

Cell Wall Active Antifungal Compounds Produced by the Marine Fungus *Hypoxylon oceanicum* LL-15G256

II. Isolation and Structure Determination

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Fermentations of the marine fungus *Hypoxylon oceanicum* (LL-15G256) were found to have potent antifungal activity. Isolation and purification of the antifungal agents provided two classes of compounds, macrocyclic polyactones and the lipodepsipeptides 15G256 γ (1), 15G256 δ (2) and 15G256 ϵ (3). The isolation and structure elucidation of the lipodepsipeptides, all containing D-glutamate, L-serine, and the rare amino acid β -ketotryptohan, are described in this paper.

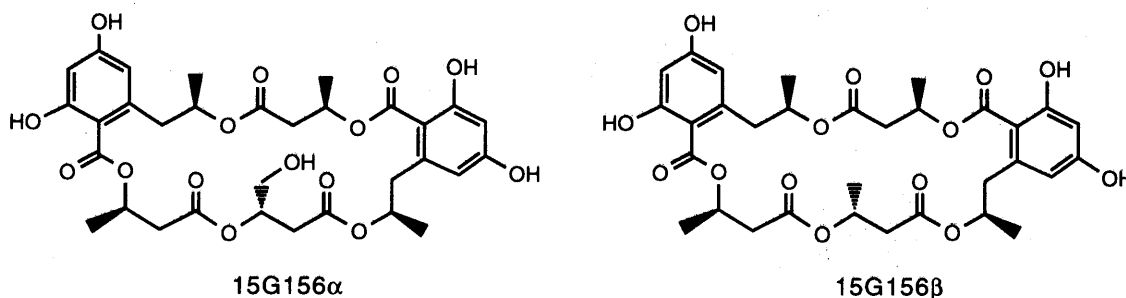
Our research program to discover novel, cell wall active, antifungal agents has been described in previous publications¹⁾ and in part in the preceding paper²⁾. Culture LL-15G256, taxonomically classified as the marine fungus *Hypoxylon oceanicum*, was found to produce antifungal activity in a screen designed to detect inhibitors of fungal cell wall formation in *Neurospora crassa*¹⁾. Bioactivity guided isolation and purification yielded three principal components named, in order of their elution from a reverse phase column, 15G256 α , 15G256 β and 15G256 γ . The UV chromophores of 15G256 α and 15G256 β were found to be identical, whereas the UV spectrum of 15G256 γ was different.

Subsequent structure elucidation of 15G256 α , origi-

nally the predominant antifungal component, and 15G256 β by NMR, MS and IR spectroscopy revealed that these compounds were macrocyclic polyactones (see Figure 1), identical with two recently reported antifungal products BK223-A and BK223-C³⁾ of the terrestrial fungi *Penicillium verruculosum* and *Scedosporium apiospermum* F26263⁴⁾. Besides having antifungal activity against phytopathogens, 15G256 α (BK223-A) was also reported as a nerve growth factor potentiator⁵⁾, and 15G256 β (BK223-C) was claimed to be an agonist for certain steroid hormones⁴⁾. A further series of these polyactones has been isolated⁶⁾ and will be reported elsewhere.

As the third component, 15G256 γ (1), was recognized

Fig. 1. Structures of the macrocyclic polyesters 15G256 α and 15G256 β which are identical with BK223-A and BK223-C³⁾, respectively.



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as an entirely different structural class of antifungal agent⁷), fermentation efforts were directed towards the improvement of production yields for this component²). Changing the medium from a Sabouraud-maltose to a glycerol-peptone base, raising the temperature to 28°C, and omitting seawater, increased the yield for 15G256 γ from 3 mg/liter to 400 mg/liter and provided this compound as the chief antifungal product. Minor components such as 15G256 δ (2) and 15G256 ϵ (3) were also generated under these conditions in good yields.

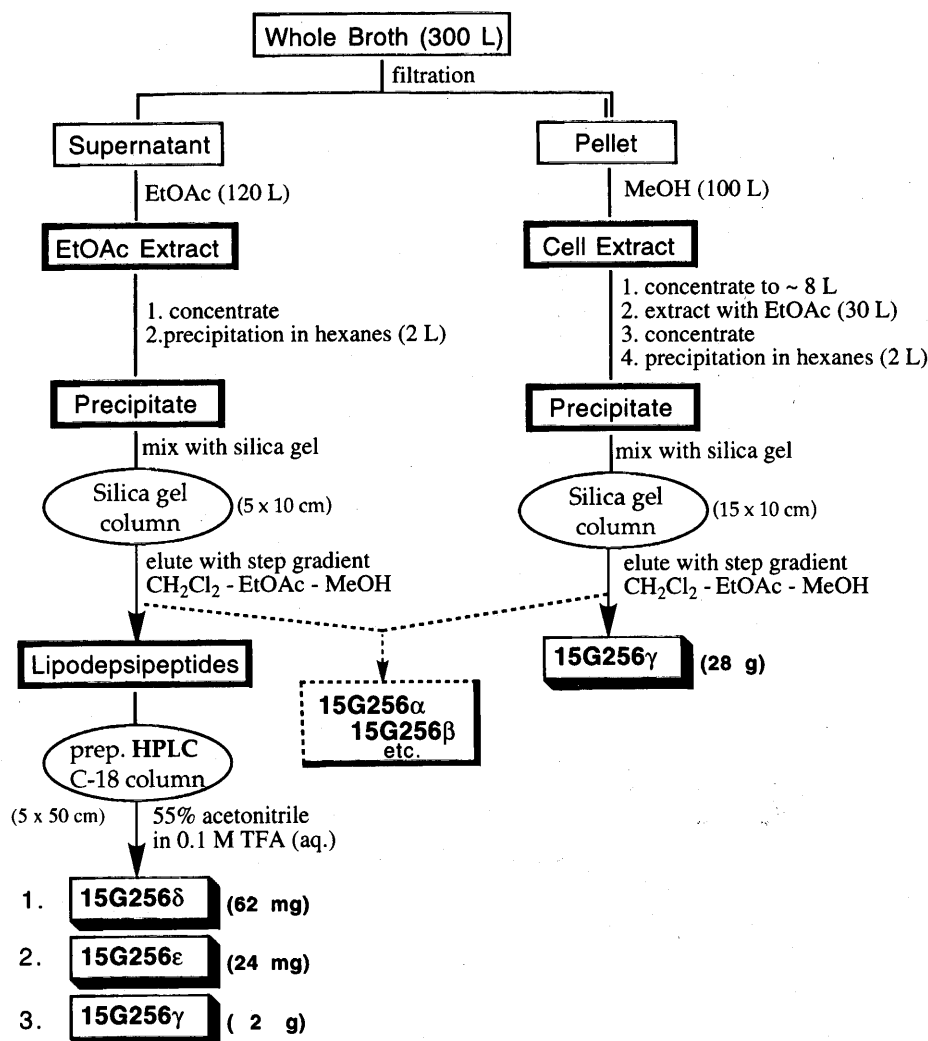
Isolation

Initial tests showed approximately equal antifungal potency in both the cell pellet and the supernatant liquid of fermentation broths. Therefore, the active compounds were recovered from both fractions through separate extractions (compare Scheme 1). The pellet was extracted by slurring with methanol or, preferably, 85% acetone followed by filtration. Subsequent concentration of the

filtrate yielded an aqueous mixture from which the active material was extracted with ethyl acetate, yielding a yellow oil upon evaporation of the solvent. Separation of the components was then accomplished by silica gel chromatography.

The supernatant was processed by extraction with ethyl acetate or, alternatively, by adsorption on Amberchrom CG 161dm and subsequent elution with methanol. Evaporation of the organic solvent gave a dark-brown, viscous oil which was then chromatographed on silica gel to separate the components. The silica gel column was eluted with solvent mixtures composed of increasing concentrations of ethyl acetate in methylene chloride (10%, 25%, 50%, and 100%), followed by a methanol wash in order to recover all applied material. The active constituents were separated on silica gel chromatography according to their polarities: 15G256 β eluted prior to 15G256 α , which was followed by 15G256 γ . 15G256 δ (2) and 15G256 ϵ (3) were not separated from 15G256 γ (1)

Scheme 1. Isolation of lipodepsipeptides from culture LL-15G256.



in this step. These compounds were eventually separated from each other and unrelated components by reverse phase chromatography using a Whatman CCS/C₈ column eluted with 72% MeOH or 60% acetonitrile mixed with 0.1 M TFA. Concentration of the active fractions yielded the lipodepsipeptides as white solids.

Structure Elucidation

The structure determination of 15G256 γ (**1**) was achieved by spectroscopic analysis. A molecular formula of C₃₃H₄₆N₄O₉ was determined by HRFAB mass spectrometry {[M+H]⁺ cal: 643.3343, found: 643.3349}. The number of carbon atoms was also evident from 33 individual ¹³C NMR signals recorded on d₆-DMSO solutions (Table 1). The chemical shifts indicated the presence of one conjugated carbonyl, five carboxyl or amide groups, eight olefinic, and nineteen aliphatic

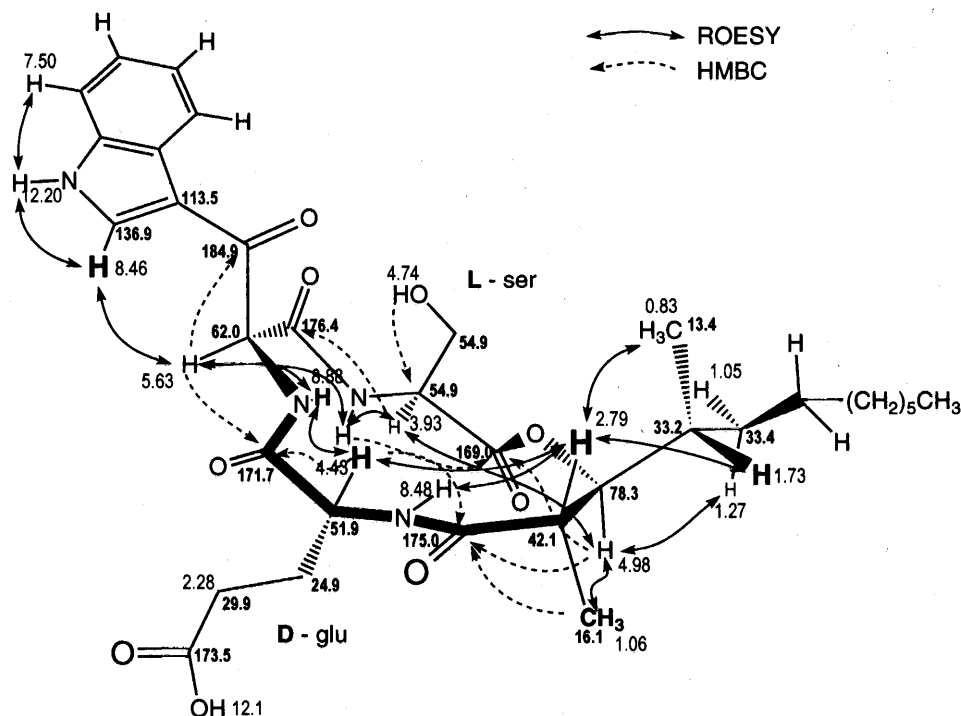
functionalities suggesting three amino acids and a fatty acid moiety. ¹H NMR data (see Table 4) in combination with DEPT, HMQC and HETCOR experiments placed 40 of the 46 hydrogen atoms on carbons, which indicated that 6 protons were connected to heteroatoms. The additional information from COSY and TOCSY experiments ascertained the presence of the substructures glutamic acid, serine and 2,4-dimethyl-3-oxy-dodecanoic acid.

Correlations from HMBC and COLOC measurements to identify the fourth substructure as β -ketotryptophan. Specifically, HMBC data confirmed indole as part of the β -ketotryptophan unit through correlations of the proton at 8.16 ppm with four relevant aromatic carbons (δ_c 136.6, 125.5, 123.3, 113.5) and the proton at 8.46 ppm to three of the same carbon atoms (δ_c 136.6, 125.5, 113.5) (see Tables 1 and 4). Although there was no HMBC

Table 1. ¹³C NMR shift values of 15G256 γ (**1**), 15G256 γ -hy (**4**, acyclic 15G256 γ), 15G256 δ (**2**) and their epimers (**a**) dissolved in d₆-DMSO.

Assignment	Mult (DEPT)	1 (γ)	1a (γ -epi)	4 γ -hy	4a (γ -hy-epi)	2 (δ)	2a (δ -epi)
W3	s	184.88	186.72	186.27	186.38	185.95	186.05
1	s	174.96	174.24	176.59	176.20	177.09	174.66
E5	s	173.54	173.54	174.04	174.12	173.63	173.71
E1	s	171.86	172.00	171.46	171.57	172.05	171.98
S1	s	169.02	168.81	171.26	171.26	169.27	170.01
W1	s	167.43	167.74	167.02	166.58	166.93	167.23
I2	d	136.88	133.88	135.82	135.69	137.10	134.00
I7a	s	136.56	136.40	136.48	136.46	136.60	136.29
I3a	s	125.51	125.33	125.64	125.64	125.53	125.47
I6	d	123.29	123.12	123.28	123.22	123.27	123.19
I5	d	122.38	122.00	122.23	122.11	122.32	122.03
I4	d	121.07	121.16	121.22	121.22	121.09	121.23
I3	s	113.45	114.68	113.59	113.55	114.15	113.74
I7	d	112.44	112.22	112.32	112.25	112.40	112.15
3	d	78.30	77.32	74.63	74.34	76.19	71.67
W2	d	62.04	58.49	59.42	59.61	60.33	59.43
S3	t	58.83	59.51	61.23	61.23	61.77	60.61
S2	d	54.92	55.01	54.85	54.85	54.55	55.55
E2	d	51.90	53.12	52.42	52.11	52.78	53.55
2	d	41.21	41.58	43.29	43.56	41.20	43.52
4	d	33.15	32.60	34.02	34.02	40.92	41.92
5	t	33.37	33.23	33.52	33.62	30.56	30.06
10	t	31.23	31.23	31.25	31.25	31.24	31.24
E4	t	29.94	30.23	30.13	30.17	30.07	30.07
7	t	29.14	29.14	29.28	29.31	29.43	29.18
8	t	28.96	28.96	28.98	29.00	28.95	28.95
9	t	28.64	28.64	28.66	28.66	28.68	28.66
6	t	26.77	26.74	26.64	26.64	26.31	24.79
E3	t	24.92	25.57	26.50	26.95	25.54	26.77
11	t	22.04	22.04	22.04	22.04	22.04	22.04
2-CH ₃	q	16.08	15.88	14.14	14.32	15.51	11.06
12	q	13.89	13.89	13.88	13.88	13.89	13.89
4-CH ₃	q (t)	13.43	13.07	12.65	12.72	65.03	62.85

Fig. 2. Selected HMBC and ROESY correlations to establish connectivities and relative configurations within the peptidic macrocycle.



between the β -keto- α -aminomethine-carboxamide function and the indole moiety, ROESY data indicated the close proximity between the indole proton at 8.46 ppm and the methine proton resonating at 5.63 ppm. The connection between these two units to form β -keto-tryptophan is clearly evidenced by the UV spectrum (compare Figure 6) which indicates a chromophore that is conjugated beyond the regular indole moiety fully consistent with β -oxotryptophan (UV_{max} of 281 nm for tryptophan vs. 310 nm for β -ketotryptophan⁸⁾a.

Finally, COLOC and HMBC measurements were utilized to establish the bonding sequence and to corroborate and refine the NMR assignments of the substructures as shown in Figure 2.

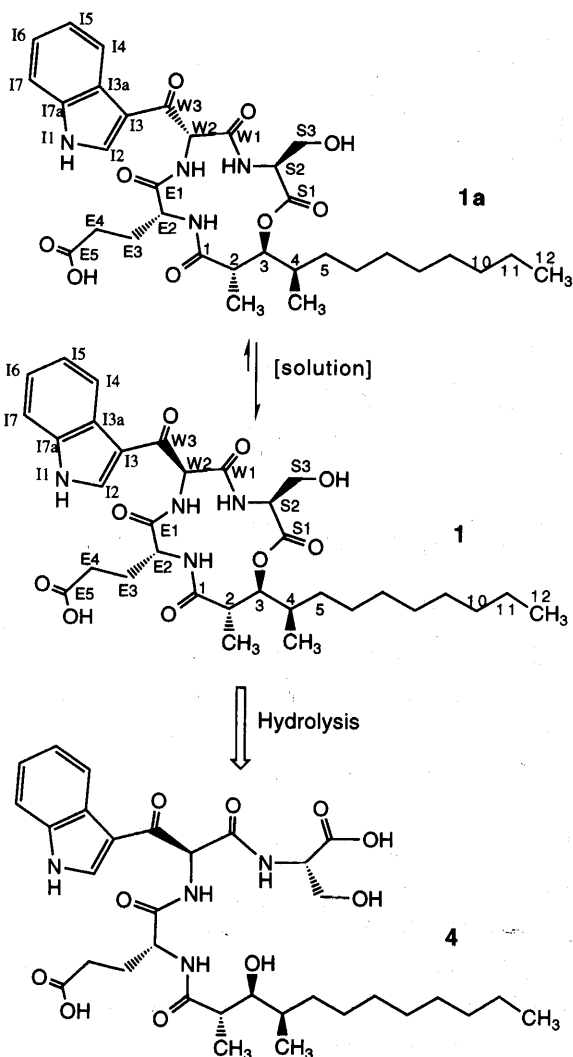
The structure of 15G256 δ (**2**) was readily elucidated by comparing the observed NMR chemical shifts and coupling patterns to those of **1** (see Tables 1, 3, 4 and 5). The FAB mass spectrum indicated a MW of 658 consistent with a molecular formula of $C_{33}H_{46}N_4O_{10}$ suggesting that **2** is an oxidized analog of **1**. Since both the 1H and DEPT NMR spectra of **2** revealed only two methyl groups instead of the expected three, a modification of one methyl group was suspected. A new

^{13}C NMR signal at 65.0 ppm correlating to proton signals at 4.26 and 3.65 ppm (in d_4 -MeOH $\delta_C = 66.9$, $\delta_H = 4.50$ and 3.77) respectively, confirmed the presence of an oxymethylene group instead of a methyl group. Couplings of these methylene protons with the vicinal proton at 1.75 ppm as revealed by COSY, placed this group at position 4 of the fatty acid chain. Thus, **2** contains a 2-methyl-3-hydroxy-4-oxymethylene-dodecanoic acid moiety instead of the 2,4-dimethyl-3-oxy-dodecanoic acid residue in 15G256 γ . Most importantly, three-bond HMBC's of the protons at both oxymethylene groups (C-4 methylene $\delta_H = 4.26$, 3.65; serine hydroxymethylene $\delta_H = 3.64$, 3.50) connected the carboxyl carbon of serine at 169.3 ppm with the new C-4 oxymethylene group of the fatty acid suggesting that the 3-hydroxyl group was no longer part of the lactone as seen in 15G256 γ (**1**). The different linkage is also apparent from the chemical shift of the oximethine proton (H-3) which resonates at 4.98 ppm for **1**, but at 3.15 ppm for **2**. The macrocycle of 15G256 δ (**2**) is therefore a fifteen-membered ring as depicted in Figure 4.

A MW of 614, consistent with a molecular composition of $C_{31}H_{42}N_4O_9$, was determined for 15G256 ϵ (**3**). A

a) The authors in ref. 8 present β -ketotryptophan in janthinocin B as a mixture of E and Z enols, but the NMR spectrum shows clearly one signal at ~ 185 ppm indicative of a conjugated ketone. The enol forms would enable an extended conjugation in the chromophore as observed for dehydrotryptophan (ref. 8) and shift the UV_{max} to ~ 340 nm.

Fig. 3. Structures of the lipodepsipeptide 15G256 γ (**1**), its epimeric form 15G256 γ -epi (**1a**), and the hydrolysate 15G256 γ -hy (**4**)



comparison of NMR data with those of **1**, indicated that **3** lacked two methylene groups. The ^1H NMR spectrum revealed a much reduced signal for the methylene protons at ~ 1.25 ppm and in the ^{13}C NMR spectrum two signals were absent at around 30 ppm. DEPT analysis indicated the presence of only two methylene carbons in this range (δ_{C} 30.40, 30.15) instead of the four observed for **1** (δ_{C} 30.50, 30.40, 30.31, 30.02). As the signal at 30.40 ppm (see Table 3) is assigned to the glutamic acid residue, the data signify that the fatty acid chain in 15G256 ϵ (**3**) is shortened by two methylene groups. Thus, the structure of 15G256 ϵ contains the 2,4-dimethyl-3-oxy-decanoic acid moiety as shown in Figure 5.

Fig. 4. Structures of the lipodepsipeptide 15G256 δ .

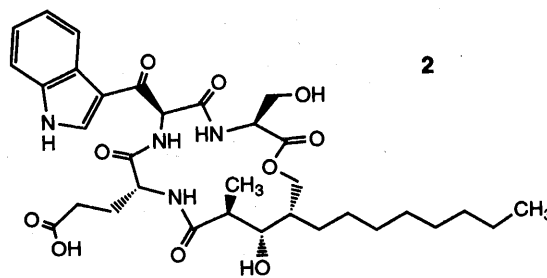
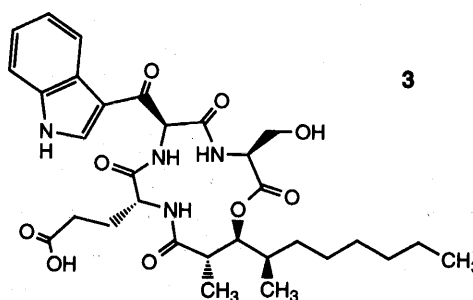


Fig. 5. Structures of the lipodepsipeptide 15G256 ϵ .

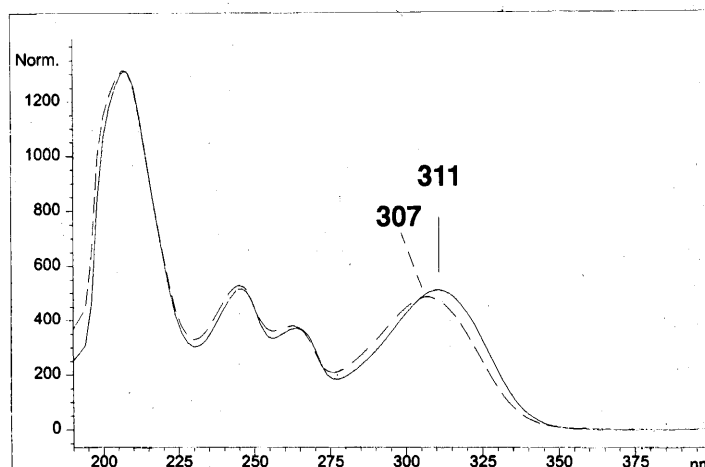


Epimerization at the α -Carbon (W-2) of β -Ketotryptophan

While the lactone bond in **1**, **2**, and **3** is relatively stable in solutions containing up to 0.5 M TFA and also survives methylation reactions (e.g. 1% H_2SO_4 in MeOH), a noticeable epimerization reaction occurs when these lipopeptides are left in solution. Hydrolytic conditions such as 4 M HCl or strong basic solutions such as 1 M NaOH, KOH or $\text{Ba}(\text{OH})_2$ cleave the lactone group within minutes and also accelerate the epimerization. Thus, **3** was treated with 1 M NaOH and $\text{Ba}(\text{OH})_2$ for 10 minutes to yield **4** and its W2 epimer **4a**. HPLC purification yielded **4** in pure form, but solutions of **4** slowly epimerized to eventually generate a 1 : 1 mixture of **4** and **4a**.

Once put into solutions purified forms of **1**, **2**, **3** and **4** readily epimerize whereas solids (powders) of these compounds do not change their composition. In DMSO solution, 15G256 γ (**1**) exists initially as a single isomer^b, and slowly undergoes epimerization at the α -carbon (W2) of β -ketotryptophan until equilibrium is reached after 1

^b This isomer exists in two rotameric forms in a ratio of 93 : 7 as observed in the ^1H NMR spectrum, indicated by the doublet signals for W-2 (H_α of the β -ketotryptophan moiety) at 5.63 ppm and 6.03 ppm respectively, or the two doublet signals for the corresponding NH at 8.88 and 9.06 ppm, respectively, through direct, but negative ROESY correlations.

Fig. 6. UV spectrum of 15G256 γ (—) and its epimer **1a** (---).

to 2 weeks at room temperature when *ca.* 25% of the unnatural isomer^c is generated. Evidence for this reaction is the appearance of a new doublet for W-2 ($H_{\alpha, \text{trp}}$) at 5.92 ppm while the signal at 5.63 ppm representing the natural form diminishes in intensity. HPLC analysis of the mixture, using MeOH as organic modifier, reveals a second peak (**1a**) with increased retention time (see Table 6). When this second peak was isolated and reanalyzed, a reversal to the original form was noted by the detection of two peaks, identical in retention time with the previous peaks for **1** and **1a**. UV spectra of the two peaks, obtained *via* diode array detection, show small but distinct differences in their maxima, the natural form (**1**) has a maximum at 311 nm whereas its epimer (**1a**) has this maximum at 307 nm (see Figure 6).

This epimerization reaction of **1** can be studied by NMR on d_4 -MeOH solutions as well. Now the intensity of the singlet for W-2 at 5.77 ppm disappears rapidly due the H-D proton exchange, which also effects the C-signal at 63.7 ppm in the ^{13}C NMR spectrum due to line broadening and changing of the signal from a singlet to a triplet that may be obscured by noise. Eventually, a second set of signals is observed, reaching again about 25% intensity compared to the original spectrum.

Similar observations were made with **2**, **3**, and **4**, but the ratios of native form to epimer at equilibrium vary for the individual species, it is 60:40 for **2** and **2a**, 75:25 for **3** and **3a**, and 50:50 for **4** and **4a**. It needs to be noted that the UV spectra of **4** and **4a** do not differ and

are identical with that of **1** (compare Figure 6).

15G256 γ -Methylester (**5**)

Although a small amount of the methyl ester of 15G256 γ was isolated from processed fermentation extracts, the occurrence of this derivative is presumed to be the result of storing crude 15G256 γ extracts as methanolic solutions, since HPLC analyses of freshly prepared fermentation extracts did not show this component. As the methyl ester derivative (**5**) was also prepared from **1** by reacting with diazomethane or acidified methanol, ample material for structure elucidation was available. In either reaction, **5** was obtained as a single, less polar reaction product with a mass of m/z 656. New signals in the ^1H and ^{13}C NMR spectrum at 3.56 ppm and 51.3 ppm respectively, indicated the presence of a methoxyl group. This group was added to the carboxyl function of the glutamate moiety as its ^{13}C -resonance shifted from 173.5 to 172.5 ppm and showed a three bond HMBC. As the remaining signals for **5** in both the ^{13}C NMR and ^1H NMR spectrum were virtually superimposable with those of 15G256 γ (**1**) (see Tables 2, 3 and 4), any alteration of the ring structure appears unlikely.

Surprisingly, purified **5** does not convert to **5a** in neutral solutions, but **5a** can be generated under acidic conditions. It therefore seems likely that epimerization in solution of **1**, **2**, **3**, and **4** is due to the presence of the acid functionality in glutamic acid.

^c This isomer also exists in two rotameric forms in a ratio of 90:10 as noted for two new doublets for W-2 at 5.92 and 5.79 ppm, respectively.

Table 2. ^{13}C NMR shift values of 15G256 γ (**1**) and its methyl ester (**5**) (solvent varies).

Assignment	Mult (DEPT)	1 (γ)	5 (γ -OMe)	5a (γ -epi-OMe)	5 (γ -OMe)
W3	s	184.88	184.88	186.72	185.60
1	s	174.96	174.95	174.24	176.61
E5	s	173.54	172.45	173.45	174.07
E1	s	171.86	171.76	171.90	172.41
S1	s	169.02	169.00	168.80	170.63
W1	s	167.43	167.41	167.72	168.57
I2	d	136.88	136.88	133.88	138.14
I7a	s	136.56	136.57	136.41	137.78
I3a	s	125.51	125.49	125.31	126.92
I6	d	123.29	123.28	123.11	124.85
I5	d	122.38	123.37	122.00	123.93
I4	d	121.07	121.06	121.17	122.63
I3	s	113.45	113.45	114.68	114.76
I7	d	112.44	112.43	112.22	113.41
3	d	78.30	78.28	77.30	80.79
W2	d	62.04	62.04	58.49	63.43
S3	t	58.83	58.83	59.51	61.59
S2	d	54.92	54.90	55.00	55.68
E2	d	51.90	51.76	53.01	54.32
E-OMe	q	—	51.31	51.32	52.32
2	d	41.21	41.21	41.58	43.34
4	d	33.15	33.12	32.58	35.70
5	t	33.37	33.35	33.21	34.50
10	t	31.23	31.22	31.23	32.69
E4	t	29.94	29.64	29.93	30.91
7	t	29.14	29.13	29.14	30.59
8	t	28.96	28.95	28.96	30.36
9	t	28.64	28.63	28.64	30.09
6	t	26.77	26.65	26.70	27.92
E3	t	24.92	24.84	25.50	26.00
11	t	22.04	22.03	22.04	23.44
2-CH ₃	q	16.08	16.03	15.89	16.98
12	q	13.89	13.88	13.88	14.43
4-CH ₃	q	13.43	13.41	13.08	14.38
	Solvent ppm	<i>d</i> ₆ -DMSO 39.50	<i>d</i> ₆ -DMSO 39.50	<i>d</i> ₆ -DMSO 39.50	<i>d</i> ₃ -MeCN 1.30, 118.25

Absolute Configuration

The absolute configurations of the amino acids serine and glutamic acid were determined by GC/MS analysis of their *n*-butyl heptafluorobutyrate derivatives using a Chirasil L-Val column. Commercial D- and L-amino acids were used as standards. The glutamic acid obtained by hydrolysis of 15G256 γ had the same retention time and mass spectrum as the standard D-glutamic acid derivative and serine coincided with the L-serine derivative. Therefore, 15G256 γ contains L-serine and D-glutamic acid as indicated in Figures 2 and 3.

The absolute configuration of β -ketotryptophan could

not be determined by the above procedure, as this unit does not survive the harsh hydrolytic conditions. Its absolute configurations was determined relative to those of D-glutamic acid and L-serine through correlations of NOE measurements in combination with computer modeling using the dynamics software QUANTA/CHARMm. Two-dimensional NMR spectroscopy methods (TOCSY, ROESY and NOESY) were utilized to obtain spin systems and proton-proton distances. These distances were used as constraints in dynamics simulations and energy minimization calculations.

Except for **4**, in the studied lipopeptides E-2 ($\text{H}\alpha$ of the glutamic acid residue) showed strong NOE's to two

Table 3. ^{13}C NMR shift values of 15G256 γ (**1**), 15G256 δ (**2**), and 15G256 ϵ (**3**).

Assignment	Mult (DEPT)	5 (γ -OMe)	1 (γ)	2 (δ)	1 (γ)	3 (ϵ)
W3	s	185.64	185.47	187.08	185.27	185.26
1	s	177.45	177.25	179.69	176.46	176.37
E5	s	174.68	175.80	176.12	173.98	173.99
E1	s	173.60	173.49	174.40	172.14	172.11
S1	s	170.70	170.60	171.14	170.35	170.30
W1	s	169.84	169.60	169.52	168.42	168.41
I2	d	137.56	137.67	137.93	137.96	137.96
I7a	s	138.41	138.21	138.53	137.79	137.78
I3a	s	127.18	127.10	127.28	127.00	126.96
I6	d	124.78	124.71	124.77	124.39	124.39
I5	d	123.75	123.69	123.72	123.41	123.42
I4	d	122.79	122.76	122.81	122.59	122.59
I3	s	115.27	115.13	115.98	114.79	114.76
I7	d	113.1	113.14	113.11	113.08	113.05
3	d	80.68	80.55	79.07	79.94	79.95
W2	d	n.d.	63.71	62.04	63.25	63.25
S3	t	61.25	61.25	63.71	61.34	61.23
S2	d	55.86	55.82	56.47	56.04	55.89
E2	d	53.79	53.83	55.05	54.00	53.81
2	d	43.69	43.59	43.37	43.17	43.13
4	d	35.70	35.54	42.95	35.14	35.14
5	t	34.96	34.86	32.35	34.56	34.56
10	t	32.96	32.86	33.04	32.59	32.55
E4	t	30.95	30.90	31.03	30.40	30.40
7	t	30.78	30.70	31.19	30.50	—
8	t	30.61	30.53	30.64	30.31	—
9	t	30.34	30.26	30.39	30.02	30.15
6	t	28.34	28.24	27.97	27.94	27.89
E3	t	26.34	26.35	26.83	26.04	25.99
11	t	23.63	23.55	23.69	23.29	23.27
2-CH ₃	q	16.49	16.52	16.28	16.82	16.77
12	q	14.39	14.40	14.38	14.32	14.30
4-CH ₃	q (t)	14.02	14.04	66.88	14.10	14.08
OMe		52.21	—			
	Solvent ppm	<i>d</i> ₄ -MeOH 49.00	<i>d</i> ₄ -MeOH 49.00	<i>d</i> ₄ -MeOH 49.00	<i>d</i> ₆ -Acetone 29.80, 206.10	<i>d</i> ₆ -Acetone 29.80, 206.10

other protons of the macrocycle, the NH of β -oxotryptophan and H-2 (but not to the 2-CH₃ group) of the fatty acid residue. Considering steric and energetic requirements, these protons are therefore on the same face of the molecule (assume *cis*-configuration) as shown in Figure 2. With no observed ROESY correlation between H-2 and H-3 and a coupling constant of around 10 Hz, the relative configuration of these two protons is required to be *trans*. The location of H-3 on the face opposite to E-2 is fully consistent with its ROESY correlation to S-2 (H α of the L-serine residue). In view of the *R* configuration of D-glutamic acid and the *S* configuration of the L-serine, the absolute configurations at C-2 and C-3 are therefore *S*.

As the amide bonded protons have ROESY's (not shown in Figure 2) between each other in both the natural form (**1**) and its epimer (**1a**), an orientation of all NH into the ring is required. The relative weak or non-existent dipolar couplings (NOE's) between the α -protons and the corresponding NH's (same amino acid), confirm the predicted *trans* conformation of these protons for all three amino acids. This may not always be apparent from their coupling constants, but is clearly evident for H α and NH of β -oxotryptophan in **1a** (see Table 5). Considering that **1** has a strong NOE between E-2 (H α of glutamic acid) and the peptidic NH of β -oxotryptophan, but **1a** does not, and that a strong ROESY correlation exists between W-2 and NH of serine for **1a**

Table 4. Observed ^1H NMR shift values of 15G256 γ (**1**), its acyclic form (**4**), 15G256 δ (**2**), and their epimers (**a**) in d_6 -DMSO.

Assignment	Signal	1 (γ)	1a (γ -epi)	4 γ -hy	4a (γ -hy-epi)	2 (δ)	2a (δ -epi)
L-Serine							
NH	d	6.97	8.00	8.44	8.63	7.69	9.07
S-2	m	3.93	4.35	4.24	4.25	4.37	4.20
S-3	t	3.37	3.45, 3.68	3.54, 3.67	3.65, 3.73	3.64, 3.50	3.75, 3.66
OH	t	4.74	4.79	~4.5	n.o.	n.o.	n.o.
(COOH)	—	—	—	~12.4	n.o.	—	—
β-Oxotryptophan							
NH	d	8.88	7.83	8.35	8.43	8.74	7.57
W-2 (indole)	d	5.63	5.92	5.92	5.90	5.70	5.94
H-1	d	12.20	12.03	12.10	12.13	12.18	12.07
H-2	d	8.46	8.27	8.53	8.61	8.47	8.56
H-4	d	8.16	8.16	8.12	8.12	8.17	8.14
H-5	m	7.22	7.22	7.22	7.20	7.20	7.22
H-6	m	7.24	7.23	7.24	7.22	7.24	7.23
H-7	d	7.50	7.48	7.50	7.48	7.50	7.48
D-Glutamic acid							
NH	d	8.48	8.16	8.21	8.06	8.59	8.54
E-2	m	4.43	3.99	4.30	4.36	4.33	4.26
E-3	m	1.83, 1.80	1.90	1.77, 1.98	1.78, 2.00	1.83, 1.80	1.83, 1.80
E-4	m	2.28, 2.16	2.32	2.29	2.29	2.31, 2.15	2.30
COOH	—	12.1	12.1	~12.4	12.4	n.o.	n.o.
2<i>S</i>,4<i>R</i>-Dimethyl-3<i>S</i>-oxy-duodecanoic acid							
2	dq	2.79	2.67	2.46	2.43	2.56	2.63
3	dd	4.98	5.07	3.46	3.44	3.15	3.54
4	m	1.73	1.73	1.46	1.46	1.75	1.49
5	m	1.27, 1.05	1.27, 1.05	1.32, 1.13	1.32, 1.13	1.23, 1.14	1.19, 1.08
6	m	1.31	1.31	1.20	1.20	1.22	1.18
7	m	1.23	1.23	1.20	1.20	1.24	1.23
8	m	1.21	1.21	1.20	1.20	1.23	1.23
9	m	1.25	1.25	1.20	1.20	1.23	1.23
10	m	1.22	1.22	1.19	1.19	1.22	1.22
11	m	1.26	1.26	1.21	1.21	1.24	1.24
12	t	0.84	0.84	0.79	0.79	0.84	0.84
2-CH ₃	d	1.06	1.06	0.91	0.91	1.15	1.05
4-CH ₂ R	d (dd)	0.83	0.83	0.76	0.76	4.26, 3.65	4.37, 3.99
		R=H	R=H	R=H	R=H	R=O	R=O

n.o. = Not observed.

Table 5. 3J coupling constants of protons within the macrolide [Hz].

Coupling	1 (γ)	1a (γ -epi)	4 γ -hy	4a (γ -hy-epi)	2 (δ)	2a (δ -epi)
(<i>ser</i>)NH-HC α	5.6	9.0	7.6	8.0	9.3	7.5
(<i>trp</i>)NH-HC α	6.4	9.2	8.2	7.6	7.1	8.2
(<i>glu</i>)NH-HC α	7.5	8.0	8.4	7.6	5.7	9.0
H2-H3	9.9	10.6	7.6	7.6	2.7	1.9
H3-H4	2.1	1.8	2.2	2.7	7.2	7.0

which in turn, is weak in **1**, and the required orientation of the NH into the ring, the absolute configuration for W2 (C α of the β -oxotryptophan moiety) is assigned to be *S* in **1** and *R* in **1a**. These observations are the same for each epimeric pair of lipodepsipeptides which consequently contain *L*- β -ketotryptophan in their naturally occurring form. Calculations suggest that the steric energy of the "unnatural" form (**1a**) is increased by about 3 kcal/mol; this may perhaps be the reason why the natural conformation is favored in solution.

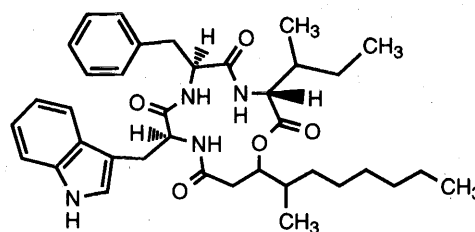
Although there is little steric hindrance for a rotation around the single bond between C-3 and C-4 of the fatty acid, inertia of the long fatty tail and energetic requirements in correlation with the observed NOE's for the substituents at C-4 and C-5 calls for a preferred conformation of the side chain. It is therefore possible to also determine the absolute configurations at C-4. As ROESY correlations were observed between H-2 and both H-4 and the 4-CH₃ group, and between the 2-CH₃ group and H-4, but not to the 4-CH₃ group, which indicates an orientation of the methyl groups in opposite directions, the absolute configuration at C-4 has to be *R*. The strong NOE's between H-3 and both protons at C-5 fully support this assignment.

Conclusion

Culture LL-15G256 produces two entirely different classes of antifungal compounds: macrocyclic polyacetones and lipodepsipeptides. Under certain fermentation conditions²⁾ the lipodepsipeptides are produced at high titers with 15G256 γ (**1**) as the predominant compound. (The structure of arthrichitin⁹⁾ which was published recently, appears to be identical with 15G256 γ , although no stereochemistry of the amino acids was reported). 15G256 ϵ (**3**) is the two-carbon lower homologue in the fatty acid side chain and 15G256 δ (**2**) represents the hydroxylated and ring expanded version of 15G256 γ . These lipodepsipeptides are relative stable compounds, but epimerize in the β -oxotryptophan moiety when left in solution presumably through keto-enol-tautomerism. Studies to stabilize or to replace this rare amino acid are ongoing.

Although the 15G256 γ , δ and ϵ components have novel structures, a similar series of lipodepsipeptides, the beauverolides, named after the producing fungus *Beauveria bassiana*, have been described¹⁰⁾. For comparison, the structure of beauverolide K_a is shown in Figure 7. All reported beauverolides (no biological activity stated) contain the fatty acid 3-hydroxy-4-methyl-

Fig. 7. Structure of beauverolide K_a.



Beauverolide K_a

C₃₇H₅₀N₄O₅

MW : 630

decanoic acid but differ in their amino acid composition, whereas the lipodepsipeptides reported here have the same amino acids but differ in the fatty acid portion.

The occurrence of 2,4-dimethyl-3-hydroxy-decanoic acid or 2,4-dimethyl-3-hydroxydodecanoic acid has not been reported, but several natural products containing 2,4-dimethyl-fatty acids are known including 2,4-dimethyldodecanoic acid which was identified as a constituent in duck wax¹¹⁾. 2,4-Dimethyloctanoic acid is the lipid portion of the majusculamides¹²⁾ and microcolins¹³⁾ where the carbons carrying the methyl groups apparently have the *R* configuration, whereas 2*S*,4*S*-dimethyldocosanoic acid¹⁴⁾ is a major component of glycolipids from *Mycobacterium tuberculosis*. Lastly, a fatty acid with three methyl groups, namely 2,4,6-trimethylnonanoic acid, is the lipid portion of the formestins¹⁵⁾.

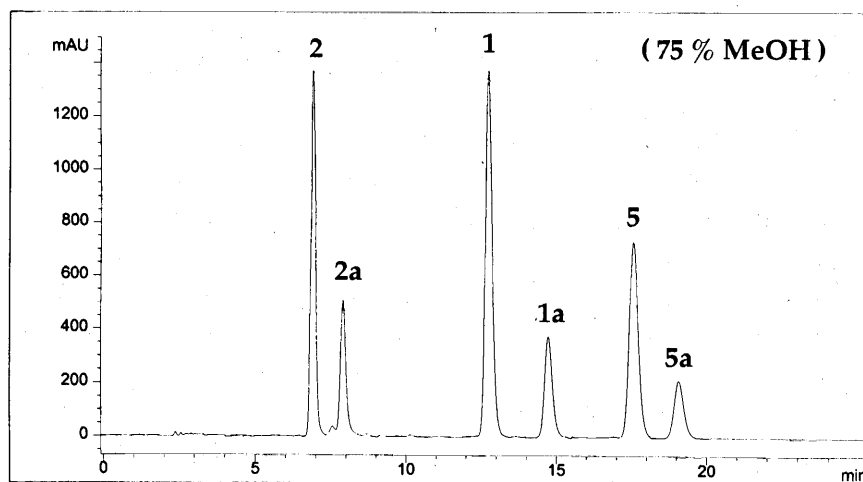
Experimental

General

A Hewlett-Packard 1090 M LC system with diode array detection (monitored at 262 nm) employing a variety of columns was used for the analysis of fractions or to check the purity of isolated components. (Columns: RAININ Microsorb (C8) or a Whatman Partisil 5 C₈ reverse phase column (5 μ , 4.6 \times 100mm), a Supelcosil LC-ABZ (5 μ , 4.6 \times 150 mm) or VYDAC 'protein and peptide' C₁₈ (4.6 \times 250 mm) all eluted isocratically with 70% MeOH/20% H₂O/10% 0.1M TFA, or 55% MeCN/35% H₂O/10% 0.1M TFA at a flow rate of 1 ml/minute. The same typical retention profiles of the lipopeptides were observed as shown in Chromatograms 1 and 2.

Preparative HPLC separations were accomplished on

Chromatogram 1. Retention profiles of selected 15G256 lipopeptides using MeOH (75%)/0.02 M TFA and a YMC ODS-A column (4.6 × 250 mm) at 40°C with a flow rate of 1 ml/minute.



Chromatogram 2. Retention profiles of selected 15G256 lipopeptides using MeCN (60%)/0.02 M TFA and a YMC ODS-A column (4.6 × 250 mm) at 40°C with a flow rate of 1 ml/minute.

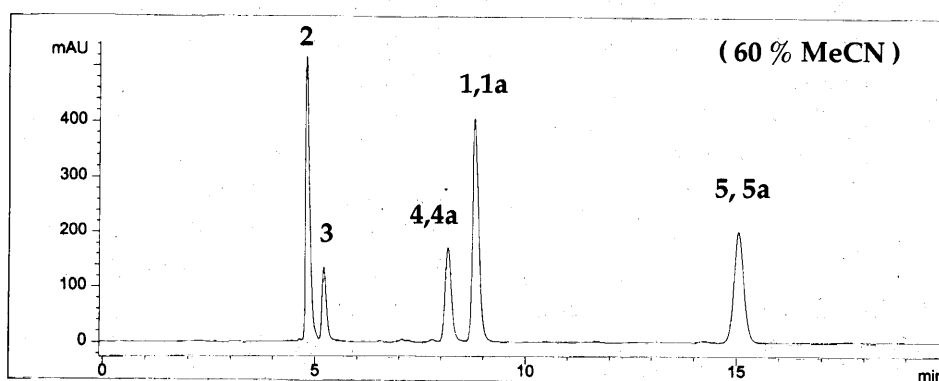


Table 6. Relative retention times of selected lipopeptides using MeOH (75%)/0.02 M TFA or MeCN (60%)/0.02 M TFA on a YMC ODS-A column (4.6 × 250 mm) at 40°C, flow rate 1 ml/minute.

	1	1a	2	2a	3	3a	4	4a
MeOH	1	1.15	0.53	0.81	0.54	n.d.	1.39	1.51
MeCN	1	1	0.56	0.56	0.59	0.59	0.93	0.93

a Whatman CCS/C₈ column (2 × 50 cm) or a MODCol® Kromasil C₁₈ column (2.54 × 25 cm) using 72% MeOH/water + 5 ml TFA/liter of solvent, with a flow rate of 9.9 ml/minute and monitored by a variable wavelength detector (LDC) at 280 or 300 nm. Solvent was delivered with a Waters 600MS HPLC pump. For larger preparations a RAININ Dynamax® C₁₈ cartridge column system (5 × 50 cm) was used with a flow rate of 40 ml/

minute and monitored by a Model UV-1 variable wavelength detector (RAININ) at 275 or 300 nm.

Silica gel chromatography was performed on self-packed open columns by step gradient elution using methylene chloride/ethyl acetate mixtures. Fractions from all columns were generally collected by hand and pooled according to peaks observed on TLC or HPLC analysis. All solvents were obtained from J. T. Baker,

Inc., and were of the highest commercially available purity.

Solutions of pure compounds were concentrated by evaporation under reduced pressure and the material was subsequently obtained as an amorphous solid by freeze-drying from *t*-BuOH solutions.

A Hewlett-Packard 5890 Series II Gas Chromatograph, fitted with a mass selective detector (series 5971) and a Chirasil L-Val column (25 m, 0.25 mm i.d., Alltech) was used for the analysis of hydrolyzed and derivatized 15G256 γ fractions to check the absolute configuration of amino acids. Retention time and fragmentation patterns were used to identify peaks. With a temperature gradient from 70°C to 200°C at a rate of 7°C/minute and a 15 minute hold time at 200°C, reproducible retention profiles were observed.

NMR spectra were obtained on a Bruker AMX 300 MHz or 600 MHz NMR instrument. Chemical shifts are given in ppm relative to the solvent signals of *d*₆-DMSO at δ 2.49 ppm (¹H) and δ 39.50 ppm (¹³C), *d*₆-acetone at δ 2.04 ppm (¹H) and δ 29.80 ppm (¹³C), *d*₃-acetonitrile at δ 1.98 ppm (¹H) and δ 1.30 ppm (¹³C), or *d*₄-MeOH at δ 3.30 ppm (¹H) and δ 49.00 ppm (¹³C) for ¹H and ¹³C NMR signals, respectively.

Antifungal activity was monitored by the *Neurospora crassa* assay¹⁾ with nikkomycin as positive control.

Isolation and Purification of the Principal Components 15G256 α , β and γ

Whole mash (5 liters) was separated into pellet and supernatant by centrifugation at 3000 rpm for 30 minutes. The pellet was extracted with 2 liters of 85% aqueous acetone while the supernatant was extracted with 2 liters of ethyl acetate. The organic extract of the supernatant and the pellet extract were processed separately under the following conditions. After evaporation of the solvent, the remaining solids (*ca.* 2 g from the pellet and *ca.* 1.2 g from the supernatant) were resuspended in methylene chloride and loaded onto a self-packed silica gel column (21 \times 300 mm). The compounds were eluted with increasing concentrations of ethyl acetate (10, 25, 50, and 100%) followed by a final column wash with 100% MeOH. From the pellet extract six fractions were collected with 15G256 β (114 mg), 15G256 α (125 mg) and 15G256 γ (52 mg) in fractions B1, D1 and E1 respectively. The silica gel chromatography of the supernatant extract did not yield satisfactorily pure material so that an additional purification step was necessary. The following silica gel fractions were obtained from the supernatant extract (A~F) and from the pellet extract (A1~F1): A

(64.1 mg); B (550 mg); C (470 mg); D+E (43.5 mg); F (250 mg). -A1 (33 mg); B1 (114 mg); C1 (423 mg); D1 (125 mg); E1 (52 mg); F1 (467 mg). As an alternative to silica gel column chromatography, preparative TLC (silica gel) developed in EtOAc/CH₂Cl₂ (1:1) can be used for the purification of the components.

Isolation and Purification of 15G256 γ (1), 15G256 δ (2), and 15G256 ϵ (3) from a 300-liter tank fermentation

Whole mash (300 liters) was mixed with 1.5% toluene and stirred for 30 minutes, and then filtered through a ceramic microfilter utilizing tangential flow. Filtrate and retentate fractions were processed separately to yield the 15G256 compounds.

The retentate was reslurried in MeOH (100 liters) and filtered. The extract was concentrated under reduced pressure to remove the bulk of the MeOH and the resulting aqueous suspension was extracted with ethyl acetate. Concentration of the latter under reduced pressure yielded a volume of about 4 liters. This was further concentrated to yield an oily residue which was stirred into hexane (2 liters) to remove nonpolar impurities resulting in the formation of a precipitate. This precipitate was slurried with 300 g silica gel in the presence of 500 ml acetone for 20 minutes, acetone was then evaporated and the powdery remain transferred to a partially loaded silica gel column to yield a final column of 15 \times 10 cm. The column was eluted sequentially with 1 liter of methylene chloride, 2 liters of 50% ethyl acetate/50% methylene chloride and 6 liters of ethyl acetate followed by a MeOH wash (2 liters). The ethyl acetate fraction yielded approximately 28 g of 15G256 γ .

The filtrate was mixed with EtOAc (120 liters) for three hours. The ethyl acetate phase was separated and concentrated under reduced pressure to yield a volume of approximately 4 liters. This mixture (containing precipitated material) was poured into EtOAc (12 liters) and washed with water. The ethyl acetate phase was then concentrated to yield an oily residue which was stirred into hexane (2 liters) to produce a precipitate. The precipitate was mixed with 10 g silica gel and acetone. The residue was collected after the evaporation of acetone and charged onto a silica gel column (5 \times 10 cm) which was then eluted with a stepwise gradient (*ca.* 250 ml each) of EtOAc in methylene chloride (5, 10, 25, 50, 75% and 3 times 100%). Fractions 7 and 8 (each ethyl acetate eluate) were concentrated to yield 4.5 g solid material. This was dissolved in 30 ml MeOH and chromatographed in three aliquots on a RAININ Dynamax[®] C₁₈ cartridge column system (5 \times 50 cm) using 55% acetonitrile in

mixture with 0.1 M TFA at a flow rate of 40 ml/minute. The effluent was monitored by a Model UV-1 variable wavelength detector at 300 nm. The fractions containing 15G256 δ , 15G256 ϵ and 15G256 γ eluted at 13 minutes, 15 minutes and 30 minutes, respectively. Yields of pure components: 15G256 δ (62 mg), 15G256 ϵ (24 mg) and 15G256 γ (2 g).

Preparation of Acyclic Lipopeptide 15G256 γ -hy (**4**) from 15G256 γ (**1**) by Base Hydrolysis

Five ml of a 1 M NaOH solution was added to 600 mg of **1** dissolved in 50 ml methanol. To this mixture was added 5 ml of an aqueous (1%) Ba(OH)₂ solution. A white precipitate formed immediately on the addition of Ba(OH)₂. This mixture was swirled for 10 minutes, a sample was then taken and analyzed by HPLC using a RAININ Mircosorb C18 (0.46 × 15 cm) column and a mixture of 70% MeOH/10% 0.1 M aqu. TFA/20% water as eluent. Instead of the 15G256 γ -peak at 8.5 minutes, a major peak at 12.1 minutes indicated the formation of the hydrolyzed product (**4**). (When 55% acetonitrile was used instead of 70% MeOH, the order of elution was reversed, **1** eluted at 6.4 minutes and **4** at 5.8 minutes) Acidification of the reaction mixture after 15 minutes with HCl dissolved the precipitate and gave a clear, slightly colored solution. The pH was adjusted to 4 and the solution was then poured into a biphasic system of ethyl acetate (120 ml) and water (50 ml) and agitated. Separation of the phases and concentration of the organic phase yielded 330 mg of glassy solid.

Compound **4** was purified by preparative HPLC using the MODCol® system isocratically eluted with 70% MeOH/0.8% TFA in water. 220 mg of **4** was recovered by pooling the appropriate fractions, evaporating the organic solvent and extracting **4** from the remaining aqueous mixture with ethyl acetate. Finally, trituration of the residue with acetonitrile produced **4** as a white solid which was subsequently dried *in vacuo*.

Preparation of 15G256 γ -OMe (**5**), the Methyl Ester of 15G256 γ (**1**)

Dry 15G256 γ (**1**) (1070 mg) was dissolved in 100 ml methanol and 1 ml of H₂SO₄ was added while swirling the reagent vessel. The reaction mixture was then let stand for 2 hours. An HPLC analysis using an analytical Supelcosil LC-ABZ C₁₈ reverse phase column eluted with 70% MeOH/10% 0.1 M TFA/20% water, revealed that the original 15G256 γ -peak with a retention time of 10.2 minutes had disappeared and a major peak with a retention time of 16.0 minutes was produced instead. The

reaction mixture was concentrated to approximately 10 ml after 400 mg solid NaHCO₃ had been added. This was then poured into ethyl acetate (100 ml) and the solution was transferred into an extraction funnel. Shaking with 25 ml of a 0.1 M NaHCO₃ solution and two water washes provided a neutral reacting ethyl acetate phase. The solvent was evaporated, the residue was then dissolved and dried from MeOH twice and finally re-dissolved in 40 ml *t*-BuOH, frozen and lyophilized. The dry product (1021 mg) was found to be the methyl ester of **1**, 15G256 γ -OMe (**5**).

Physico-chemical Properties

15G256 γ (**1**): C₃₃H₄₆N₄O₉, MW 642, (see Figure 3).

$[\alpha]_D^{25} = +22.9 \pm 1$ ($c = 1.07\%$, MeOH); UV (MeOH) λ_{\max} nm (ϵ) 208 (41,710), 246 (16,820), 264 (12,275), 311 (16,100); CD (MeOH) λ_{ext} (nm)/ $\Delta\epsilon = 327$ (2.95), 286 (−1.42), 245 (−4.38), 226 sh (−2.92, [rel. val.]); IR ν_{\max} (KBr) 3335, 3121, 2957, 2927 (s), 2855 (s), 1723 (s), 1681 (s), 1664 (s), 1645 (s), 1534, 1518 (s), 1459, 1430, 1376 (s), 1319, 1266, 1245, 1227, 1194, 1115, 1057, 748 cm^{−1}; ¹H NMR (see Table 4); ¹³C NMR (see Tables 1, 2 and 3); MS(FAB) $[M+H]^+ = m/z$ 643.5; MS(FAB) $[M+Na]^+ = m/z$ 665.4; HRFAB-MS $[M+H]^+ = m/z$ 643.3349, calcd. for C₃₃H₄₇N₄O₉ = 643.3343 ($\Delta = -0.6$ mmu); El. Anal.: C 61.70% (theor 61.68%), H 7.83% (theor 7.17%), N 7.37% (theor 8.72%).

15G256 γ -epi (**1a**): C₃₃H₄₆N₄O₉, MW 642, (see Figure 3).

UV (MeOH) λ_{\max} nm (ϵ) 208 (41,710), 245 (17,000), 264 (12,600), 307 (14,800); ¹H NMR (see Table 4); ¹³C NMR (see Tables 1, 2 and 3).

15G256 δ (**2**): C₃₃H₄₆N₄O₁₀, MW 658, (see Figure 4).

UV (MeOH) λ_{\max} nm (ϵ) 208 (33,720), 246 (13,600), 262 (9,925), 310 (13,100); ¹H NMR (see Table 4); ¹³C NMR (see Table 2 and 3); MS(FAB) $[M+H]^+ = m/z$ 659.3; MS(FAB) $[M+Na]^+ = m/z$ 681.3; HRFAB-MS $[M+Na]^+ = m/z$ 681.3119, calcd. for C₃₃H₄₆N₄O₁₀Na = 681.3112 ($\Delta = -0.7$ mmu).

15G256 ϵ (**3**): C₃₁H₄₂N₄O₉, MW 614, (see Figure 5).

UV (MeOH) λ_{\max} nm (ϵ) 208 (41,000), 246 (16,200), 264 (11,800), 311 (15,400); IR ν_{\max} (KBr) 3350, 3121, 3061, 2956 (s), 2928 (s), 2855 (s), 2489, 1723 (s), 1645 (s), 1518 (s), 1455, 1432, 1375 (s), 1339, 1316, 1245, 1192, 748 cm^{−1}; ¹H NMR (see Table 4); ¹³C NMR (see Table 3); MS(FAB) $[M+H]^+ = m/z$ 615.2; MS(FAB) $[M+Na]^+ = m/z$ 637.3; HRFAB-MS $[M+Na]^+ = m/z$ 637.2840, calcd. for C₃₁H₄₂N₄O₉Na = 637.2849 ($\Delta = +0.9$ mmu).

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