also had an ether extractable compound. Hence mulundocandin, like other echinocandins[2], is a cyclic peptide with a lipophilic side chain. This is further confirmed by the IR spectrum[1] (3333, 2941, 1640, 1613, 1515, 1413, 1220 and 1060 cm^{-1}) and ^1H NMR spectrum[1] (7.24~7.99 ppm, 5 amidic protons exchanging with D_2O; 1.18~1.32 ppm, 18 aliphatic protons) of mulundocandin.

Amino acid analysis of mulundocandin showed the presence of serine, threonine and hydroxyproline. Since unusual amino acids are also present in echinocandin type of antibiotics such amino acids have to be identified by alternate methods.

Acid Hydrolysis of Mulundocandin (**1**)

Concentration of the ethereal extract of the acid hydrolysate (6 N HCl, 110°C, 20 hours) furnished a fatty acid (**2**) which upon treatment with ethereal diazomethane gave the corresponding ester (**3**).



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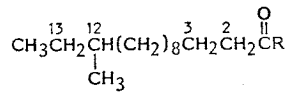


2 R = OH

3 R = OCH_3

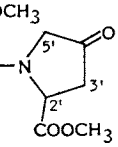
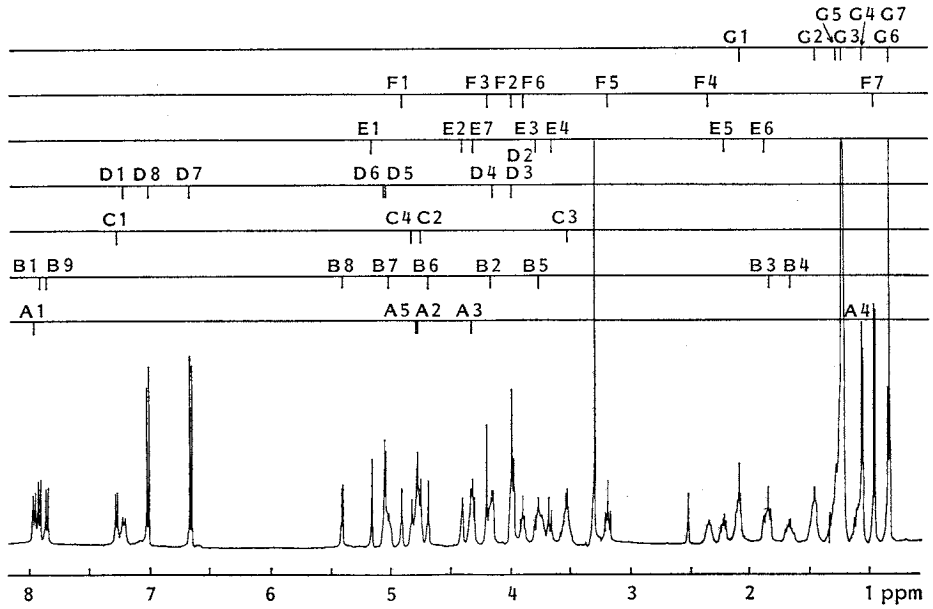
4 R = 

Fig. 1. 400 MHz ^1H NMR spectrum of mulundocandin.

All spin systems identified in a COSY-spectrum are indicated by bars centered at the chemical shift of the signal.



GC-MS of the ester **3** gave the molecular ion at *m/z* 256 corresponding to the molecular formula $C_{14}H_{29}COOCH_3$. As revealed by the ^1H NMR spectrum, the methyl ester **3** has a CH_2CH_3 group (0.84, t, J=7 Hz, 3H) and a $CHCH_3$ group (0.82, d, J=7 Hz, 3H). The fatty acid **2** is then a branched

Table 1. Proton-proton correlation spectrum of mulundocandin.

Spin	Multiplicity	Chemical shift (ppm)	Intensity	Coupling partner
A1	d	7.99	1	A2
A2	dd	4.80	1	A1, A3
A3	m	4.32	1	A2, A4, A5
A4	d	1.08	3	A3
A5	d	4.81	1	A3
B1	d	7.94	1	B2
B2	ddd	4.18	1	B1, B3, B4
B3	m	1.84	1	B2, B4, B5
B4	m	1.66	1	B2, B3, B5
B5	m	3.76	1	B3, B4, B6, B7
B6	d	4.69	1	B5
B7	m	5.02	1	B5, B8, B9
B8	d	5.43	1	B7
B9	d	7.87	1	B7
C1	d	7.30	1	C2
C2	m	4.78	1	C1, C3
C3	m	3.53	2	C2, C4
C4	t	4.83	1	C3
D1	d	7.24	1	D2
D2	m	3.98	1	D1, D3
D3	m	3.98	1	D2, D4, D6
D4	dd	4.17	1	D3, D5, D7, D8
D5	d	5.07	1	D4
D6	d	5.08	1	D3
D7	d	6.69	2	D4, D8, D9
D8	d	7.05	2	D4, D7, D9
D9	s	9.26	1	D7, D8
E1	d	5.12	1	E2
E2	m	4.41	1	E1, E3, E5, E6
E3	dd	3.78	1	E2, E4
E4	dd	3.68	1	E2, E3, E5
E5	m	2.21	1	E2, E4, E6, E7
E6	m	1.85	1	E2, E5, E7
E7	dd	4.32	1	E5, E6
F1	d	4.92	1	F2
F2	m	4.00	1	F1, F3, F4
F3	d	4.21	1	F2
F4	m	2.23	1	F2, F5, F6, F7
F5	m	3.19	1	F4, F6
F6	m	3.90	1	F4, F5
F7	d	0.97	3	F4
G1	m	2.09	2	G2
G2	m	1.46	2	G1, G3
G3	m	1.26	17	G2, G4, G5, G6, G7
G4	m	1.11	1	G3, G5
G5	m	1.30	1	G3, G4
G6	d	0.84	3	G3
G7	t	0.84	3	G3

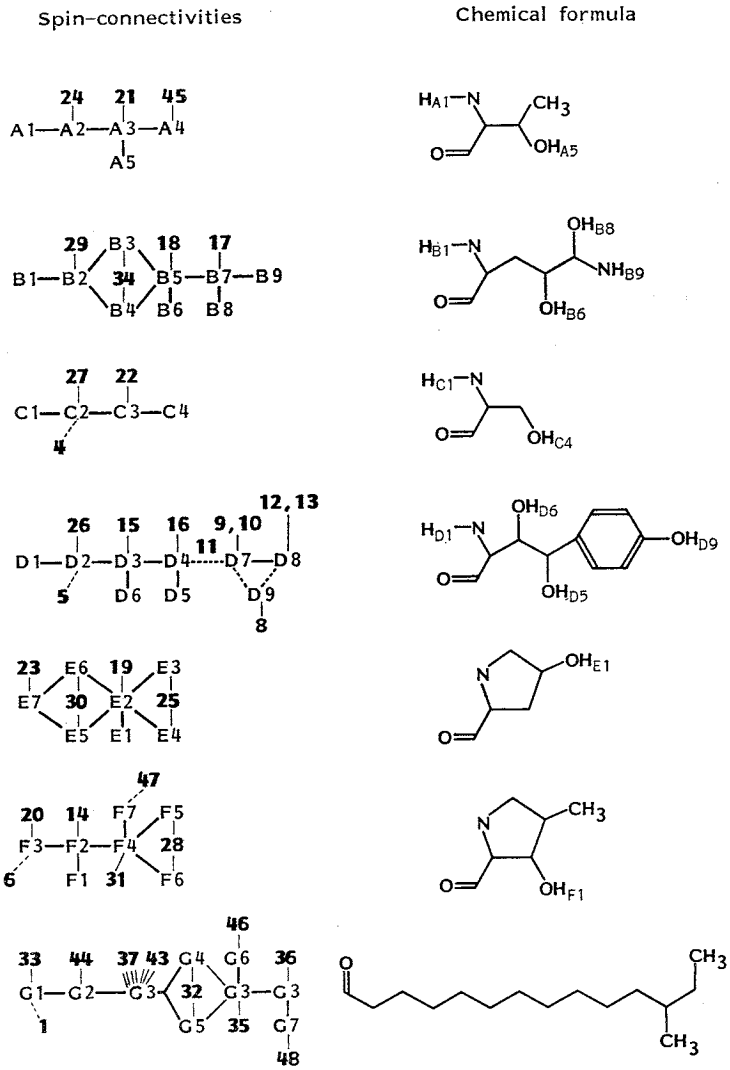
chain acid—methyl substituted tetradecanoic acid—and the methyl substituent is linked to any carbon between C-2 to C-12.

Resolving the methylene envelope in the ^{1}H NMR spectrum of **3** with lanthanide shift reagent [Eu(fod)$_3$] ruled out any substitution up to C-8. The position of the methyl substitution was inferred based on the comparison of the chemical shifts of C-10 to C-15 in the ^{13}C NMR spectrum with the calculated chemical shift values[8]. With the methyl substituent at C-12 the chemical shifts for C-10 to C-15 agree well with the calculated values obtained from the appropriate branched long chain hydrocarbons. Any other position of the group gives quite different chemical shifts for C-10 to C-14.

Shorter hydrolysis period (6 N HCl, 100°C, 2 hours) and esterification with diazomethane enabled

Fig. 2. Spin-connectivities detected in a two dimensional COSY-spectrum and a proton-carbon heteronuclear correlation spectrum are depicted on the left side.

Each spin system, consisting of several protons and carbons (signal numbers in the ^{13}C NMR spectrum are indicated in bold numerals on the left side), can be correlated with a chemical formula by interpretation of spin-connectivities and chemical shift as is shown on the right side.



isolation of an additional lipophilic compound **4** by preparative HPLC using silica gel. The compound **4** showed weak ninhydrin color reaction and was found to be the methyl ester of *N*-(12-methyltetradecanoyl)-4-oxoproline. 4-Oxoproline is known to be produced during acid hydrolysis of 4,5-dihydroxyornithine moiety present in echinocandin type of antibiotics[4]. The isolation of **4** established the point of linkage of the fatty acid part with the peptide - amino acid portion.

Compound **5** was isolated by successive chromatographic purification (Sephadex LH-20, silica gel, preparative HPLC) of the crude acid hydrolysate obtained under much milder conditions (6 N HCl, PrOH, 25°C for 3 days/37°C for 1 day, freeze-drying). Acids such as **5** are present in the echinocandin type of antibiotics[4]. However compound **5** appears to be an artifact since there is no evidence for the presence of a OC_3H_7 group in mulundocandin (**1**). It is conceivable that the benzylic ether is formed from the propanol used in the reaction.

The above experiments indicated the nature of only some of the constituents of mulundocandin. Also the sequence of the amino acids remained to be established. The structure of the individual amino acids and their sequence were then elucidated using conventional NMR experiments.

The proton-proton correlation spectrum (COSY)[5~7] (Fig. 1 and Table 1), established the different spin systems (Fig. 2) and thus the structure of all the constituents of mulundocandin. The proton-carbon correlation spectrum[8,9] and the distortionless enhancement by polarization transfer (DEPT) spectrum[10,11] were used to assign the ^{13}C NMR signals of mulundocandin (Table 2). These results

Table 2. ^{13}C NMR spectrum of mulundocandin.

Signal	Multiplicity[a]	Chemical shift (ppm)	Connected to/assignment[b]	Signal	Multiplicity[a]	Chemical shift (ppm)	Connected to/assignment[b]
1	s	171.97	G1/carbonyl[c]	25	t	55.70	E3+E4
2	s	171.62	Carbonyl	26	d	53.58	D2
3	s	170.44	Carbonyl	27	d	51.91	C2
4	s	170.37	C2/carbonyl[c]	28	t	51.06	F5+F6
5	s	169.41	D2/carbonyl[c]	29	d	49.48	B2
6	s	168.88	F3/carbonyl[c]	30	t	37.26	E5+E6
7	s	168.23	Carbonyl	31	d	37.04	F4
8	s	156.53	Hydroxyphenyl	32	t	35.96	G4+G5
9	d	132.45	D7	33	t	35.07	G1
10	d	132.45	D7	34	t	34.60	B3+B4
11	s	128.11	Hydroxyphenyl	35	d	33.69	G3
12	d	114.61	D8	36	t	29.35	G3
13	d	114.61	D8	37	t	29.03	G3
14	d	74.99	F2	38	t	29.02	G3
15	d	73.59	D3	39	t	28.90	G3
16	d	73.18	D4	40	t	28.86	G3
17	d	72.26	B7	41	t	28.83	G3
18	d	69.21	B5 (+B6)	42	t	28.50	G3
19	d	69.13	E2	43	t	26.42	G3
20	d	68.79	F3	44	t	25.31	G2
21	d	66.15	A3	45	q	19.39	A4
22	t	62.28	C3	46	q	19.03	G6
23	d	60.72	E7	47	q	11.14	F7
24	d	56.26	A2	48	q	10.73	G7

[a] According to the DEPT spectrum.
[b] According to proton-carbon heteronuclear correlation spectrum.
[c] See Table 4.

Table 3. NOE studies of mulundocandin.

Irradiation	Response	Intensity enhancement (%)
A1	C1	3.5
	C2	3.4
	B2	16.7
B1	B3	3.0
	B4	4.6
	B5	7.8
	G1	9.4
B9	B7	5.0
	B8	2.4
	F2	4.4
	F3	28.6
C1	C2	7.5
	C3	3.0
	D2, D3	7.3
	A1	2.1
C3	F2	1.2
	F6	2.2
	B6	1.5
D1	E7	8.2
	D2, D3	12.0
	D4	5.0

confirmed the structure of the constituents of mulundocandin. The sequence of the subunit was determined by nuclear Overhauser effect (NOE)-difference spectroscopy[12,13] and by long range proton-carbon correlation spectroscopy[14].

Irradiating the amide protons and observing the enhancement of spatially proximate protons (Table 3) led to the sequence of the spin systems. The NOE experiments also established conformational restrictions within the molecule, some of which are depicted in Fig. 3.

The proton-carbon correlation *via* small couplings[14] optimized for $J=12.5$ Hz (Table 4) confirmed this sequence, as three carbonyl-signals gave correlation peaks to a neighbouring amide proton as well as to a C-H in a different spin system. This established the connectivities of spin systems B. . .G, B. . .F and C. . .D which are all in agreement with the sequence elucidated from NOE experiments.

Fig. 3. The sequence of all spin-system-subunits, established using the selective steady state NOE's (Table 3) and supported by proton-carbon long range couplings of several amide protons to the neighbouring carbonyl (Table 4), are depicted.

Arrows indicate NOE's, which can be interpreted as spatial proximity of the protons involved—thick lines are used if the distance is small (large NOE) and thiner lines are used if the distance is large (small NOE).

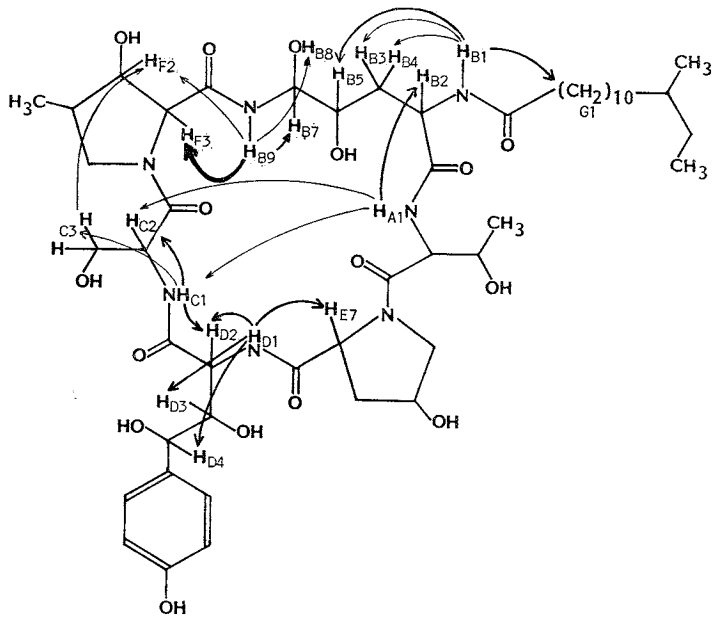


Table 4. Long range carbon-proton correlation of mulundocandin.

Signal	Chemical shift (ppm)	Correlations to protons	Assignment
1	171.97	G1, G2, B1	C=O, 12-methyltetradecanoic acid
4	170.37	C2	C=O, serine
5	169.41	C1, D2	C=O, 2-amino-3,4-dihydroxy-4-(4-hydroxyphenyl)butyric acid
6	168.88	B9, F3	C=O, 3-hydroxy-4-methylproline

Interpretation of the experiments described above unambiguously establishes the constitutional formula of mulundocandin as **1**. This structure of mulundocandin is not known for any antibiotic of the echinocandin type reported in the literature. Hence mulundocandin (**1**) is a new member of the family of the echinocandin type of antibiotics.

Experimental

General

Diazomethane solution in ether was prepared according to the published procedure[15]. GC analysis was performed using Perkin-Elmer 900 gas chromatograph with 3% OV-1 on Gas-chrome Q column maintained at 170°C; the injector part and detector temperature were 200°C. MS were obtained using AEI MS-902S spectrometer equipped with an on-line data system DS-50 SM. The NMR spectra were recorded in DMSO-d_6 on Bruker AM 400 WB spectrometer operating at 400.13 MHz for protons and 100.62 MHz for carbons. The 270 MHz ^{1}H NMR spectra and 68 MHz ^{13}C NMR spectra were recorded on Brucker AM-270 spectrometer.

Acid Hydrolysis of Mulundocandin

(a) Preparation of **3**: Mulundocandin (560 mg) and 4 ml of 6 N hydrochloric acid were heated at 110°C for 20 hours in a sealed vial. After cooling, the vial was opened and the reaction mixture was diluted with water (10 ml). It was then extracted with distilled diethyl ether (3×40 ml). The ethereal extracts were combined, dried over anhydrous sodium sulfate and concentrated under reduced pressure to obtain **2** (100 mg). It was then dissolved in diethyl ether and treated with excess ethereal diazomethane solution. After 1 hour at 5~10°C the ethereal solution was concentrated under reduced pressure to obtain a beige-colored liquid **3** (100 mg). GC analysis showed **3** (retention time: 8 minutes 12 seconds) to be accompanied by another minor compound (retention time: 6 minutes 30 seconds).

EI-MS *m/z* 256 (26% relative intensity, M^+), 199 (20%, $M-C_4H_9$), 143 (18%, $(CH_2)_6COOCH_3^+$), 87 (62%, $CH_2=CHC(OH)OCH_3^+$), 74 (100%, $CH_2=C(OH)OCH_3^+$); ^{1}H NMR (270 MHz, $CDCl_3$) δ 0.82 (d, J=7 Hz, CH_3CH), 0.84 (t, J=7 Hz, CH_3CH_2), 1.1 (m, CH_2), 1.25~1.30 (m, 8×CH_2+CH), 1.4 (m, 3-CH_2), 2.28 (t, J=7 Hz, 2-CH_2), 3.65 (s, OCH_3); ^{13}C NMR (68 MHz, $CDCl_3$) δ 51.3 (OCH_3), 174.1 (C=O), 34.2 (C-2), 25.0 (C-3), 29.2~29.7 (C-4. . .C-9), 27.1 (C-10, calcd 27.1), 36.1 (C-11, calcd 36.7), 34.5 (C-12, calcd 34.6), 30.0 (C-13, calcd 29.7), 11.3 (C-14, calcd 11.1), 19.3 (C-15, calcd 19.0).

(b) Preparation of **3** and **4**: Mulundocandin (150 mg) was dissolved in 2 ml of 6 N hydrochloric acid and left standing for 2 hours at 100°C in a sealed vial. The preparation was then cooled to 0°C and extracted with diethyl ether. To the washed and dried ether extract an ethereal diazomethane solution was added. After 5 minutes the volatile components were removed in mild vacuum. The residue, dissolved in 0.2 ml CH_2Cl_2, was applied onto a column (4 mm×10 cm) filled with silica gel (Grace GmbH, Worms, 60 Å HPLC, 5 μm). The column was then eluted with CH_2Cl_2 at a pressure of ~70 bar. Along with the compound **3** (12.7 mg), another compound **4** (1.2 mg) showing a weak ninhydrin positive reaction was isolated.

EI-MS *m/z* 367.2717 (11% relative intensity, M^+, calcd 367.2723 for $C_{21}H_{37}NO_4$), 308 (6%, $M-COOCH_3$), 225 (4.5%, $C_{14}H_{29}CO^+$), 198 (5%, $M-C_{12}H_{25}$), 185 (65%, $M-C_{13}H_{26}$), 143 (25%, $M-C_{14}H_{28}CO$), 84 (100%, amine fragment of oxoproline); IR (KBr) ν_{max} cm^{-1} 1660 (amide), 1750

(ester), 1770 (5-membered ring carbonyl); ^{1}H NMR (270 MHz, $CDCl_3$) δ 0.82 (d, J=7 Hz, CH_3CH), 0.84 (t, J=7 Hz, CH_3CH_2), 1.1 (m, CH_2), 1.25~1.35 (m, 8×CH_2+CH), 1.65 (m, 3-CH_2), 2.27 (t, J=7 Hz, 2-CH_2), 2.6 and 2.9 (ABX system, J_{AB}=18 Hz, J_{AX}=3 Hz, J_{BX}=10 Hz, 3'-CH_2), 3.75 (s, OCH_3), 3.97 and 4.03 (AB system, J_{AB}=16 Hz, 5'-CH_2), 5.0 ppm (dd, J=3 and 10 Hz, 2'-CH).

(c) Isolation of **5** from Mild Hydrolysis Reaction: Mulundocandin (400 mg) was dissolved in 40 ml of 6 N hydrochloric acid and 5 ml of propanol. The reaction mixture under argon was allowed to stand for 3 days at room temperature and for 1 day at 37°C. The solvent was then removed by freeze-drying. The reaction mixture was initially purified on a Sephadex LH-20 column (3.5×85 cm) in methanol. An enriched fraction (76 mg) was chromatographed on silica gel using methanol in chloroform (20% methanol in chloroform increased to 30% methanol in chloroform) as eluent. The main hydrolysis product, showing λ_{max} at 276, was then purified by preparative HPLC (Lichrosorb RP-18), E. Merck, Darmstadt; the mobile phase was 0.5% TFA in water to which a gradient of the same solvent with 20% CH_3CN has been added) to obtain **5** (1.3 mg). The structure of the compound was based on EI-MS spectra of its silylation product.

EI-MS m/z 557.2841 (1.2%, M^+, calcd 557.2844 for $C_{25}H_{51}NO_5Si_4$), 440 (9%, M−$COOSi(CH_3)_3$), 339 (18%, $(CH_3)_3SiOC_6H_4CHOC_3H_7CHOSi(CH_3)_3^+$), 320 (5%, $^+CHOSi(CH_3)_3CHNHSi(CH_3)_3$-$COOSi(CH_3)_3$), 237 (100%, $(CH_3)_3SiOC_6H_4CHOC_3H_7^+$), 218 (45%, $^+CHNHSi(CH_3)_3COOSi(CH_3)_3$). All fragments have been confirmed by high resolution MS (HR-MS).

NMR Experiments

(a) COSY-spectrum: The spectrum was recorded with the standard pulse scheme $\pi/2$-t_1-$\pi/4$-t_2 ($\pi/2$=9.0 μseconds) and phase cycling of both radio-frequency (rf)-pulses[6,7]. The data matrix of 1,024×2,048 points resulted from 1,024 spectra, 4K each, with a maximum acquisition time of 284.6 mseconds in t_1 and 573 mseconds in t_2.

(b) Proton-carbon Correlation Spectrum: The spectrum was recorded with the standard pulse-scheme $\pi/2$ (^{1}H)-$t_1/2$-π (^{13}C)-$t_1/2$-Δ-$\pi/2$ (^{1}H)+$\pi/2$ (^{13}C)-Δ-t_2 (BB dec) and phase cycling of all rf-pulses[16] ($\pi/2$(^{1}H)=32 μseconds, $\pi/2$(^{13}C)=13.6 μseconds). The data matrix of 512×4,096 points resulted from 512 spectra, 4K each, with a maximum acquisition time of 213.5 mseconds in t_1 and 275 mseconds in t_2. Δ was optimized for a proton-carbon coupling constant of 152 Hz (3.3 mseconds).

The same pulse sequence was used for detecting long range connectivities (Δ=40 mseconds optimal for a proton carbon coupling constant of 12.5 Hz). The data matrix of 512×1,024 points resulted from 512 spectra, 1K each, with a maximum acquisition time of 192.3 mseconds in t_1 and 512 mseconds in t_2.

(c) Selective Steady State NOE Difference Spectra: Saturation of the indicated signals was obtained by 10 seconds low power irradiation during the relaxation time. Spectra without irradiation were subtracted from these free induction decays (FID) and gave, after Fourier transformation, the difference spectra. Intensities are calculated with respect to the saturated signal (=100%).

Acknowledgment

We are thankful to Dr. H. H. SCHÖNE, Pharma Biochemie, Hoechst AG., Frankfurt for the amino acid analysis, Dr. G. C. S. REDDY and Mr. R. G. BHAT for technical assistance and Dr. P. K. INAMDAR for GC analysis.

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