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Directed Biosynthesis of New Enniatins

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New cyclohexadepsipeptides of the enniatin type with potential anthelmintic properties were produced by two different strategies: 1. *In vitro* synthesis by use of the multienzyme enniatin synthetase, and 2. *in vivo* precursor feeding of enniatin producing strains *Fusarium scirpi* and *Fusarium sambucinum*. The compounds were analyzed by HPLC, various NMR measurements and mass spectrometry. The three *N*-methyl L-amino acid positions in the enniatin B molecule could be gradually replaced by other (*N*-methyl) L-amino acids, *e.g.* alanine, cysteine, threonine and serine. The latter two amino acids yield new enniatins with functional groups in the hydrophobic side chains. Similarly the three D-2-hydroxyisovalerate residues, present in all naturally occuring enniatins, could be substituted by D-2-hydroxybutyric acid and D-lactic acid. Despite its lower yield the *in vitro* synthesis has the advantage of a broader variety of products formed.

Enniatins are cyclic hexadepsipeptides with ionophoretic properties[1], consisting of alternating units of D-2-hydroxyisovaleric acid (D-Hiv) and *N*-methyl L-amino acids with a branched hydrocarbon side chain, *e.g.* *N*-methyl L-isoleucine, *N*-methyl L-valine and *N*-methyl L-leucine[2] (Fig. 1). The pharmaceutical interest in these compounds is based on their antifungal, antibacterial, and, as recently demonstrated, anthelmintic properties[3]. Furthermore, enniatins are potent inhibitors of the mammalian cholesterol acyl transferase[4]. Enniatins also bind to the γ-aminobutyric acid receptor and cause non-specific rat cortical membrane disruption *in vitro*[5].

The synthesis of enniatin was performed by the multifunctional enzyme enniatin synthetase (ESyn)[2,6], which belongs to the class of *N*-methyl cyclopeptide synthetases. These enzymes are peptide synthetases with integrated *N*-methyltransferase domains like *e.g.* cyclosporin synthetase[7]. Enniatins are synthesized from their primary precursors D-Hiv and a branched chain L-amino acid under consumption of ATP following a thioltemplate mechanism[8]. In the first step of biosynthesis the substrates are activated as thioesters *via* adenylation. On this stage *N*-methylation of the covalently bound substrate L-amino acid occurs prior to peptide bond formation yielding a dipeptidol unit. *S*-adenosyl-L-methionine (AdoMet) serves as the methyl group donor. Enniatin biosynthesis proceeds in an iterative process by successive condensation of three dipeptidol building units to form a linear hexadepsipeptide which in the last step cycles to give enniatin[8].

Due to the rather low substrate specificity of ESyn for L-amino acids, complex mixtures of naturally occuring enniatins differing in the amino acid portion could be isolated. The enzyme is capable of synthesizing enniatins A~C and also mixed-type enniatins containing different amino acids[9,10]. As shown by PIEPER *et al.*[11], ESyns from

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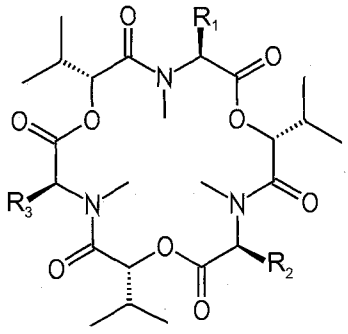
Fig. 1. Structures of enniatins.



Enniatin A: R_1=R_2=R_3=*sec*-butyl; Enniatin A1: R_1=isopropyl, R_2=R_3=*sec*-butyl; Enniatin B: R_1=R_2=R_3=isopropyl; Enniatin B1: R_1=R_2=isopropyl, R_3=*sec*-butyl

various *Fusarium* strains differ in their specificity for the substrate L-amino acid. The enzyme from the enniatin B producing fungus *Fusarium scirpi*, for instance, exhibits high affinity for L-valine, the constituent amino acid of enniatin B, whereas the multienzyme from the enniatin A producer *Fusarium sambucinum* preferably incorporates L-leucine and L-isoleucine.

A key enzyme in enniatin bioynthesis is D-2-hydroxyisovalerate dehydrogenase, that supplies ESyn with D-Hiv[12]. Consequently, all naturally occuring enniatins contain this D-2-hydroxy carboxylic acid.

The present paper describes the synthesis of new enniatin homologues by feeding submerged cultures of different *Fusarium* strains and by *in vitro* synthesis using the multifunctional enzyme ESyn.

Materials and Methods

Chemicals and Radioisotopes

Commercial sources of reagents were as follows: AdoMet and ATP were from Sigma, Deisenhofen, Germany. Tris was from Applichem, Darmstadt, Germany. [^{14}C-methyl]-AdoMet (53 Ci/mol), [U-^{14}C]-L-valine (257 Ci/mol), [U-^{14}C]-L-leucine (370 Ci/mol) and [U-^{14}C]-L-isoleucine (250 Ci/mol) were purchased from Amersham, Braunschweig, Germany.

All other chemicals were of the purest available grades from standard commercial sources.

HPLC/MS Analysis

For separation and purification of new enniatins a Waters HPLC system, consisting of two pumps (model 510), an autosampler type 717, and a PDA detector 996, was used. The analysis was carried out either isocratically with 75% methanol on a Merck RP-18 column (5 μm, 4.6×250 mm; mobile phase, MeOH/H_2O 75 : 25; flowrate, 1 ml/minute; column temperature, 50 °C) or by a linear gradient for 60 minutes (10 to 95% MeOH) using a Nova-Pak® column (C_{18}, 4 μm, 4.6×250 mm; flowrate, 1 ml/minute). Identification of the products was done by retention time and UV spectra (210 nm). Single peaks were analyzed by EI-MS using a Finnigan MAT 8200 mass spectrometer at 70 eV.

NMR Studies

The various ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra were obtained on a Bruker AMX-400 MHz spectrometer. Chemical shifts are expressed in δ values (ppm) with $CHCl_3$ as an internal standard and coupling constants are given in J (Hz).

Analysis by TLC

Products were separated on silica gel F_{254} plates (Merck, Darmstadt, Germany) with EtOAc - MeOH - H_2O, 100 : 5 : 1 (in volume) as the solvent system. After TLC separation, radioactive products were extracted from the silica gel plates with ethanol and applied to HPLC as described above. Konica X-ray films A3 were applied for autoradiography.

Growth of Organisms

500 ml Erlenmeyer flasks containing 100 ml of FCM medium consisting of molasses 3% and cornsteep liquor 1% were inoculated with 10^9 spores of either *Fusarium scirpi* (ETH 1536) or *Fusarium sambucinum* (BBA 63933). After cultivation for 72 hours at 28°C on a rotary shaker (120 rpm), the mycelium was used as inoculum for the main culture. A 2-ml aliquot of the preculture was transferred to 100 ml of the production medium FDM consisting of saccharose 2.5%, $NaNO_3$ 0.425%, NaCl 0.5%, $MgSO_4 \cdot 7H_2O$ 0.25%, KH_2PO_4 0.136%, $FeSO_4$ 0.003% and $ZnSO_4$ 0.00084%. After incubation for 72 hours at 28°C on a rotary shaker (120 rpm) cells were harvested by suction filtration on a Büchner funnel, freeze-dried and stored at −80°C.

Feeding Experiments and Purification of Crude Enniatins

To 3-day old cultures of *F. scirpi* and *F. sambucinum* substrates for enniatin production (D-2-hydroxy carboxylic acid or L-amino acid) were separately added to give a final

concentration of 10 mM. The cultivation was continued for another 2 days at 28°C.

The mycelium was separated from the medium by suction filtration on a Büchner funnel and extracted three times by grinding with 50-ml portions of acetone. The medium was extracted three times with 75 ml ethyl acetate each. All organic phases were pooled, dried over magnesium sulfate, and evaporated. The products were dissolved in 1~2 ml of chloroform and purified by chromatography on an aluminium oxide column (20×30 cm) using a linear gradient of chloroform from 20~100% in carbon tetrachloride. Enniatin-containing fractions were evaporated and applied to HPLC as described above.

Purification of Enniatin Synthetase

Enzyme purification was carried out according to ZOCHER *et al.*[6]. Ultrogel AcA 34 fractions were used for *in vitro* synthesis.

Protein Determination

Protein concentrations were determined by the dye-binding method of BRADFORD[13] using bovine serum albumin as a standard.

In Vitro Synthesis

For *in vitro* synthesis of [^{14}C]-radiolabeled enniatins containing different L-amino acids 10~20 μg of partially purified ESyn (in 100 mM Tris-HCl pH 8.0, 1 mM EDTA, 4 mM dithiothreitol, 1 mM benzamidine, 10% glycerol) were incubated in the presence of 1 mM D-Hiv, 5 mM ATP, 10 mM $MgCl_2$, 0.15 μCi [^{14}C-methyl]-AdoMet, and the substrate L-amino acid(s) (each 10 mM) in a final volume of 175 μl. After incubation for 30 minutes at 27°C the reaction was stopped by addition of 2 ml of water, and the product was extracted with 2 ml of ethyl acetate. The organic phase was evaporated, and the residue was subjected to TLC as described above. Radioactive products were detected by autoradiography. Structure determination was done by mass spectrometry and/or ^{13}C NMR and ^{1}H NMR.

For the *in vitro* synthesis of [^{14}C]-radiolabeled enniatins differing in their D-2-hydroxy carboxylic acid moiety, L-valine or L-isoleucine (1 mM) was used as the substrate L-amino acid. The final concentration of D-2-hydroxy carboxylic acid was 10 mM.

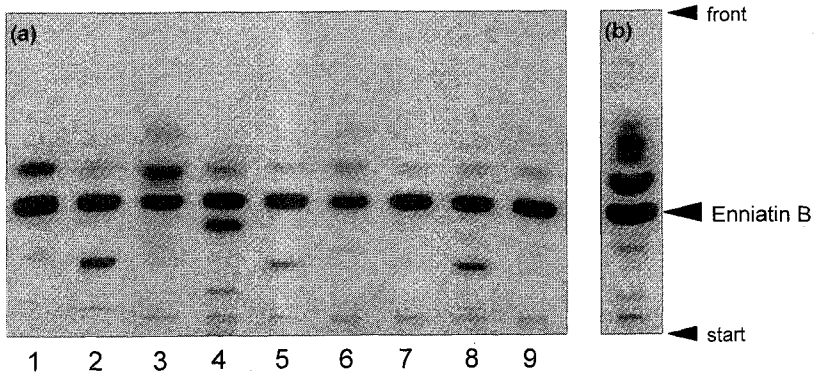
Synthesis of non-labeled enniatins followed the same protocol using 1 ml of desalted crude extract or concentrated partially purified enzyme. In this case unlabeled AdoMet was added to give a final concentration of 5 mM.

Results and Discussion

In Vitro Studies

Previous studies on the substrate specificity of ESyn revealed that the *N*-methyl L-valine moiety in enniatin B

Fig. 2. *In vitro* synthesis of enniatins containing different *N*-methyl L-amino acids (autoradiograph of TLC separation of ethyl acetate extractable compounds).



ESyn from *F. scirpi* was incubated in the presence of 1 mM D-Hiv, 5 mM ATP, 10 mM $MgCl_2$, 10 mM L-amino acid, and 0.15 μCi [^{14}C-methyl]-AdoMet.

The solvent system was EtOAc - MeOH - H_2O, 100 : 5 : 1 (in volume).

(a) Lane 1: L-valine (control); in lane 2~9 L-valine was replaced by (2) L-alanine, (3) L-cysteine, (4) L-methionine, (5) *O*-methyl-L-serine, (6) β-chloro-L-alanine, (7) β-thienyl-L-alanine, (8) L-threonine, and (9) L-citrulline.

(b) L-valine was replaced by L-serine.

could be replaced by L-α-aminobutyric acid (L-Abu), L-norvaline, and L-leucine[14]. In order to increase the diversity of enniatin depsipeptides we tested further L-amino acids for their suitability to serve as substrates for ESyn. For these experiments we supplied the enzyme with various L-amino acids (L-alanine, L-methionine, *O*-methyl-L-serine, L-cysteine, L-serine, L-threonine, β-chloro-L-alanine, β-thienyl-L-alanine, and L-citrulline), in the presense of D-Hiv, [^{14}C-methyl]-AdoMet and ATP. TLC separation of the ethyl acetate extracts and subsequent autoradiography showed the formation of novel compounds with Rf values differing from that of enniatin B (Fig. 2). The compounds were isolated by HPLC and subjected to ^{1}H NMR (^{13}C NMR) and EI-MS analyses. In the course of these experiments we found that (*N*-methyl) L-alanine, L-serine and L-threonine could be incorporated into the corresponding enniatins (Table 1).

Our results show that incorporation of amino acids is restricted to compounds carrying short aliphatic side chains (C_1 - C_4), whereas amino acids with bulky side chains are not accepted by the enzyme. Interestingly, also L-threonine and L-serine with a hydroxyl group in the side chain could be incorporated.

Enniatins containing D-lactic acid (D-Lac) are of special interest because of their anthelmintic properties[3,15,16]. Therefore we tested the ability of ESyn to incorporate D-Lac and other homologous D-2-hydroxy carboxylic acids (D-2-hydroxy-3-methyl-*n*-valeric acid, D-2-hydroxy-*n*-valeric acid, D-2-hydroxybutyric acid (D-Hbu), and D-hydroxisobutyric acid) into the enniatin B molecule. As

Table 1. *In vitro* synthesis of new enniatins.

	Enniatin	MW [g/mol]	MS data (*m/z*)	Precursor added	R_1	R_2	R_3	R_4	R_5	R_6
A	D-Hiv$_2$-D-Lac-(L-Melle)$_3$	653.8	653* 654** 676***	D-Lac	$^{sec}C_4H_9$	$^{sec}C_4H_9$	$^{sec}C_4H_9$	$^{iso}C_3H_7$	$^{iso}C_3H_7$	CH_3
	D-Hiv-D-Lac$_2$-(L-Melle)$_3$	625.8	625* 626**		$^{sec}C_4H_9$	$^{sec}C_4H_9$	$^{sec}C_4H_9$	$^{iso}C_3H_7$	CH_3	CH_3
B	D-Hiv$_2$-D-Lac-(L-Melle)$_2$-L-MeAla	611.7	612** 635***	D-Lac and L-Ala	$^{sec}C_4H_9$	$^{sec}C_4H_9$	CH_2OH	$^{iso}C_3H_7$	$^{iso}C_3H_7$	$^{iso}C_3H_7$
	D-Hiv$_2$-D-Lac-L-Melle-(L-MeAla)$_2$	569.7	569*		$^{sec}C_4H_9$	CH_2OH	CH_2OH	$^{iso}C_3H_7$	$^{iso}C_3H_7$	$^{iso}C_3H_7$
C	D-Lac$_3$-(L-Melle)$_2$-L-MeAbu	569.7	569*	D-Lac and L-Abu	$^{iso}C_3H_7$	$^{iso}C_3H_7$	$^{iso}C_3H_7$	$^{iso}C_3H_7$	$^{iso}C_3H_7$	CH_3
D	D-Hiv$_2$-D-Lac-(L-Melle)$_2$-L-MeThr	641.8	643**	D-Lac and L-Thr	$^{iso}C_3H_7$	$^{iso}C_3H_7$	$^{iso}C_3H_7$	$^{iso}C_3H_7$	CH_3	CH_3
	D-Hiv$_2$-D-Lac-L-Melle-(L-MeThr)$_2$	629.8	629* 630**		$^{iso}C_3H_7$	$^{iso}C_3H_7$	$^{iso}C_3H_7$	CH_3	CH_3	CH_3
E	D-Hiv$_3$-(L-Melle)$_2$-L-MeThr	669.9	671**	L-Thr	$^{iso}C_3H_7$	$^{iso}C_3H_7$	$^{iso}C_3H_7$	$^{iso}C_3H_7$	$^{iso}C_3H_7$	CH_2CH_3
	D-Hiv$_3$-L-Melle-(L-MeThr)$_2$	657.8	658**		$^{iso}C_3H_7$	$^{iso}C_3H_7$	$^{iso}C_3H_7$	$^{iso}C_3H_7$	CH_2CH_3	CH_2CH_3
F	D-Hiv$_3$-(L-Melle)$_2$-L-MeSer	655.8	657**	L-Ser	$^{iso}C_3H_7$	$^{iso}C_3H_7$	CH_3	$^{iso}C_3H_7$	$^{iso}C_3H_7$	$^{iso}C_3H_7$
	D-Hiv$_3$-L-Melle-(L-MeSer)$_2$	629.8	630*		$^{iso}C_3H_7$	$^{iso}C_3H_7$	CH_2CH_3	$^{iso}C_3H_7$	$^{iso}C_3H_7$	$^{iso}C_3H_7$
G	D-Lac$_3$-(L-MeVal)$_3$	555.6	555*	D-Lac	$^{iso}C_3H_7$	$^{iso}C_3H_7$	CH_2OH	$^{iso}C_3H_7$	$^{iso}C_3H_7$	$^{iso}C_3H_7$
H	D-Hiv$_3$-(L-MeVal)$_2$-L-MeThr	641.8	643**	L-Thr	$^{iso}C_3H_7$	CH_2OH	CH_2OH	$^{iso}C_3H_7$	$^{iso}C_3H_7$	$^{iso}C_3H_7$
	D-Hiv$_3$-L-MeVal-(L-MeThr)$_2$	643.8	644**		CH_2OH	CH_2OH	CH_2OH	$^{iso}C_3H_7$	$^{iso}C_3H_7$	$^{iso}C_3H_7$

* EI-MS corresponds to M^+, ** LC-MS (ESI) corresponds to $[M+H]^+$, *** LC-MS (ESI ro APCI) corresponds to $[M+Na]^+$.

Enniatin synthetase (ESyn) from *F. sambucinum* was used in A, B, C, D, E, and F.

ESyn from *F. scirpi* was used in H and G.

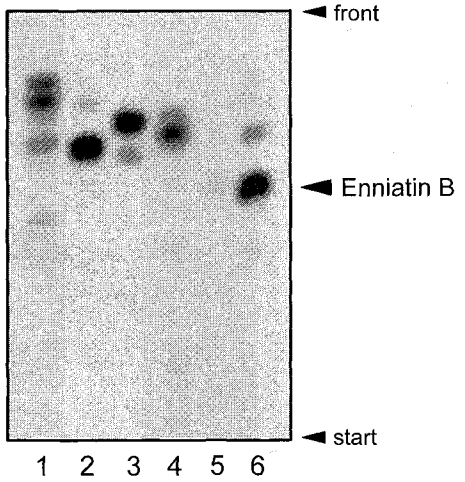
A~H: Product identification was done by EI-MS, A and G: Additional analysis by ^{1}H NMR and ^{13}C NMR.

shown in Fig. 3 all D-2-hydroxy carboxylic acid containing compounds tested gave new radioctive products with higher Rf values than enniatin B.

Upscaling of the enzymatic synthesis and subsequent HPLC separation yielded indeed various D-Lac-containing and the corresponding enniatin B homologues as measured by MS and ^{1}H NMR analysis (Table 1). As can be seen synthesis of asymmetrically substituted enniatins containing one or two units of D-Lac and the total exchange of D-Hiv by D-Lac could be observed (Table 1, Fig. 4a). The structure of the corresponding enniatin homologue (D-Lac-L-MeVal)$_3$ was elucidated on the basis of its EI-MS, ^{1}H and ^{13}C NMR spectra. The EI-MS spectrum of the compound showed a molecular ion M^+ of *m/z* 555 associated with the fragment ions at *m/z* 482, 353, 268, 168 and 86. The initial fragmentation of the enniatin is accompanied by the loss of the [D-Lac]-fragment containing one more hydrogen atom, the resulting fragment ion is *m/z* 482. The further loss of the [L-MeVal]-fragment for the enniatin generates the fragment ion at *m/z* 353 with only very low intensity. The ^{1}H and ^{13}C NMR spectral data are summarized in Table 3. The simplicity of the NMR spectra is indicative of the symmetric and cyclic nature of the depsipeptide. The ^{13}C NMR spectrum ($CDCl_3$) showed only 9 resonances, in contrast to the 27 carbons in the molecule (Table 3). The ^{1}H NMR spectrum in $CDCl_3$ displayed 7 proton signals (Table 3). The results on the basis of ^{1}H/^{13}C NMR spectra supported the molecular formula of the new enniatin consisting of three D-Lac and three L-MeVal residues. In the case of the asymmetrically substituted enniatin A homologue D-Hiv-L-MeIle-D-Lac-L-MeIle-D-Lac-L-MeIle (Fig. 4b) the ^{13}C NMR spectrum ($CDCl_3$) confirming the structure showed 32 resonances (Table 3).

From kinetic studies *in vitro* using the 2-hydroxy carboxylic acids described above we found a slow down of the reaction rate of product formation when D-Lac was used (data not shown).

Fig. 3. *In vitro* synthesis of enniatins containing different D-2-hydroxy carboxylic acids (autoradiograph of TLC separation of ethyl acetate extractable compounds).



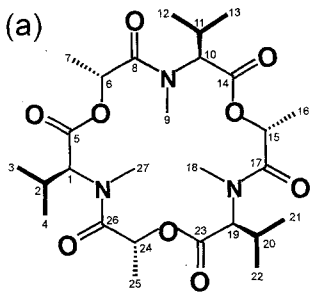
ESyn from *F. scirpi* was incubated in the presence of 1 mM L-valine, 5 mM ATP, 10 mM $MgCl_2$, 10 mM D-2-hydroxy carboxylic acid, and 0.15 μCi [^{14}C-methyl]-AdoMet.

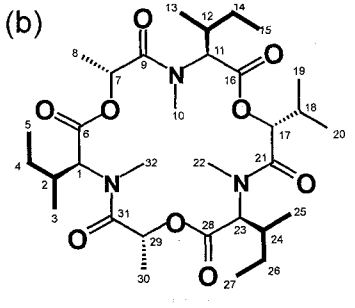
In lane 1~5 D-Hiv was replaced by (1) D-2-hydroxy-*n*-valeric-acid, (2) D-α-hydroxy-β-methyl-*n*-valeric acid, (3) D-2-hydroxybutyric acid, (4) D-Lac, and (5) L-Lac.

Lane 6: D-Hiv (control).

The solvent system was EtOAc - MeOH - H_2O, 100 : 5 : 1 (in volume).

Fig. 4. Structures of the new compounds *cyclo*(*N*-methyl-L-valyl-D-lactyl-*N*-methyl-L-valyl-D-lactyl-*N*-methyl-L-valyl-D-lactyl) (a) and *cyclo*(*N*-methyl-L-isoleucyl-D-hydroxyisovaleryl-*N*-methyl-L-isoleucyl-D-lactyl-*N*-methyl-L-isoleucyl-D-lactyl) (b).





In Vivo Studies

In addition to the *in vitro* studies we demonstrated the production of new enniatins by feeding submerged cultures of enniatin producing *Fusaria* with homologous substrates. The D-Hiv homologues used were DL-2-hydroxy-*n*-valeric acid, D-2-hydroxy-3-methyl-*n*-valeric acid, DL-Hbu, and D-Lac and the L-amino acid substrates externally added were L-Abu, L-alanine, L-cysteine, L-threonine, L-serine and L-allylglycine. The obtained crude enniatins were isolated by HPLC. Spectroscopical analysis (MS, ^{1}H NMR) revealed that indeed both the D-2-hydroxy carboxylic and the *N*-methyl L-amino acid moiety could be exchanged (Table 2).

The D-2-hydroxy carboxylic acid compounds D-2-hydroxybutyric acid and D-Lac as well as the L-amino acid components L-alanine, L-Abu, L-serine, and L-threonine could be introduced into the enniatin molecule (Table 2). In the case of *F. scirpi* the incorporation of *N*-methylalanine into the enniatin B homologue D-Hiv-L-MeVal-D-Hiv-L-MeVal-D-Hiv-L-MeAla was confirmed by EI-MS and ^{1}H NMR (Table 2). The ^{1}H NMR spectrum of the compound displayed 12 signals of which however only one proton signal (δ 1.45, 3H, d, J=7.0 Hz) could clearly be classified as CH_3 protons of *N*-methylalanine. Most of the newly formed products were hybrid enniatins with one or two units replaced by new substrates. However, products containing triple substitutions of one component were also formed.

Table 2. *In vivo* biosynthesis of new enniatins by precursor feeding submerged *Fusarium* cultures.

	Enniatin	MW [g/mol]	MS data (*m/z*)	Precursor added	R_1	R_2	R_3	R_4	R_5	R_6
A	D-Hiv$_2$-D-Lac-(L-Melle)$_3$	653.8	653* 654** 676***	D-Lac	$^{sec}C_4H_9$	$^{sec}C_4H_9$	$^{sec}C_4H_9$	$^{iso}C_3H_7$	$^{iso}C_3H_7$	CH_3
	D-Hiv-D-Lac$_2$-(L-Melle)$_3$	625.8	625* 626** ^{1}H/^{13}C NMR		$^{sec}C_4H_9$	$^{sec}C_4H_9$	$^{sec}C_4H_9$	$^{iso}C_3H_7$	CH_3	CH_3
B	D-Hiv$_3$-(L-Melle)$_2$-L-MeSer	655.8	657**	L-Ser	$^{sec}C_4H_9$	$^{sec}C_4H_9$	CH_2OH	$^{iso}C_3H_7$	$^{iso}C_3H_7$	$^{iso}C_3H_7$
	D-Hiv$_3$-L-Melle-(L-MeSer)$_2$	629.8	629*		$^{sec}C_4H_9$	CH_2OH	CH_2OH	$^{iso}C_3H_7$	$^{iso}C_3H_7$	$^{iso}C_3H_7$
C	D-Hiv$_2$-D-Lac-(L-MeVal)$_3$	611.7	611*	D-Lac	$^{iso}C_3H_7$	$^{iso}C_3H_7$	$^{iso}C_3H_7$	$^{iso}C_3H_7$	$^{iso}C_3H_7$	CH_3
	D-Hiv-D-Lac$_2$-(L-MeVal)$_3$	583.7	583*		$^{iso}C_3H_7$	$^{iso}C_3H_7$	$^{iso}C_3H_7$	$^{iso}C_3H_7$	CH_3	CH_3
	D-Lac$_3$-(L-MeVal)$_3$	555.6	555* ^{1}H/^{13}C NMR		$^{iso}C_3H_7$	$^{iso}C_3H_7$	$^{iso}C_3H_7$	CH_3	CH_3	CH_3
D	D-Hiv$_2$-D-Hbu-(L-MeVal)$_3$	625.8	625*	DL-Hbu	$^{iso}C_3H_7$	$^{iso}C_3H_7$	$^{iso}C_3H_7$	$^{iso}C_3H_7$	$^{iso}C_3H_7$	CH_2CH_3
	D-Hiv-D-Hbu$_2$-(L-MeVal)$_3$	611.7	611*		$^{iso}C_3H_7$	$^{iso}C_3H_7$	$^{iso}C_3H_7$	$^{iso}C_3H_7$	CH_2CH_3	CH_2CH_3
E	D-Hiv$_3$-(L-MeVal)$_2$-L-MeAla	611.7	611* ^{1}H NMR	L-Ala	$^{iso}C_3H_7$	$^{iso}C_3H_7$	CH_3	$^{iso}C_3H_7$	$^{iso}C_3H_7$	$^{iso}C_3H_7$
F	D-Hiv$_3$-(L-MeVal)$_2$-L-MeAbu	625.8	625*	L-Abu	$^{iso}C_3H_7$	$^{iso}C_3H_7$	CH_2CH_3	$^{iso}C_3H_7$	$^{iso}C_3H_7$	$^{iso}C_3H_7$
G	D-Hiv$_3$-(L-MeVal)$_2$-L-MeSer	627.8	628**	L-Ser	$^{iso}C_3H_7$	$^{iso}C_3H_7$	CH_2OH	$^{iso}C_3H_7$	$^{iso}C_3H_7$	$^{iso}C_3H_7$
	D-Hiv$_3$-L-MeVal-(L-MeSer)$_2$	615.7	616**		$^{iso}C_3H_7$	CH_2OH	CH_2OH	$^{iso}C_3H_7$	$^{iso}C_3H_7$	$^{iso}C_3H_7$
	D-Hiv$_3$-(L-MeSer)$_3$	603.7	604** 627***		CH_2OH	CH_2OH	CH_2OH	$^{iso}C_3H_7$	$^{iso}C_3H_7$	$^{iso}C_3H_7$

* EI-MS corresponds to M^+, ** LC-MS (ESI) corresponds to $[M+H]^+$, *** LC-MS (ESI ro APCI) corresponds to $[M+Na]^+$.

F. sambucinum was used in A and B.

F. scirpi was used in C, D, E, F, and G.

A~G: Product identification was done by EI-MS, A, C, and E: Additional analysis by ^{1}H NMR and/or ^{13}C NMR.

Table 3. ^{1}H (400 MHz) and/or ^{13}C (100 MHz) NMR spectral data for the new compounds *cyclo*(*N*-methyl-L-valyl-D-lactyl-*N*-methyl-L-valyl-D-lactyl-*N*-methyl-L-valyl-D-lactyl) (**1**) and *cyclo*(*N*-methyl-L-isoleucyl-D-hydroxyisovaleryl-*N*-methyl-L-isoleucyl-D-lactyl-*N*-methyl-L-isoleucyl-D-lactyl) (**2**) in $CDCl_3$.

	1		**2**
Carbon No.	^{1}H chemical shifts (ppm)[a]	^{13}C chemical shifts (ppm)[b]	^{13}C chemical shifts (ppm)[b]
1	4.43 (1H, d, *J*=9.8 Hz)	63.1	60.5
2	2.27 (1H, m)	27.8	32.3
3	0.83 (3H, d, *J*=6.7 Hz)	18.5	15.3
4	1.03 (3H, d, *J*=6.5 Hz)	20.1	25.0
5		169.8	10.3
6	5.62 (1H, q, *J*=6.5 Hz)	66.3	170.1
7	1.45 (3H, d, *J*=6.5 Hz)	16.5	66.1
8		169.2	16.8
9	3.06 (3H, s)	32.9	169.0
10	4.43 (1H, d, *J*=9.8 Hz)	63.1	31.6
11	2.27 (1H, m)	27.8	65.1
12	1.03 (3H, d, *J*=6.5 Hz)	20.1	34.1
13	0.83 (3H, d, *J*=6.7 Hz)	18.5	16.7
14		169.8	24.9
15	5.62 (1H, q, *J*=6.5 Hz)	66.3	10.6
16	1.45 (3H, d, *J*=6.5 Hz)	16.5	170.6
17		169.2	74.0
18	3.06 (3H, s)	32.9	29.9
19	4.43 (1H, d, *J*=9.8 Hz)	63.1	18.4
20	2.27 (1H, m)	27.8	18.0
21	1.03 (3H, d, *J*=6.5 Hz)	20.1	169.1
22	0.83 (3H, d, *J*=6.7 Hz)	18.5	31.2
23		169.8	59.5
24	5.62 (1H, q, *J*=6.5 Hz)	66.3	34.7
25	1.45 (3H, d, *J*=6.5 Hz)	16.5	16.0
26		169.2	24.4
27	3.06 (3H, s)	32.9	11.4
28			169.8
29			67.5
30			15.8
31			169.2
32			35.6

[a] Chemical shifts are shown with reference to $CHCl_3$ as 7.29 ppm.

[b] Chemical shifts are shown with reference to $CHCl_3$ as 77.2 ppm.

Coupling constants expressed in Hz are given in parentheses

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