Enniatins of *Fusarium* sp. Strain F31 and Their Inhibition of *Botrytis cinerea* Spore Germination

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A spectrum of enniatins was isolated from *Fusarium* sp. strain F31 by bioassay-guided isolation directed against *Botrytis cinerea*. Two new enniatins, J_2 (**7**) and J_3 (**8**), were co-isolated and both contained, in addition to three hydroxyisovaleric acid units, *N*-methylated L-alanine, L-valine, and L-isoleucine units, differing only in their primary sequence. Two other enniatins, named enniatin J_1 (**1**) and enniatin K_1 (**6**), each containing two *N*-Me-L-Val units and one *N*-Me-L-Ala or α-*N*-Me-L-butyric acid unit, respectively, were isolated for the first time without directed biosynthesis. The enniatin structures were elucidated by spectroscopic and chemical methods, and the absolute configuration of the amino acids (L) and hydroxyisovaleric acid (D) was consistent with all previously isolated enniatins. The known enniatins B (**2**), B_1 (**4**), B_2 (**5**), and B_4 (**3**) were also isolated. The minimum inhibitory concentration of pure enniatins against *Botrytis cinerea* was 75 μg/mL.

The fungal phytopathogen *Botrytis cinerea* Pers.:Fr. (Sclerotiniaceae) causes gray mold to a wide range of host species and poses serious problems in agriculture, horticulture, and silviculture.[1] In Swedish forest nurseries with dense and humid growth and storage conditions, seedlings are highly susceptible to infection by *B. cinerea*.[2−4] At present, the disease is managed mainly by a combination of synthetic chemical fungicides, such as tolylfluanid, and sanitation measures to reduce *Botrytis* inoculum. Alternative methods can alleviate concerns over fungicide use in relation to human health, the environment, and development of resistance to fungicides by the pathogen.[5,6] Biological control by means of microorganisms is an approach that has been successful in several crop systems,[7] but has not been tested yet in forest nursery production systems. A microcosm bioassay screening for *in planta* biocontrol of *B. cinerea*[8] by microorganisms inhabiting the phyllosphere of conifer seedlings resulted in a diverse strain collection of different fungal species.[9] Among the species, the *Fusarium* sp. strain F31 (Nectriaceae) suppressed *B. cinerea* infection *in planta* and also presented inhibition zones in dual culture plates with *B. cinerea*, suggesting production of antifungal metabolites.

Fusarium species produce a wide variety of antibiotic compounds,[10,11] including the enniatins, an interesting group with a wide range of activities, e.g., phytotoxic,[12] antimicrobial,[13] insecticidal,[14] and inhibition of acyl-Co-A: cholesterol acyltransferase (ACAT).[15] The well-established cationophoric properties of enniatins could be one mechanistic explanation of the broad spectrum of activity.[16] Enniatins are cyclohexadepsipeptides produced nonribosomally by the multifunctional enzyme enniatin synthetase.[17] Their basic structure is three D-hydroxy acid residues and three L-amino acid residues alternately linked with ester and amide bonds forming an 18-membered ring. The enniatins are structurally related to the cyclodepsipeptides beauvericin and destruxins. The hydroxy acids reported from natural enniatins are hydroxyisovaleric acid (HyIv) from *Fusarium* spp.[13,14,18] and *Halosarpeia* sp.[19] or combinations of HyIv and 2-hydroxy-3-methylpentanoic acid from *Verticillium hemipterigenum*.[20] Amino acids found to be incorporated include Val, Ile, and Leu, and different fungal species show preferences for particular amino acids that are incorporated in the enniatin structure.[21] The amino acid recognition site of enniatin synthetase does not show an absolute specificity, but in vitro and in vivo directed biosyntheses have shown incorporation of alanine and α-aminobutyric acid units in enniatins.[22] Extensive synthetic efforts have also produced enniatins with incorporation of a variety of amino and hydroxy acids.[23]

In this paper we present the bioassay-guided isolation and structural determination of enniatins from *Fusarium* sp. strain F31.

Results and Discussion

Liquid cultures of *Fusarium* sp. strain F31 were filtered to remove mycelia, and the filtrates were fractionated by solid-phase extraction (SPE). The 95% MeCN eluate inhibited germination of *Botrytis* spores in a microtiter plate bioassay. Gradient preparative HPLC of the 95% fraction presented chromatographic peaks with t_R 15−18 min with two distinct regions of activity coinciding with fractions A and B (Figure 1).

The material in fraction A was subjected to HRFABMS, which resulted in a pseudomolecular ion at *m/z* 612.3824 $[M + H]^+$ (calcd for $C_{31}H_{53}N_3O_9$+H 612.3860). In the ^{1}H NMR spectrum, there were signals corresponding to three Me groups at δ_H 3.13 (3H, s), 3.16 (3H, s), and 3.21 (3H, s), which in HSQC presented cross-peaks at δ_C 32.4, 31.8, and 31.8, respectively, suggestive of methyl groups attached to nitrogens. Six proton signals were found at δ_H 4.68−5.30, and except for the quartet at δ_H 4.76 (1H, q, J = 7.5 Hz) all were doublets (5 × 1H, d, J = 8.3−10.0 Hz). The signals at δ_H 4.68−5.30 were likely candidates for the six H-2 (H_α) in an enniatin ring skeleton. COSY and HSQC-DEPT NMR data were used to assign three hydroxyisovaleric acid (HyIv) units with H-2 at δ_H 5.04, 5.04, and 5.30 connecting to C-2 (C_α) at δ_C 76.0, 76.5, and 75.6, respectively, and also

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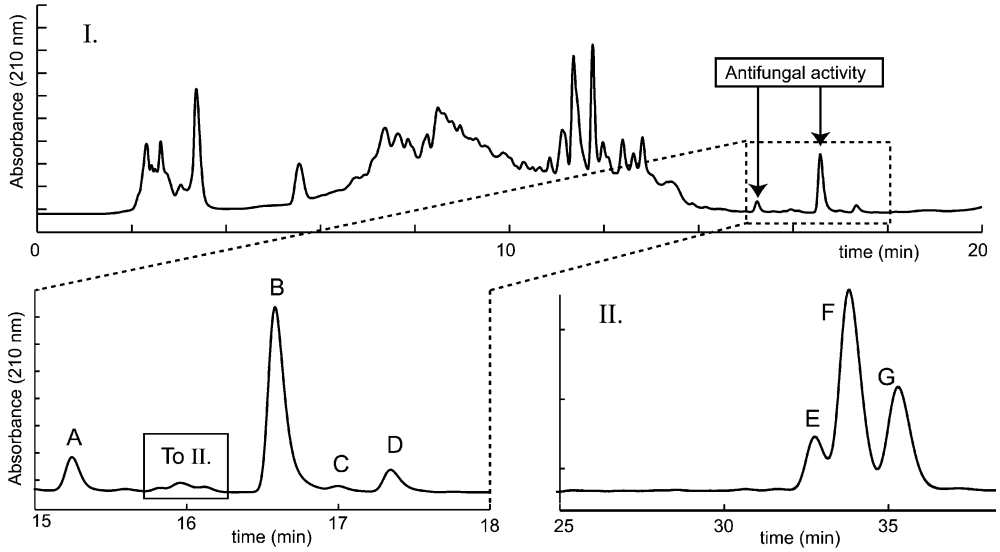


Figure 1. Chromatographic separation of enniatins. I: Gradient preparative HPLC of 95% MeCN SPE fraction at 20−100% MeCN in H_2O in 10 min with enniatin elution interval magnified at lower left. II: Isocratic preparative HPLC of indicated fractions eluted with 63% MeCN in H_2O.

two spin systems in agreement with two Val units (H-2 at δ_H 4.68 and 4.91; C-2 at δ_C 62.8 and 61.9). The final H-2 at δ_H 4.76 (C-2 at δ_C 54.2) was connected only to a methyl group at δ_H 1.42 (3H, d, J = 7.5 Hz), suggesting an Ala unit (Table 1). To determine the absolute configuration of the subunits, a small sample from fraction A was hydrolyzed and the HyIv was separated from the amino acids by extraction with ethyl acetate. The configuration of HyIv was investigated by an enantiomeric resolution method by acidic esterification with (*S*)-butanol and trimethylsilylation of the hydroxy group followed by GC/MS analysis. Comparison with derivatives of HyIv standards established the D configuration. The absolute configuration of the amino acids was determined by the advanced Marfey method.[24,25] The amino acids were derivatized and analyzed by LCMS and compared with derivatives of amino acid standards. The amino acids all had the L configuration.

When performing ESIMS on the sodium adduct pseudomolecular ion of depsipeptides, ring opening is mediated by cleavage of the ester bonds in the backbone.[26] In the case of enniatins, the MS^2 spectrum contains a superposition of three linear acylium ions derived from ring opening at each of the ester bonds. The product ions can be isolated and sequenced by further MS cycles, as fragments are lost from the acylium end.[26] Fraction A was subjected to ESIMS, and when selecting the pseudomolecular ion at *m/z* 634.5 $[M + Na]^+$, the MS^2 spectrum contained product ions of *m/z* 549.4 and 521.5. This corresponds to loss of *N*-Me-Ala (85 amu) and *N*-Me-Val (113 amu), respectively (Table 2 [**1**]). Further, MS^3 on *m/z* 549.4 and 521.5 yielded product ions at *m/z* 449.3 and 421.3, respectively, which is in agreement with loss of HyIv (100 amu). Performing MS^4 on *m/z* 421.3 showed two product ions at *m/z* 336.1 and 308.1 equaling loss of *N*-Me-Ala and *N*-Me-Val, respectively, while MS^4 on *m/z* 449.3 resulted in a product ion at *m/z* 336.2 corresponding to loss of *N*-Me-Val. The alternating loss of amino acid and HyIv units in MS^{2-4} demonstrates that the compound in fraction A has a primary sequence of alternating HyIv and amino acid units.

The structure was then defined as cyclo-(D-HyIv-*N*-Me-L-Val-D-HyIv-*N*-Me-L-Val-D-HyIv-*N*-Me-L-Ala), given the name enniatin J_1 (**1**). Enniatin J_1 was previously synthesized[23] and also isolated from *Fusarium scirpi* by directed biosynthesis[22] and identified by EIMS and ^1H NMR. This is the first naturally occurring derived isolation of enniatin J_1 without feeding experiments and the first extensive spectroscopic description of the compound.

The material from fraction B gave a simple ^1H NMR spectrum, and $ESIMS^{2-4}$ of the pseudomolecular ion at *m/z* 662.6 $[M + Na]^+$ indicated a structure of alternating units of 100 and 113 amu (Table 2) with a high degree of structural and conformational symmetry of the compound. Further data from NMR analysis were identical to that reported for enniatin B (**2**),[27] and HRFABMS analysis supported the findings. As *Fusarium* often produce mixtures of enniatins, the remaining fractions C and D were investigated, and after further chromatographic isolation, fractions E, F, and G were analyzed. The compounds in fractions C, D, and G were identified as the previously found enniatins B_4 (**3**), B_1 (**4**), and B_2 (**5**), respectively, by interpretation of MS^{1-4} data (Table 2) together with comparison of experimental and literature NMR and HRFABMS data.[18,27]

Analysis of the compound in fraction E with HRFABMS gave a pseudomolecular ion at *m/z* 626.4086 $[M + H]^+$ (calcd for $C_{32}H_{55}N_3O_9$+H 626.4017), and $ESIMS^{2-4}$ of the sodium adduct ion indicated the presence of three HyIv units and three amino acid units of 113, 113, and 99 amu (Table 2 [**6**]). The ^1H NMR spectrum of the compound contained three methyl singlets in the region δ_H 3.17−3.26, suggesting full *N*-methylation, and additional NMR data made elucidation of three spin systems in agreement with three HyIv units possible: H-2 at δ_H 5.08, 5.21, and 5.29 connecting to C-2 at δ_C 76.2, 76.3, and 75.8, respectively (Table 1). Two additional spin systems were identified as two Val units (H-2 at δ_H 4.56 and 4.78; C-2 at δ_C 63.6 and 62.7). The last H-2 at δ_H 4.95 (C-2 at δ_C 59.3) coupled to a methylene group (H-3 at δ_H 1.88 and 2.06; C-3 at δ_C 22.3), which in turn was connected to a terminal methyl group (H-4 at δ_H 0.99; C-4 at δ_C 11.4). The last spin system was consequently assigned to an α-aminobutyric acid unit (Abu). After determination of absolute configuration the structure was demonstrated to be cyclo-(D-HyIv-*N*-Me-L-Val-D-HyIv-*N*-Me-L-Val-D-HyIv-*N*-Me-L-Abu) and given the name enniatin K_1 (**6**). Enniatin K_1 was previously obtained from *F. scirpi* by directed biosynthesis but identified and characterized only by EIMS.[22]

The material in fraction F showed a pseudomolecular ion at *m/z* 626.3972 $[M + H]^+$ (calcd for $C_{32}H_{55}N_3O_9$+H 626.4017) in HRFABMS. $ESIMS^{2-4}$ on the sodium adduct

Table 1. NMR Data (MeOH-d_4, 600/150 MHz) for Enniatins J_1 (**1**), J_2+J_3 (**7**+**8**), and K_1 (**6**)

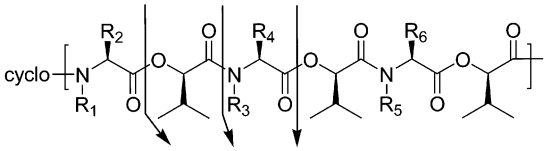
	enniatin J_1		enniatin J_2/J_3		enniatin K_1	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
Ala						
2-CH	4.76 (q, J = 7.5 Hz)	54.2	4.84 (q, J = 7.6 Hz)	54.6		
			4.82 (q, J = 7.6 Hz)	54.6		
3-CH_3	1.42 (d, J = 7.5 Hz)	14.1	1.48 (d, J = 7.6 Hz)	14.8		
			1.48 (d, J = 7.6 Hz)	14.8		
1-C=O		171.5		172.3		
				172.3		
N-CH_3	3.13	32.4	3.17	32.5		
			3.17	32.5		
Val						
2-CH	4.91 (d, J = 9.9 Hz)	61.9	4.87 (d, J = 10.1 Hz)	62.9	4.78 (d, J = 10.0 Hz)	62.7
	4.68 (d, J = 10.0 Hz)	62.8	4.65 (d, J = 10.1 Hz)	63.7	4.56 (d, J = 9.9 Hz)	63.6
3-CH	2.25	29.0	2.30	29.3	2.29	28.6
	2.21	28.3	2.27	29.0	2.29	28.6
4-CH_3	1.04, 0.91	19.7, 19.7	1.10, 0.97	20.6, 20.6	1.10, 0.98	20.1, 20.1
	1.03, 0.90	19.9, 19.9	1.10, 0.97	20.6, 20.6	1.11, 1.00	20.1, 20.6
1-C=O		171.0		171.9		171.6
		171.4		172.1		171.9
N-CH_3	3.21	31.8	3.26	32.5	3.24	31.8
	3.16	31.8	3.23	32.3	3.26	32.0
Abu						
2-CH					4.95 (d, J = 9.7 Hz)[a]	59.3
3-CH_2					2.06, 1.88	22.3
4-CH_3					0.99	11.4
1-C=O						*b*
N-CH_3					3.17	31.3
Ile						
2-CH			5.00 (d, J = 9.5 Hz)	61.2		
			4.81 (d, J = 10.2 Hz)	61.7		
3-CH			2.11	35.5		
			2.09	35.0		
3′-CH_3			1.06	16.7		
			1.05	16.7		
4-CH_2			1.21, 1.50	27.0		
			1.20, 1.52	27.2		
5-CH_3			0.92	10.8		
			0.90	10.8		
1-C=O				172.0		
				172.3		
N-CH_3			3.24	32.5		
			3.21	32.4		
Hylv						
2-CH	5.30, 5.04, 5.04 (all d, J = 8.3 Hz)	75.6, 76.5, 76.0	5.35*, 5.32**, 5.14** 5.12**, 5.08*, 5.07* (all d, *J = 7.5 Hz, **J = 7.6 Hz)	76.3, 76.3 77.1 77.1, 76.6, 76.6	5.29, 5.21, 5.08 (all d, J = 7.2 Hz)	75.8, 76.3, 76.2
3-CH	2.17, 2.15, 2.13	31.1, 30.6, 30.5	2.20, 2.20, 2.19	31.8, 31.8, 31.3	2.19, 2.19, 2.16	31.0, 31.0, 31.0
			2.19, 2.17, 2.17	31.3, 30.0, 30.0		
4-CH_3	0.98, 0.99, 0.99	18.5, 18.4, 18.0	1.00, 1.00, 1.03	19.0, 19.0, 18.7	1.02, 1.03, 1.02	18.2, 18.1, 18.0
			1.03, 1.02, 1.02	18.7, 18.4, 18.4		
4′-CH_3	0.92, 0.96, 0.95	18.5, 18.4, 18.0	1.00, 1.00, 1.03	19.0, 19.0, 18.7	1.01, 1.03, 1.02	18.2, 18.1, 18.1
			1.03, 1.02, 1.02	18.7, 18.4, 18.4		
1-C=O		171.0, 171.4, 171.5		172.1, 172.3, 172.3 172.2, 172.3, 172.3		172.7, 172.9, 172.9

[a] Strong coupling only to δ_H 1.88. [b] No cross-peak from δ_H 4.95 found in HMBC.

ion at *m/z* 648.5 $[M + Na]^+$ indicated three amino acid units of 85, 113, and 127 amu, respectively, in addition to three units of 100 amu (Table 2 [**7**, **8**]). The ^1H NMR spectrum was more complicated than for the other enniatins with 12 signals in the H-2 region (δ_H 4.65−5.35), i.e., twice as many as expected for a single enniatin. The *N*-methyl region contained resonances corresponding to an integral of 18 protons, and all other signals also indicated two overlaid sets of enniatin signals with all H-2 signals at equal intensities. Identification of the spin systems resulted in six HyIv units (H-2 at δ_H 5.07−5.35), two Ala units (H-2 at δ_H 4.82 and 4.84 with the methyl H-3 signal at δ_H 1.48 for both), and two Val units (H-2 at δ_H 4.65 and 4.87). The last two H-2 at δ_H 4.81 and 5.00 were each part of an s-butyl spin system, i.e., representing two Ile spin systems (Table 1). The data from HRFABMS together with NMR analysis strongly suggested the composition of the molecules in the sample to be three HyIv units and one unit each of *N*-Me-Ala, *N*-Me-Val, and *N*-Me-Ile. Investigation of absolute configuration demonstrated D configuration of HyIv and L configuration of *N*-Me-Ala, *N*-Me-Val, and *N*-Me-Ile. Thus, the spectroscopic complexity was not due to differences in the absolute configuration of the HyIv or amino acids.

Alternatively, the spectroscopic complexity could be due to the presence of two different conformers of a single enniatin. If this was the case, a change in solvent or temperature should change the appearance of the spectrum. However, performing NMR analysis in pyridine-d_5, $CDCl_3$, and MeOH-d_4 as well as ^1H NMR at 70 and 30 °C

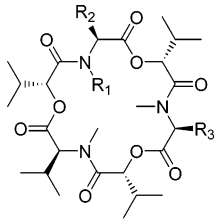
Table 2. ESIMS[1–4] Data from Analysis of Isolated Enniatins[a]



	[M+Na]	MS^2	MS^3	MS^4	R_1	R_2	R_3	R_4	R_5	R_6
		549.4	449.3	336.2	Me	Me	Me	i-Pr	Me	i-Pr
Enniatin J_1 (**1**)	634.5	521.5	421.3	336.1	Me	i-Pr	Me	Me	Me	i-Pr
				308.1	Me	i-Pr	Me	i-Pr	Me	Me
Enniatin B (**2**)	662.6	549.3	449.3	336.3	Me	i-Pr	Me	i-Pr	Me	i-Pr
		549.3	449.3	336.3	Me	i-Bu	Me	i-Pr	Me	i-Pr
Enniatin B_4 (**3**)	676.5	563.3	463.3	350.2	Me	i-Pr	Me	i-Pr	Me	i-Bu
				336.2	Me	i-Pr	Me	i-Bu	Me	i-Pr
		549.3	449.3	336.2	Me	s-Bu	Me	i-Pr	Me	i-Pr
Enniatin B_1 (**4**)	676.5	563.3	463.1	350.2	Me	i-Pr	Me	i-Pr	Me	s-Bu
				336.2	Me	i-Pr	Me	s-Bu	Me	i-Pr
		549.3	449.3	336.3	H	i-Pr	Me	i-Pr	Me	i-Pr
Enniatin B_2 (**5**)	648.6	535.3	435.3	336.2	Me	i-Pr	H	i-Pr	Me	i-Pr
				322.2	Me	i-Pr	Me	i-Pr	H	i-Pr
		549.3	449.2	336.2	Me	Et	Me	i-Pr	Me	i-Pr
Enniatin K_1 (**6**)	648.6	535.3	435.3	336.2	Me	i-Pr	Me	Et	Me	i-Pr
				322.2	Me	i-Pr	Me	i-Pr	Me	Et
		563.3	463.2	350.2	Me	Me	Me	i-Pr	Me	i-Bu
				336.2	Me	Me	Me	i-Bu	Me	i-Pr
Enniatin J_2 (**7**)	648.5	535.3	435.2	308.2	Me	i-Pr	Me	i-Bu	Me	Me
Enniatin J_3 (**8**)				350.2	Me	i-Pr	Me	Me	Me	i-Bu
		521.3	421.3	336.2	Me	i-Bu	Me	Me	Me	i-Pr
				308.1	Me	i-Bu	Me	i-Pr	Me	Me

[a] Fragmentation in MS^2 causes ring opening at all three ester bonds, meaning that MS^{2-4} spectra contain superimpositions of fragment ions from three linear acylium ions.

Chart 1. Enniatins Isolated from *Fusarium* sp. Strain F31



		R_1	R_2	R_3
Enniatin J_1	(**1**)	Me	i-Pr	Me
Enniatin B	(**2**)	Me	i-Pr	i-Pr
Enniatin B_4	(**3**)	Me	i-Pr	i-Bu
Enniatin B_1	(**4**)	Me	i-Pr	s-Bu
Enniatin B_2	(**5**)	H	i-Pr	i-Pr
Enniatin K_1	(**6**)	Me	i-Pr	Et
Enniatin J_2	(**7**)	Me	s-Bu	Me
Enniatin J_3	(**8**)	Me	Me	s-Bu

in DMSO-d_6 did not result in any changes that could indicate the presence of two conformers. These results suggested that the NMR complexity of the sample depends on the presence of two isomers, J_2 and J_3 (**7** and **8**), with differing primary sequences. Chromatographic resolution of the mixture was not successful.

An alternative way of verifying the presence of J_2 and J_3 was to hydrolyze the compounds at a single locus followed by sequencing of the isolated linear depsipeptides by $ESIMS^{1-3}$. Similar hydrolysis site specificity of J_2 and J_3 and MS^{2-3} fragmentation paths of any isolated linear depsipeptides were to be expected. A small amount of fraction F was subjected to partial acidic hydrolysis, and workup of the hydrolysis mixture was done by HPLC. Five fractions containing substances with *m/z* values corresponding to addition of H_2O to the structure (*m/z* 644.3 [M + H]$^+$) were isolated, and the major fractions four and five were further analyzed by $ESIMS^{1-3}$ (Figure 2). In MS^2 on the pseudomolecular ion both fractions yielded a product ion at *m/z* 544.3 corresponding to loss of an HyIv unit. Fraction four also gave a product ion at *m/z* 299.2, equal to HyIv, *N*-Me-Ala, and *N*-Me-Val, implying the Val and Ala units to be linked to the same HyIv unit, i.e., being geminal neighbors. Along the same lines, fraction five gave a fragment ion at *m/z* 313.2, implying *N*-Me-Ile and *N*-Me-Ala to be geminal neighbors. A subsequent MS step on *m/z* 544.3 gave rise to product ions in agreement with loss of *N*-Me-Ile for fraction four (*m/z* 417.3) and *N*-Me-Val for fraction five (*m/z* 431.3). Fragmentation of these ions both yielded product ions correlating to loss of *N*-Me-Ala, at *m/z* 332.2 and 346.1, respectively. Due to the alternating hydroxy acid–amino acid sequence of the enniatins, the sequential losses of HyIv and then two amino acid units in MS^{1-3} implied that the lost amino acid units were positioned at opposite ends of the linear structures; that is, they could not be geminal neighbors. In fraction four, Ile and Ala had to be separated by Val, and in fraction five, Val and Ala had to be separated by Ile. For each fraction, the retrieved data agreed with two possible primary sequences that theoretically were derived from two different fragmentation paths. The difference between the two paths consisted of which end of the compound lost the first amino acid. One fragmentation path identified the sequence in fraction four as Ile-Val-Ala, and in fraction five as Val-Ile-Ala. The other path in turn identified the sequence as Ala-Val-Ile for fraction four and Ala-Ile-Val for fraction five. Thus, without need to confirm which of the two fragmentation paths the linear depsipeptides followed, the MS^{1-3} data were sufficient to verify that the compounds in fractions four and fraction five derived from enniatins with opposite primary sequences. Consequently, the fraction F sample was shown to contain equal amounts of the novel compounds enniatin J_2 [cyclo-(D-HyIv-*N*-Me-L-Val-D-HyIv-***N*-Me-L-Ile-**D-HyIv-***N*-Me-L-Ala**)] and enniatin J_3 [cyclo-(D-HyIv-*N*-Me-L-Val-D-HyIv-***N*-Me-L-Ala-**D-HyIv-***N*-Me-L-Ile**)].

Two alternative primary sequences of enniatins containing three different amino acids were also suggested[13] for the previously isolated enniatin E, reported to contain one unit each of *N*-methylated Val, Leu, and Ile. As J_2 and J_3 were present in equal amounts, no preference in the biosynthesis of either primary sequence was apparent. Enniatins have been shown to be biosynthesized by condensation of three amino acid–hydroxy acid dipeptidols with amino acid recognition already in the dipeptidol formation step.[17] The biosynthesis of equal amounts of **7** and **8** is in agreement with amino acid selectivity at dipeptidol formation but no further selectivity at each step in the subsequent condensation of the dipeptidol units.

Minimum inhibitory concentration (MIC, defined as full inhibition of *B. cinerea* spore germination) was found at 75 μg/mL for enniatin B_1 and 100 μg/mL for a commercial enniatin mixture used as reference (Table 3). At these concentrations, sample wells had similar absorbance values at 620 nm (A_{620}) as control wells containing liquid Hagem medium only, and observation of these wells with a microscope revealed no germination of spores of *B. cinerea*.

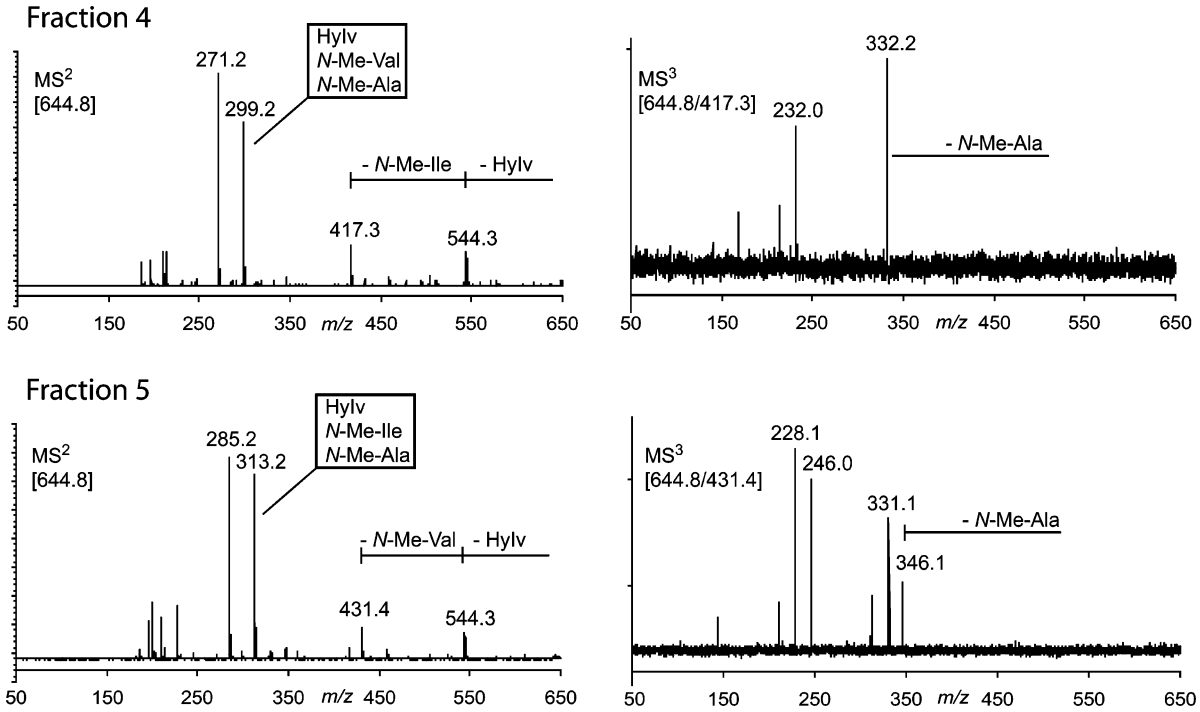


Figure 2. ESIMS$^{1-3}$ analysis of the linear depsipeptides from fractions four and five obtained by hydrolysis of HPLC fraction F. Subsequent loss of HyIv and two amino acid units implies the amino acids to be positioned at separate ends of the linear structure. Data also show that *N*-Me-Val is the central amino acid unit in fraction four and *N*-Me-Ile central in fraction five.

Table 3. Enniatin MIC and Detectable Inhibition Concentration (μg/mL) for Spore Inhibition of *Botrytis cinerea*, Determined by Recording Absorbance Values at 620 nm (A_{620}) and Ocular Observation Using a 4-Rank Scale

	MIC			detectable inhibition		
	μg/mL	A_{620}[a]	scale[b]	μg/mL	A_{620}[a]	scale[b]
enniatin B	>100	*c*		100	0.070	1
enniatin B_1	75	0.044	3	25	0.053	1
enniatin B_2	>100	*c*		>100	*c*	
enniatin B_4	>100	*c*		50	0.052	1
enniatin J_1	>100	*c*		>100	*c*	
enniatin K_1	>100	*c*		100	0.074	1
standard[d]	100	0.046	3	10	0.065	1
control wells						
liquid Hagem		0.043	3		0.043	3
liquid Hagem + spores		0.107	0		0.107	0

[a] Average absorbance values at 620 nm of sample wells in duplicate after incubation for 24 h. [b] 0: fungal growth comparable to growth in control wells containing Hagem medium and spores; 1: reduced fungal growth; 2: spore germination but no clearly visible hyphal growth; 3: total inhibition of spore germination. [c] At an enniatin concentration of 100 μg/mL, absorbance values and ocular observations were similar to values recorded for control wells containing Hagem and spores. [d] Commercial enniatin mixture.

Enniatin B_1 and the commercial enniatin mixture showed a partial inhibition at concentrations of 25 and 10 μg/mL, respectively (Table 3), with A_{620} values between those of control wells with liquid Hagem only and control wells containing Hagem and spores of *B. cinerea*. Observation of these sample wells with a microscope showed germination of the spores, but fungal growth and hyphal density were not as fully developed as in the control wells containing only Hagem and pathogen spores. All other enniatins tested did not fully inhibit spore germination at concentrations up to 100 μg/mL, although partial inhibition was found for enniatin B, B_4, and K_1 (Table 3). The enniatin J_2 and J_3 mixture was not tested for MIC. Production of antifungal enniatins might be one mechanism behind the suppressive effect by *Fusarium* sp. strain F31 on infection by *B. cinerea in planta* observed in the microcosm bioassay.[9] However production of enniatins by the *Fusarium* strain *in planta* has yet to be demonstrated.

Experimental Section

General Experimental Procedures. SPE was done with 1 g or 10 g prepacked columns or columns packed in-house with bulk C18 material [all Isolute C18 (EC), International Sorbent Technology, Hengoed, UK]. Preparative HPLC was run on a Gilson 305/306 pump system (Gilson, Inc., Middleton, WI) at 10 mL/min flow with a Gilson 118 UV detector monitoring at 210 nm. Fractions were collected with a Gilson 215 liquid handler, and collection was done in polypropylene, 2 mL deep well plates (Thermo Hypersil-Keystone, Cheshire, UK). For the mobile phase MeCN (LiChrosolv, Merck KgaA, Darmstadt, Germany) and deionized, filtered water (Millipore, Billerica, MA) were used. ESI mass spectra were obtained on an Esquire ion-trap MS (Bruker Daltonik GmbH., Bremen, Germany). MS$^{2-4}$ was performed on selected sodium or proton adduct ions. LCMS was done with the same mass spectrometer coupled to a HP1100 LC system (Hewlett-Packard, Palo Alto, CA). High-resolution FABMS was performed on a JEOL SX/SX102A four-sector tandem mass spectrometer (JEOL Ltd., Akishima, Japan) with glycerol as matrix and PEG as internal standard. GCMC was performed on an HP5890-HP5970 instrument (Hewlett-Packard, Palo Alto, CA). ^{1}H and ^{13}C NMR data were acquired on Bruker DRX-600 and DRX-400 NMR spectrometers (Bruker Biospin GmBH, Rheinstetten, Germany) equipped with a 2.5 mm SEI microprobe (^{1}H/^{13}C) and a 5 mm QXI probe (^{1}H/^{13}C/^{31}P/^{15}N), respectively. Depending on HDO peak interference, NMR experiments were recorded at 20 or 30 °C if not stated otherwise. For structure elucidation, NMR experiments were performed on samples in MeOH-d_4 and in $CDCl_3$ for comparison with literature values when applicable. Chemical shifts were determined relative to internal CD_2HOD (δ_C 49.15; δ_H 3.31), TSP [sodium 3-(trimethylsilyl)propionate (δ_H 0.00)], and MeOH (δ_C 50.05) or $CHCl_3$ (δ_C 77.23; δ_H 7.27). Standard pulse sequences supplied by Bruker were used for determination of ^{1}H and ^{13}C frequencies and

connectivities. For complete structure elucidation, 1D ^{1}H, COSY, TOCSY, HSQC-DEPT, and HMBC were applied.

Microorganisms and Growth Conditions. *Fusarium* sp. strain F31 was isolated from needles of *Pinus sylvestris* L. (Pinaceae) seedlings originating from a Swedish forest nursery.[9] Needle segments were placed on 2% water agar in Petri dishes at 20 ± 1 °C. Growing fungal colonies were transferred to and subsequently kept on Hagem medium.[28,29] The *Fusarium* strain was identified by morphological characteristics and by partial sequencing of the internally transcribed spacer (ITS) regions and the 5.8S gene using universal primers ITS1 and ITS4.[30] The strain is deposited at the fungal culture collection in the Department of Forest Mycology and Pathology, SLU, Sweden, and the ITS sequence at GenBank (accession number AY442182). To produce liquid cultures for enniatin isolation, 250 mL of liquid Hagem medium in 500 mL Erlenmeyer flasks was inoculated with six agar plugs (3 mm diameter) taken from the leading edge of a fresh culture grown on Hagem medium in 9 cm diameter Petri dishes. Culture flasks were incubated for 14 days at 20 ± 2 °C on a rotary shaker (120 rpm).

Botrytis cinerea strain 98G1, originating from infected *P. sylvestris* seedlings from a Swedish forest nursery, was grown on Hagem medium. The isolate was identified by morphological characteristics and by partial sequencing of the ITS regions and the 5.8S gene using primers ITS1 and ITS4.[30] The strain is deposited at the fungal culture collection in the Department of Forest Mycology and Pathology, SLU, Sweden, and the ITS sequence at GenBank (accession number AY442181). To produce conidia, cultures were grown on tomato leaf agar[31] in Petri dishes for 5 days in darkness at 20 ± 1 °C to allow for vegetative growth, followed by 4 days beneath near-UV light tubes (Philips TL 36W/08) with a 12 h photoperiod at 20 ± 2 °C to induce sporulation. Sporulating cultures were kept in darkness at 20 ± 1 °C for not longer than 25 days. To recover conidia, Petri dishes were flooded with liquid Hagem and spores rubbed off gently with a glass rod. The suspension was filtered through a 50 μm mesh to remove mycelial fragments. Spore concentration was estimated by use of a haemacytometer.

Spore Inhibition Bioassay and MIC. Antifungal activity in chromatographic isolation was followed by a method modified from Thaning et al.[32] A replicate of the HPLC fractions was done by transferring aliquots corresponding to 40 mL of culture filtrate from the deep-well plates to 96-well microtiter plates. The solvents were evaporated in a fume hood overnight. A spore suspension with a concentration of 10^6 spores/mL was prepared by dispersing harvested spores of *B. cinerea* in liquid Hagem medium.[28,29] Each sample well was inoculated with 100 μL of spore suspension. Control wells comprised spores suspended in liquid Hagem medium and wells containing only the medium. Directly after inoculation, the absorbance values of the individual wells at 620 nm (A_{620}) were recorded with an automated plate reader (Labsystems Multiskan RC, Helsinki, Finland). Spore germination and fungal growth were monitored after incubation for 24 h at 20 ± 1 °C in darkness, both by ocular observation of the microtiter plate with a stereomicroscope and by recording the A_{620} with the plate reader. A_{620} values were compared with the recordings just after inoculation to determine whether fungal growth had occurred. For ocular observations, a four rank scale was used to compare fungal growth in wells containing HPLC fractions with fungal growth in control wells. The bioassay-guided isolation of enniatins was repeated at least four times. MIC was determined by performing the bioassay as described above with concentrations of 10, 25, 50, 75, and 100 μg/mL of isolated enniatins as well as a commercial enniatin mixture containing enniatins A, 3%; A_1, 20%; B, 19%; and B_1, 54% (enniatin microbial, lot no. 064H4104, Sigma, St Louis, MO). All MIC tests were performed in duplicate.

Sample Workup and Isolation. Cultures were filtered under reduced pressure with filter paper to remove the mycelium, and the filtrate (4.5 L) was loaded on a 160 g solid-phase extraction (SPE) column. The column was packed and activated with 500 mL of MeCN and equilibrated with 375 mL of H_2O before sample loading. Hydrophilic components were washed out with 375 mL of aqueous 20% MeCN. The lipophilic fraction was eluted with 375 mL of aqueous 95% MeCN and concentrated under reduced pressure. The SPE eluate was subjected to gradient preparative HPLC with aqueous 20−100% MeCN in 10 min followed by 100% MeCN for 8 min, yielding enniatins J_1 (**1**, 2.5 mg), B (**2**, 14.2 mg), B_4 (**3**, 0.3 mg), and B_1 (**4**, 2.9 mg). All fractions were tested with the spore inhibition bioassay. Fractions 68−71 were subjected to a second isocratic run using aqueous 63% MeCN, yielding K_1 (**6**, 0.1 mg), J_2+J_3 (**7**+**8**, 0.3 mg), and B_2 (**5**, 0.3 mg). The column used in step one was a Reprosil-pur C18 (100 × 20 mm) with guard column (30 × 20 mm, 5 μm, Dr. A. Maisch High Performance LC-GmbH, Ammerbuch, Germany) and in step two a Kromasil C18 (150 × 21.2 mm, 5 μm, HiChrom, Reading, UK).

Enniatin J_1 (1): colorless oil; ^{1}H NMR (MeOH-d_4, 600 MHz), see Table 1; ^{13}C NMR (MeOH-d_4, 150 MHz), see Table 1; diagnostic HMBC connectivities, HyIv (3 units): H-2 to C-1, C-3, C-4, and C-4′, *N*-Me-Ala (1 unit): H-2 to C-1, C-3, and N-CH_3 (δ_C 32.4), *N*-Me-Val (2 units): H-2 (δ_H 4.91 and 4.68) to C-1, C-3, C-4, C-4′, and N-CH_3 (δ_C 31.8 and 31.8), N-CH_3 (δ_H 3.21 and 3.16) to C-2 (δ_C 61.9 and 62.8); HRFABMS *m/z* 612.3824 $[M + H]^+$ (calcd for $C_{31}H_{53}N_3O_9$+H 612.3860); HyIv analysis t_R 7.39 min (D-HyIv); advanced Marfey analysis t_R and diagnostic ion 4.19 min, *m/z* 378 (*N*-Me-L-Ala) and 5.53 min, *m/z* 406 (*N*-Me-L-Val).

Enniatin J_2 and J_3 (7 and 8): colorless oil; ^{1}H NMR (MeOH-d_4, 600 MHz), see Table 1; ^{13}C NMR (MeOH-d_4, 150 MHz), see Table 1; diagnostic HMBC connectivities, HyIv (6 units): H-2 to C-1, C-3, C-4, and C-4′, *N*-Me-Ala (2 units): H-2 to C-1, C-3, and N-CH_3 (δ_C 32.5), N-CH_3 (δ_H 3.17) to C-2 (δ_C 54.6), *N*-Me-Val (2 units): H-2 (δ_H 4.87 and 4.65) to C-1, C-3, C-4, C-4′, and N-CH_3 (δ_C 32.5 and 32.3), N-CH_3 (δ_H 3.26 and 3.23) to C-2 (δ_C 62.9 and 63.7), *N*-Me-Ile (2 units): H-2 (δ_H 5.00 and 4.81) to C-1, C-3, C-4, and N-CH_3 (δ_C 32.5 and 32.4), N-CH_3 (δ_H 3.24 and 3.21) to C-2 (δ_C 61.2 and 61.7); HRFABMS *m/z* 626.3972 $[M + H]^+$ (calcd for $C_{32}H_{55}N_3O_9$+H 626.4017); HyIv analysis t_R 7.43 min (D-HyIv); advanced Marfey analysis t_R and diagnostic ion 4.15 min, *m/z* 378 (*N*-Me-L-Ala), 5.50 min, *m/z* 406 (*N*-Me-L-Val), and 5.96 min, *m/z* 420 (*N*-Me-L-Ile).

Enniatin K_1 (6): colorless oil; ^{1}H NMR (MeOH-d_4, 600 MHz), see Table 1; ^{13}C NMR (MeOH-d_4, 150 MHz), see Table 1; diagnostic HMBC connectivities, HyIv (3 units): H-2 to C-4 and C-4′, *N*-Me-Abu (1 unit): N-CH_3 (δ_H 3.17) to C-1 (amide, δ_C 172.9) and C-2 (δ_C 59.3), *N*-Me-Val (2 units): H-2 (δ_H 4.78 and 4.56) to C-1, N-CH_3 (δ_H 3.24 and 3.26) to C-1 (amide, δ_C 172.9 and 172.9) and C-2 (δ_C 62.7 and 63.6); HRFABMS *m/z* 626.4086 $[M + H]^+$ (calcd for $C_{32}H_{55}N_3O_9$+H 626.4017); HyIv analysis t_R 7.43 min (D-HyIv); advanced Marfey analysis t_R and diagnostic ion 4.90 min, *m/z* 392 (*N*-Me-L-Abu) and 5.52 min, *m/z* 406 (*N*-Me-L-Val).

Enniatin Hydrolysis and Hydroxy Acid Analysis. Enniatin J_1 (**1**), J_2+J_3 (**7**+**8**), and K_1 (**6**), 0.1 mg of each, were hydrolyzed in 250 μL of 6 M HCl at 110 °C for 14 h. The hydrolysate was extracted with 3 × 300 μL of EtOAc. To the dried organic phase 50 μL of (*S*)-butanol and 10 μL of acetyl chloride were added. The mixture was allowed to stand for 40 min at 110 °C in a screw-capped test tube to yield the (*S*)-butyl HyIv ester. The reaction mixture was dried under a stream of compressed air, after which 100 μL of EtOAc and 100 μL of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide were added and allowed to stand for 40 min at 55 °C. Samples of commercial L-HyIv and racemic HyIv were derivatized similarly. The derivatives were analyzed by GC/MS on a fused silica column (BP5; 0.25 μm, 30 m × 0.25 mm) using a temperature gradient (100 °C for 5 min, 100−110 °C in 10 min, 110−170 °C in 8 min). The injector was held at 240 °C and the detector at 250 °C. Samples (1 μL) were injected in splitless mode, and He was used as a carrier gas at 1 mL/min. The standard derivatives of D- and L-HyIv acid eluted at t_R 7.43 and 7.79 min, respectively.

Absolute Configuration of Amino Acids by Advanced Marfey Analysis. The aqueous phase of the hydrolysate

described above was dried under reduced pressure and redissolved in 50 μL of water. To the solution, 50 μL of a 1% solution of *N*-α(1-fluoro-2,4-dinitrophenyl-5-L-alaninamide) in acetone and 50 μL of 1 M $NaHCO_3$ were added. After standing for 1 h at 40 °C the reaction was quenched with 50 μL of 1 M HCl. The samples were diluted with 600 μL of MeCN and filtered through a 0.45 μm HPLC filter, and 5 μL was injected for LCMS analysis. Separation was performed on a Discovery C18 (EC) column (100 × 2.1 mm, 5 μm, Supelco, Bellafonte, PA) with a gradient chromatographic run, 15% to 71% B in A in 8 min at 0.4 mL/min flow rate (solvent A: 10 mM ammonium acetate buffer adjusted to pH 5.5 with formic acid; solvent B: MeCN). The MS scanned *m/z* range 100−600 in positive mode and t_R of derivatives were based on peaks in the reconstructed ion chromatogram of the extracted sodium adduct pseudomolecular ions. The eluate was also monitored by UV detection at 340 nm. Commercial standards of L- and DL-*N*-Me-Val, *N*-Me-L-Ile, L- and DL-*N*-Me-Ala, synthesized L- and DL-*N*-Me-Abu, and racemized commercial *N*-Me-L-Ile were derivatized and analyzed in the same way. t_R and diagnostic ion of standards: *N*-Me-L-Ala 4.13 min, *N*-Me-D-Ala, 4.69 min, *m/z* 378 $[M + Na]^+$; *N*-Me-L-Abu 4.82 min, *N*-Me-D-Abu 5.37 min, *m/z* 392 $[M + Na]^+$; *N*-Me-L-Val 5.45 min, *N*-Me-D-Val, 6.08 min, *m/z* 406 $[M + Na]^+$; *N*-Me-L-Val 5.95 min, *N*-Me-D-Val, 6.60 min, *m/z* 420 $[M + Na]^+$.

Synthesis of *N*-Methyl-L-α-aminobutyric Acid.[33] Commercial *N*-Boc-L-α-aminobutyric acid (1 mmol) was dissolved in 5 mL of anhydrous DMF. Ag_2O (0.92 mg, 4 mmol) and 0.6 mL of MeI (8 mmol) were added to the solution, which was allowed to stand at 45 °C for 18 h under constant stirring. Excess MeI was evaporated at 45 °C before filtration through a sintered glass filter. The filter was washed with 2 mL of DMF, and 20 mL of $CHCl_3$ was added to the solution, inducing a cloudy precipitate. The organic phase was washed with water several times and then evaporated under reduced pressure to yield *N*-Boc-*N*-methyl-L-α-aminobutyric acid methyl ester as an oily material. The Boc protecting group was removed under acidic conditions: 6 mL of 3 M HCl for 4 h at 22 °C. After evaporation and repeated solvation in H_2O the methyl ester was cleaved by 4 mL of 1 M NaOH at 70 °C for 2 h. The solution was washed with 3 × 2 mL of EtOAc and finally diluted with EtOH before evaporation.

***N*-Me-L-α-aminobutyric acid:** white powder; ESIMS *m/z* 118.2 $[M + H]^+$; ^1H NMR (D_2O, 400 MHz) δ 3.56 (1H, t, J = 5.8 Hz, H-2), 2.72 (3H, s, N−CH_3), 1.91 (2H, m, H-3), 0.96 (3H, t, J = 7.5 Hz, H-4); ^{13}C NMR (D_2O, 100 MHz) δ 175.0 (C-1), 65.9 (C-2), 32.8 (N−CH_3), 23.7 (C-3), 9.5 (C-4).

Racemization of *N*-Me-L-Ile and *N*-Me-L-Abu.[34] Enantiomerically pure amino acid (500 μmol) and 75 μmol of 4-hydroxybenzaldehyde (10 mg) were dissolved in 3 mL of glacial HOAc. The solution was heated at 100 °C for 4 h, after which it was filtered and evaporated under reduced pressure. The residue was dissolved in 5 mL of H_2O and subsequently dried and the procedure repeated twice.

Partial Hydrolysis and Sequencing of Enniatins. To 0.25 mg of fraction F was added 500 μL of 2 M HCl. The mixture was allowed to stand at 100 °C for 5.5 h and then evaporated under reduced pressure. The filtered reaction mixture was purified by HPLC on a Discovery C18 (EC) column (150 × 4.6 mm, 5 μm, Supelco, Bellafonte, PA) with a linear gradient run: isocratic 57% B for 16 min, to 68% B in 6 min, 6 min hold at 68% B, to 74% B in 6 min (A: H_2O with 0.1% formic acid; B: MeOH/H_2O (95:5) with 0.1% formic acid). Flow rate was 1 mL/min, and the eluate was followed by UV detection (230 nm) and post-collection ESIMS monitoring. The major fractions four and five were concentrated under reduced pressure and each dissolved in 20 μL of A/B (50:50) and subjected to ESIMS up to the third generation. The samples were introduced by direct inlet, 60 μL/h, scan range *m/z* 50−800, with maximum acquisition time 30 ms.

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