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Synthesis and anthelmintic activity of cyclohexadepsipeptides with cyclohexylmethyl side chains

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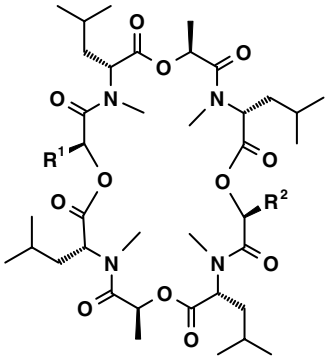
Abstract—Cyclohexadepsipeptides (CHDPs) with cyclohexylmethyl side chains represent novel enniatins with in vivo activity against the parasitic nematode *Haemonchus contortus* Rudolphi in sheep. It was found that the replacement of benzylic by cyclohexylmethyl side chains on the enniatin skeleton can increase anthelmintic efficacy. Here we report on a simple total synthesis of the precursors for this type of CHDPs and an efficient chemical transformation of the benzylic into the corresponding cyclohexylmethyl side chains.

Helminths, especially parasitic nematodes, cause significant problems for the health and well being of animals and humans. Gastrointestinal nematodes like *Haemonchus contortus* Rudolphi occur worldwide and parasitize the abomasus of domestic animals such as cattle and sheep.[1] Currently, four distinct anthelmintic classes are used for broad spectrum control of parasitic nematodes in veterinary medicine:[2] (a) benzimidazole derivatives, (b) levamisole, (c) pyrantel and (d) macrocyclic lactones.[3] However, the emerging resistance of parasites towards these traditional anthelmintics is becoming a serious problem.[3a] Therefore, the search for novel anthelmintic drugs is of increasing importance.[4]

In recent years,[5] the 24-membered cyclooctadepsipeptides (CODPs) represent the most promising substance class among the newly described anthelmintics. The chemical variation of the potent anthelmintic PF1022A **1**[6] led to the *semi*-synthetic derivative emodepside **2** (Bay 44-4400)[7], which has been commercialized as Profender spot-on® for cats in combination with praziquantel[8] (Fig. 1).

In studying the anthelmintic efficacy of the structurally closely related 18-membered cyclohexadepsipeptides (CHDPs), the so-called enniatins, we became interested in the synthesis of *semi*-synthetic CHDPs with regard to their efficacy against *H. contortus* in sheep.[9]

Some years ago we found, that the replacement of one *N*-methyl-(*S*)-isoleucine in 5-position (MeIle[5]) of the in vitro active naturally occurring enniatin A **5** by *N*-methyl-(*S*)-phenylalanine (MePhe[5]), as a structural unit of the in vivo active, naturally occurring



1	R^1, R^2 = Bn	(PF1022A)
2	R^1, R^2 = 4-*N*-Mor-benzyl	(Emodepside)
3	R^1 = Chm, R^2 = Bn	(PF1022AHH)
4	R^1, R^2 = Chm	(PF1022ADH)

Figure 1. Structure of the cyclooctadepsipeptides (**1**–**4**).

Keywords: Cyclohexadepsipeptides; Enniatin; Nematode; Haemonchus contortus; Conformer; *cis*-Amide bond; Total synthesis.
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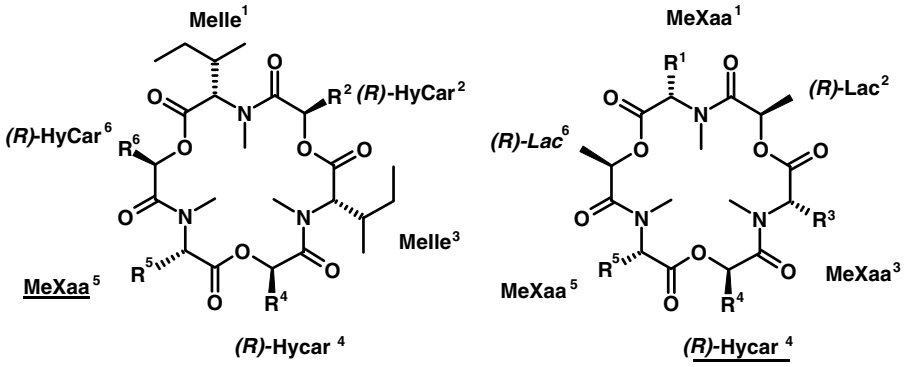


5 $R^{2,4,6}$ = *i*Pr, R^5 = *s*Bu (Enniatin A)
6 $R^{2,4,6}$ = *i*Pr, R^5 = Bn
7 $R^{2,4,6}$ = *i*Pr, R^5 = Chm
8 $R^{2,4,6}$ = Me, R^5 = Bn
9 $R^{2,4,6}$ = Me, R^5 = Chm

10 $R^{1,3,5}$ = *i*Bu, R^4 = Bn
11 $R^{1,3,5}$ = *i*Bu, R^4 = Chm
12 $R^{1,3}$ = Me, R^5 = *s*Bu, R^4 = Bn
13 $R^{1,3}$ = Me, R^5 = *s*Bu, R^4 = Chm

Figure 2. Structures of the enniatines (**5**–**13**).

Table 1. In vivo anthelmintic activities against *H. contortus* in sheep, ratio of conformers and lipophilicities of the CHDPs with cyclohexylmethyl side chain **7**, **9** and **11** in comparison with the known CHDP analogues **6**, **8** and **10**.

CHDP No.	MeXaa[5] versus (*R*)-HyCar[4]	Ratio of conformers[a]	Lipophilicity log *P*[b]	Anthelmintic activity against *H. contortus*
6	MePhe[5]	[e]	5.46	5.00[c]/3[d]
7	MeCha[5]	24:1	6.30	0.50/2
8	MePhe[5]	19:1	4.28	0.25/1
9	MeCha[5]	24:1	4.91	0.25/3
10	(*R*)-PhLac[4]	9:1	4.63	5.00/0
11	(*R*)-ChxLac[4]	9:1	5.57	0.25/2

[a] NMR spectra were recorded in $CDCl_3$.
[b] log *P*-value from HPLC (pH 2.3).
[c] Dose in mg test substance kg^{-1} body weight.
[d] 0 = ⩽50% egg reduction; 1 = 50–75% egg reduction; 2 = 75–95% egg reduction; 3 = ⩾95% egg reduction.
[e] Not determined.

beauvericin[10], to give **6** significantly improved the anthelmintic activity (Fig. 2 and Table 1).

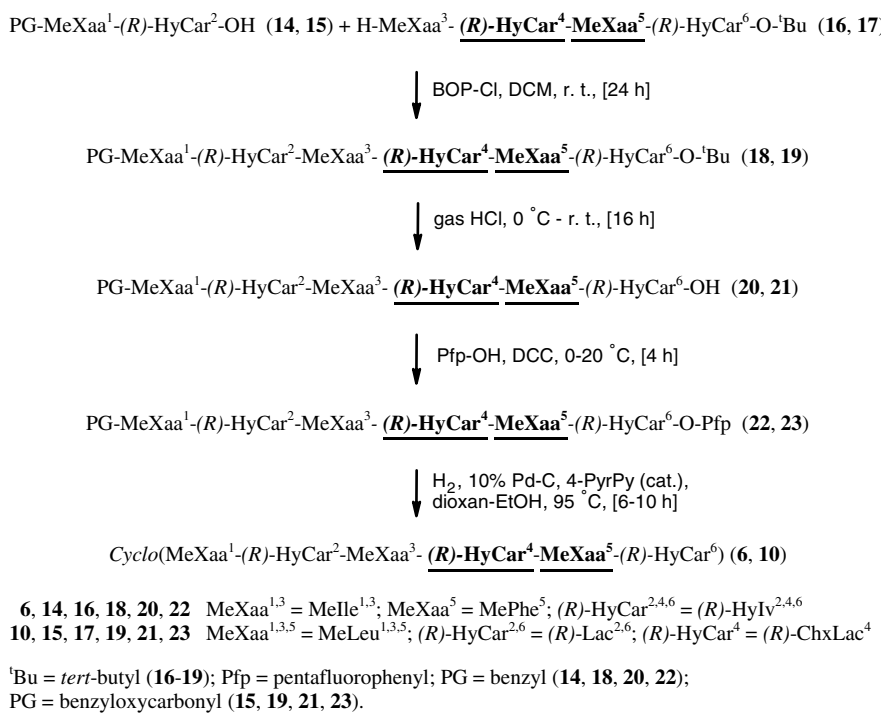
In order to better understand the effect of the benzyl substituents within the CHDPs with regard to their anthelmintic activities, we then focussed our attention on the replacement of this aromatic structural element by its more lipophilic cyclohexylmethyl (Chm) analogue. Such modification may alter the physicochemical behaviour of a molecule and ultimately its biological profile. For example, the replacement of one or two (*R*)-phenyllactic acid fragments (*R*-PhLac; R^1 and/or R^2 = Bn) in PF1022A **1** by (*R*)-ChxLac (R^1 and/or R^2 = Chm) leads to a drastic decrease of anthelmintic activity. Both the hexahydro- and dodecahydro derivative PF1022AHH **3** (R^1 = H, R^2 = Chm) and PF1022ADH **4** (R^1, R^2 = Chm) are up to 10-fold less active than **1** (Fig. 1).[11]

Particular cycloalkyl-substituted enniatins have been claimed as antiprotozoal drugs.[12] In this paper, we present a simple total synthesis of MePhe- and (*R*)-PhLac-containing CHDPs and the efficient PtO_2-catalyzed hydrogenation of the benzylic side chain yielding the corresponding MeCha as well as (*R*)-ChxLac substitution.

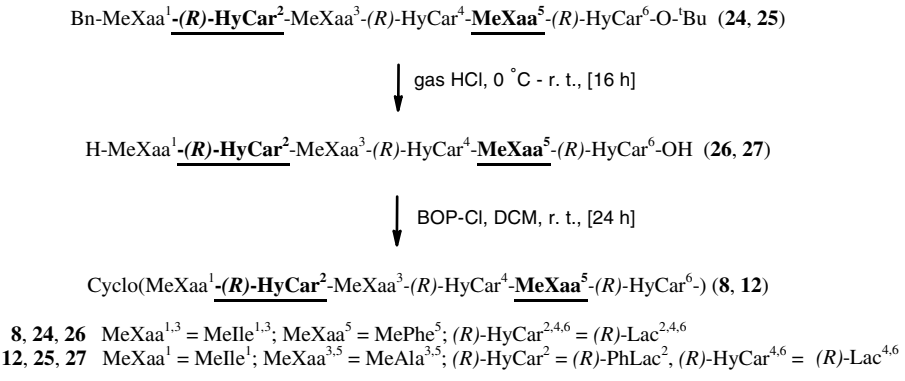
The method for preparing the parent CHDPs (**6**, **8**, **10** and **12**) involved formation of the depsipeptide hexamers (**22**, **23**, **26** and **27**)[13] from three dimeric fragments by a [2 + 4]-fragment condensation reaction, for example, by using the N-terminal protected didepsipeptides (**14**, **15**) and the O-terminal protected tetradepsipeptide fragments (**16**, **17**) in a convergent strategy as described by Jeschke et al.[14] Several other methods are known for the synthesis of CHDPs.[15]

The macrocyclization was accomplished by two different strategies in highly dilute solution, namely by (a) cyclization of the N-terminal protected linear hexadepsipeptide pentafluorophenyl (Pfp) ester (**22**, PG = Bn; **23**, PG = Z) in 17–63% yield (Scheme 1)[16,17] and (b) by ring closure of the N- and O-terminal deprotected hexadepsipeptides (**26**, **27**) affording the CHDPs **6**[18], **8**[19], **10**[20,21] and **12**[22] as shown in Scheme 2.

The latter methodology, in which the phosphonium coupling reagent bis(2-oxo-3-oxazolidinyl)-phosphonic chloride (BOP-Cl) and *N*,*N*-diisopropylethylamine (DIEA) were used, was a helpful simplification and permitted preparation of enniatins on larger scales.



Scheme 1. Synthesis of the CHDPs **6** and **10** by macrocyclization of the N-terminal protected hexadepsipeptide pentafluorophenyl esters **22** and **23**.



Scheme 2. Synthesis of the CHDPs **8** and **12** by macrocyclization of the N- and O-terminal deprotected hexadepsipeptides **26** and **27**.

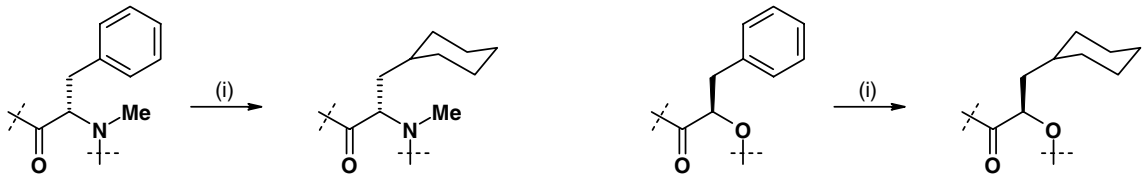
Subsequent hydrogenation of the CHDPs **6**, **8**, **10** and **12** over PtO_2 catalyst in the presence of acetic acid/water (1.4:1.0) at 50 °C under pressure (hydrogen 4–5 bar, 18 h, autoclave reaction) resulted in enniatins **7**, **9**, **11** and **13** without racemization, that contain a Chm side chain and were easily purified via preparative HPLC (Scheme 3).[23]

An efficient hydrogenation was observed when using around 1/6 amount of PtO_2 catalyst.

The structural assignments of the novel CHDPs (**7**, **9**, **11** and **13**) were based on the fragmentation pattern of molecular ion peaks $[M]^+$ in the EI mass spectra (additional molecular weight $m/z = 6$).

The fragmentation path in EI mass spectra exhibits the expected ring opening at ester and/or amide bonds.[24] In the ^{13}C NMR spectra all fragments could be assigned (absence of aromatic carbon signals).

Sheep (*Ovis aries* L, Merino or Schwarzkopf breed, 25–35 kg body weight) were infected experimentally with 5000 *H. contortus* Rudolphi L_3 larvae and treated with the test substance after the end of the prepatency period of the parasite. The test compounds were administered orally in gelatine capsules. Anthelmintic effects of the test substances were measured as a function of the reduction in faecal egg counts. For the purpose of counting eggs, freshly obtained faeces from experimentally infested animals were prepared using the McMaster method as modified by Wetzel.[25] The egg counts were determined at regular intervals before and after treatment. The anthelmintic evaluation was expressed as a function of the egg reduction as follows: $3 \geqslant 95\%$, 2 = 75–95%, 1 = 50–75% and $0 = \leqslant 50\%$ egg reduction.



MeXaa[5]: -MePhe[5]- **(6, 8)** -MeCha[5]- **(7, 9)** (*R*)-HyCar[4]: -(*R*)-PhLac[4]- **(10, 12)** -(*R*)-ChxLac[4]- **(11, 13)**

(i) H_2 - PtO_2(cat.), Ac-OH - H_2O (1.4:1.0), 50 °C, [18 h], 4-5 bar (autoclave)

Scheme 3. Synthesis of the CHDPs with cyclohexylmethyl side chain (**7**, **9**, **11** and **13**) by *N*-methyl-(*S*)-phenylalanine or (*R*)-phenyllactic acid modification in **6**, **8** and **10**, **11**.

The CHDPs **7** and **9** exhibited a 24:1 mixture of conformers in $CDCl_3$ because of the Chm side chain. On the other hand the CHDP **11** showed a 9:1 mixture of conformers in $CDCl_3$ as outlined in Table 1. The ratio of conformers of all Chm-substituted enniatins is similar to their parent compounds (**6**, **8**, **10** and **12**).

Further spectroscopic analysis of the most active CHDP **9** using a combination of 2D NMR (^1H–^1H NOESY, ^1H–^1H COSY, ^1H–^{13}C HMBC, ^1H–^{13}C HMQC) techniques showed in $CDCl_3$ solution one major conformer with an unsymmetrically folded conformation lacking a *cis*-amide bond, which corresponds to that of anthelmintically active enniatins.[14]

The CHDPs **7**, **9** and **11** tested in vivo were found to be active against the gastrointestinal nematode *H. contortus* in sheep at 0.2–0.5 mg/kg as outlined in Table 1.

In comparison to the parent CHDPs (**6**, **8**, **10** and **12**), all Chm-substituted enniatins show a strong shift of the octanol–water partition coefficients ($\log P$ values)[26] (cf. 0.63–0.94, Table 1). The CHDP **7** ($\log P = 6.30$) with MeCha in 5-position showed 10-fold greater activity against *H. contortus* than compound **6**. In addition, the MeCha derivative **9** ($\log P = 4.28$) displayed higher activity at 0.2 mg/kg b.w. against this parasitic nematode compared to its MePhe counterpart **8**. On the other hand, the CHDP **11** ($\log P = 5.57$) with (*R*)-configurated ChxLac in 5-position displayed more than 20-fold higher activity against *H. contortus* than compound **10** ($\log P = 4.63$). This fact is surprising because the replacement of one (*R*)-PhLac fragment in CODPs such as PF1022A **1** by (*R*)-ChxLac leads to a drastic decrease of the anthelmintic activity.

The reason for this effect seems to be not only a change in lipophilicity but also in steric parameters.

In conclusion, this paper describes the efficient synthesis of novel Chm side-chain containing CHDPs **7**, **9**, **11** and **13**, exhibiting in vivo anthelmintic activities against the gastrointestinal nematode *H. contortus* in sheep. It was found that the replacement of benzylic side chains (MePhe vs (*R*)-PhLac) by their more lipophilic hydrogenated analogues (MeCha vs (*R*)-ChxLac) leads to higher efficacy. Furthermore, the results demonstrate that MeCha in 5-position of the CHDP **9** can stabilize the major conformer with an unsymmetrically folded conformation lacking a *cis*-amide bond. In contrast to 24-membered CODPs, for example, the hexahydro derivative PF1022AHH **3**, hydrogenation of the (*R*)-PhLac fragment in 18-membered CHDPs can improve the anthelmintic activity as demonstrated for **11**, respectively. This result may be of importance for the design of further anthelmintically active CODPs.

Acknowledgments

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17. General method for macrocyclization of the N-terminal protected hexadepsipeptide pentafluorophenyl esters. N-terminal protected linear hexadepsipeptide pentafluorophenyl ester (1.08 mmol) in 50 ml of absolute dioxan was injected uniformely during the course of 6 h at an internal temperature of 95 °C into a rapidly stirred suspension of 1.5 g of 10% Pd–C in 550 ml of absolute dioxan containing 12 ml ethanol and 4-pyrrolidino-pyridine (1.08 mmol). During this process, hydrogen was passed through the reaction solution. Stirring was continued for a further 4 h at 95 °C, and for 12 h at room temperature. Then the reaction solution was filtered and the filtrate was concentrated in vacuo. The oily residue was taken up in chloroform and washed twice with 5% citric acid, $NaHCO_3$ solution and twice with water. The organic phase was dried over sodium sulfate, the filtrate was concentrated in vacuo and the residue purified by silica gel chromatography to give the MePhe or (*R*)-PhLac-containing enniatins.
18. *Cyclo*(*N*-methyl-(*S*)-isoleucinyl-(*R*)-2-hydroxy-isovaleryl-*N*-methyl-(*S*)-isoleucyl-(*R*)-2-hydroxy-isovaleroyl-*N*-methyl-(*S*)-phenylalanyl-(*R*)-2-hydroxy-isovaleroyl) **6** (yield: 39%): ^{13}C NMR (100 MHz, $CDCl_3$) δ 2× 10.6, 15.7, 15.9, 2× 18.2, 4× 18.3, 18.6, 19.0 (CH_3), 25.0, 25.2, 29.4, 29.7, 30.3, 35.0 (CH), 31.4, 31.8, 36.0 (NCH_3), 59.6, 60.5, 62.6 (CH–N), 74.8, 75.2 (CH–O), 169.3, 170.3, 170.4 (CO–O), 169.1, 169.2, 169.7 (CO–N). EI-MS: *m/e* 715 (M^+, 27).
19. Synthesis of *cyclo*(*N*-methyl-(*S*)-isoleucyl-(*R*)-lactyl-*N*-methyl-(*S*)-isoleucyl-(*R*)-lactyl-*N*-methyl-(*S*)-phenylalanyl-(*R*)-lactyl) **8**. DIEA (0.24 g, 1.92 mmol) and BOP-Cl (0.235 g, 0.92 mmol) were added at 0 °C to a solution of *N*-methyl-(*S*)-isoleucyl-(*R*)-lactyl-*N*-methyl-(*S*)-isoleucyl-(*R*)-lactyl-*N*-methyl-(*S*)-phenylalanyl-(*R*)-lactic acid (0.50 g, 0.769 mmol) in CH_2Cl_2 (DCM) (500 mL) and the mixture was stirred for 24 h at room temperature. Then a further amount of DIEA (0.24 g, 1.92 mmol) and BOP-Cl (0.235 g, 0.92 mmol) was added at 0 °C and stirring continued for 24 h at room temperature. The reaction solution was washed twice with water, and the organic phase separated off and dried over Na_2SO_4. The filtrate was concentrated in vacuo and the residue purified by silica gel chromatography (cyclohexane/ethyl acetate, 2:1) to give *cyclo*(*N*-methyl-(*S*)-isoleucyl-(*R*)-lactyl-*N*-methyl-(*S*)-isoleucyl-(*R*)-lactyl-*N*-methyl-(*S*)-phenylalanyl-(*R*)-lactyl) (1.3 g, 67%). ^{13}C NMR (100 MHz, $CDCl_3$) δ 2× 10.6, 15.3, 16.2 (CH_3), 24.4, 24.5 (CH_2), 32.2, 34.2 (CH), 31.0, 31.3, 36.0 (NCH_3), 34.8 (CH_2-phenyl), 60.0, 60.1, 64.4 (CH–N), 66.3, 65.6, 67.8 (CH–O), 126.6, 128.5, 129.4, 137.7 (C-phenyl), 169.8, 169.6, 170.2 (CO–O), 168.9, 169.0, 169.6 (CO–N). EI-MS: *m/e* 631 (M^+, 23).
20. *Cyclo*(*N*-methyl-(*S*)-isoleucinyl-(*R*)-lactyl-*N*-methyl-(*S*)-isoleucyl-(*R*)-phenyllactyl-*N*-methyl-(*S*)-isoleucyl-(*R*)-lactyl) **10** (yield: 52–63%): ^{13}C NMR (100 MHz, $CDCl_3$) δ 2× 16.4, 21.7, 21.8, 22.9 (CH_3), 2× 22.8, 23.0, 24.6, 2× 24.9, 37.6 (CH_2), 36.8, 2× 37.4 (CH), 31.0, 31.7, 32.8 (NCH_3), 55.3, 54.6, 56.5 (CH–N), 67.0, 67.1, 70.2 (CH–O), 126.9, 128.4, 129.6, 135.5 (arom-C), 170.3, 170.5, 170.6 (CO–O), 168.8, 2× 169.7 (CO–N). EI-MS: *m/e* 673 (M^+, 41).
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22. *Cyclo*(*N*-methyl-(*S*)-alanyl-(*R*)-lactyl-*N*-methyl-(*S*)-isoleucyl-(*R*)-phenyllactyl-*N*-methyl-(*S*)-alanyl-(*R*)-lactyl) **12** (yield: 42%): ^{13}C NMR (100 MHz, $CDCl_3$) δ 10.7, 13.7, 15.4, 16.0, 16.3, 16.4 (CH_3), 23.8, 37.7 (CH_2), 31.7 (CH), 29.7, 29.8, 30.1 (NCH_3), 53.7, 54.0, 59.9 (CH–N), 67.6, 68.7, 71.2 (CH–O), 127.2, 128.4, 129.3, 135.3 (arom-C), 169.3, 170.9, 171.5 (CO–O), 168.2, 168.9, 171.2 (CO–N). EI-MS: *m/e* 589 (M^+, 26).
23. General procedure for hydrogenation of MePhe or (*R*)-PhLac-containing enniatins. Enniatin (0.8 mmol) was hydrogenated at 50 °C in water (8.4 mL) in presence of 0.1 g PtO_2 as catalyst for 18 h at 4–5 bar (autoclave). Then the reaction solution was filtered, the filtrate concentrated in vacuo and the residue purified by silica gel chromatography (cyclohexan/ethyl acetate, 2:1) to give the Chm-substituted enniatins.
Cyclo(*N*-methyl-(*S*)-isoleucinyl-(*R*)-lactyl-*N*-methyl-(*S*)-isoleucyl-(*R*)-lactyl-*N*-methyl-(*S*)-cyclohexylmethylalanyl-(*R*)-lactyl) **9** (yield: 100%): ^{13}C NMR (100 MHz, $CDCl_3$)δ 10.6, 10.8, 2× 15.7, 16.2, 16.5, 16.9, (CH_3), 24,4, 24.7 (CH_2), 33.3, 34.2 (CH), 31.1, 32.5, 32.6 (NCH_3), 26.0, 26.1, 26.3, 32.5, 33.5, 35.8 (Chm), 56.2, 60.1, 61.9 (CH–N), 66.6, 66.8, 67.3 (CH–O), 169.9, 170.0, 170.6 (CO–O), 168.9, 169. 3, 169.5 (CO–N). EI-MS: *m/e* 637 (M^+, 25).

Cyclo(*N*-methyl-(*S*)-isoleucinyl-(*R*)-2-hydroxy-isovaleroyl-*N*-methyl-(*S*)-isoleucyl-(*R*)-2-hydroxy-isovaleroyl-*N*-methyl-(*S*)-cyclohexylmethylalanyl-(*R*)-2-hydroxy-isovaleroyl)**7** (yield: 38%) EI-MS: *m*/*e* 721 (M^+, 25).

Cyclo(*N*-methyl-(*S*)-leucinyl-(*R*)-lactyl-*N*-methyl-(*S*)-leucyl-(*R*)-cyclohexyllactyl-*N*-methyl-(*S*)-leucinyl-(*R*)-lactyl) **11** (yield: 79%): ^{13}C NMR (100 MHz, $CDCl_3$) δ 16.6, 16.9, 21.7, 21.8, 21.9, 22.7, 2× 22.9 (CH_3), 26.0, 26.1, 26.3, 32.6, 33.6, 37.1, 37.2, 37.5, 38,2 (CH_2), 2× 24.9, 25.0, 33.8 (CH), 31.7, 32.1, 32.3 (NCH_3), 55.4, 55.7, 56.1 (CH–N), 66.9, 67.2, 68.3 (CH–O), 169.4, 2× 170.5 (CO–O), 169.5, 169.6, 170.6 (CO–N). EI-MS: *m*/*e* 679 (M^+, 32).

Cyclo(*N*-methyl-(*S*)-alanyl-(*R*)-lactyl-*N*-methyl-(*S*)-isoleucyl-(*R*)-cyclohexyllactyl-*N*-methyl-(*S*)-alanyl-(*R*)-lactyl) **13** (yield: 38%): ^{13}C NMR (100 MHz, $CDCl_3$) δ 10.8, 16.0 (CH_3), 23.9 (CH_2), 33.7 (CH), 29.8, 30.4, 33.9 (NCH_3), 25.9, 26.0, 26.3, 29.9, 32.1, 33.9, 37.9 (Chm), 53.8, 54.1, 60.1 (CH–N), 67.6, 68.6, 68.8 (CH–O), 170.8, 171.6, 172.4 (CO–O), 168.2, 168.6, 168.9 (CO–N). EI-MS: *m*/*e* 595 (M^+, 2).

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