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Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 16 (2006) 4410–4415

Synthesis and anthelmintic activity of substituted (*R*)-phenyllactic acid containing cyclohexadepsipeptides

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Received 25 April 2006; revised 12 May 2006; accepted 13 May 2006
Available online 14 June 2006

Abstract—The substituted (*R*)-phenyllactic acid containing cyclohexadepsipeptides (CHDPs) represent novel enniatin derivatives with strong in vivo activities against the parasitic nematode *Haemonchus contortus* Rudolphi in sheep. 2D NMR spectroscopic analysis revealed for the substituted (*R*)-phenyllactic acid containing CHDPs one major conformer with an unsymmetrically folded conformation lacking a *cis*-amide bond. A correlation between the substitution pattern and its anthelmintic activity was found. Here we report on a simple total synthetic pathway of the precursor for this particular type of CHDPs and an efficient modification of the benzylic side chain (*R*-PhLac[2]).

Parasitic nematodes cause significant problems to the health and life of many plants and animals, and also of humans. Gastrointestinal nematodes like *Haemonchus contortus* Rudolphi occur worldwide and parasitize the abomasus of domestic animals such as cattle and sheep.[1] Therefore, the search of novel anthelmintic drugs plays an important role in veterinary medicine[2] because a serious problem is the emerging resistance of parasites towards traditional anthelmintics such as benzimidazole derivatives, levamisole and macrocyclic lactones.[3] The 24-membered cyclooctadepsipeptides (CODPs) represent the most promising substance class within the newly described anthelmintics in recent years.[4] This class constitutes a large family of peptide-related compounds derived from 2-hydroxy-(*R*)-carboxylic acids (*R*-HyCar) and *N*-methyl-(*S*)-amino acids (MeXaa) joined by amide and ester linkages. The broad chemical variation of the potent anthelmintic PF1022A[5] led to *semi*-synthetic derivatives containing as (*R*)-HyCar one or two (*R*)-4-*N*-morpholino-phenyl-lactic acids (*R*-4-*N*-MorPhLac) like **1**[6] and emodepside (Bay 44-4400) **2**[7], respectively. The latter is highly active against a broad spectrum of intestinal and extraintestinal nematodes such as filarial parasites and is commercialized as Profender® (2005, Bayer HealthCare Animal Health) in combination with praziquantel.[8]

To obtain more insight into the anthelmintic efficacy of the structurally closely related 18-membered cyclohexadepsipeptides (CHDPs), the so-called enniatins, we became interested in the preparation of semi-synthetic enniatin structures with regard to their efficacy against *H. contortus* in sheep.[9] Recently, the replacement of one *N*-methyl-(*S*)-isoleucine (MeIle) of the naturally occuring enniatin A by *N*-methyl-(*S*)-alanine (MeAla), as exemplified by **3**, has been reported to be 10-fold more active than the natural enniatins against *H. contortus*. A correlation between the nature of different CHDP major conformers and their anthelmintic activities was described.[10]

Structure of the cyclooctadepsipeptides (**1**–**2**) and enniatins (**3**–**7**).

It was found that those CHPDs with strong in vivo activity exist in $CDCl_3$ solution as one major conformer either with one *cis*-amide bond or with an unsymmetrically folded conformation lacking a *cis*-amide bond like **3**.

Keywords: Cyclohexadepsipepdides; CHDPs; Total synthesis; Anthelmintics; Parasitic nematode *Haemonchus contortus*; Conformers; *cis*-Amide bond.
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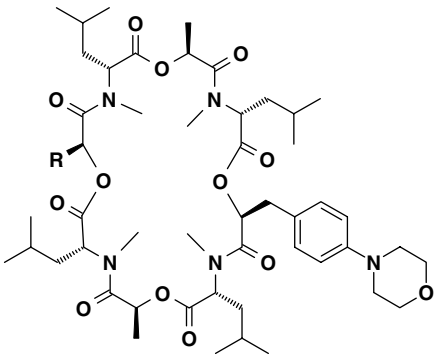
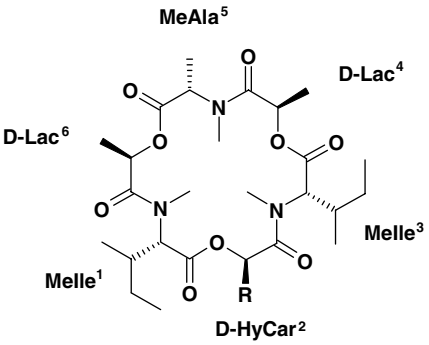
doi:10.1016/j.bmcl.2006.05.040



1 R = Me
2 R = 4-*N*-Mor-benzyl
Emodepside (Bay 44-4400)



3 R = Me
4 R = benzyl
5c R = 4-NO_2-benzyl
6a R = 2-NH_2-benzyl
6b R = 3-NH_2-benzyl
6c R = 4-NH_2-benzyl
7 R = 4-*N*-Mor-benzyl

In order to better understand the effect of the unique (*R*)-4-*N*-MorPhLac moiety within the 18-membered macrocycle in connection with its biological activity, we have now focussed our attention on the 2-position of the CHDP **3**. In fact, we found the 2-position in **3** is not important for its high binding affinity. Therefore, as part of our ongoing efforts to find novel anthelmintic drugs, we started to investigate CHDP derivatives of **3** containing substituted 2-hydroxy-(*R*)-phenyllactic acids (*R*-PhLac) in 2-position such as 2-hydroxy-(*R*)-4-nitrophenyllactic acid (*R*-4-NO_2PhLac) and 2-hydroxy-(*R*)-2-, 3- or 4-amino-phenyllactic acids (*R*-2-, 3- or 4-NH_2PhLac), respectively.[11] In this paper, we report the total synthesis of a (*R*-PhLac[2])-containing CHDP and its efficient modification of the benzylic side chain with respect to the 4-*N*-morpholino substitution.

The method for preparing the CHDP **4** involved formation of the depsipeptide hexamers (**10**–**12**)[12] from three dimeric fragments by a [2 + 4]-fragment condensation reaction, for example, by using the N-terminal protected didepsipeptides (**8**) and the O-terminal protected tetradepsipeptide fragment **9**, in a convergent strategy as already described by Jeschke et al.[7] Several further methods are known for syntheses of CHDPs.[13]

The macrocyclization was accomplished by ring closure of the N- and O-terminal deprotected hexadepsipeptides (**12**) under high dilution conditions using the phosphonium coupling reagent bis(2-oxo-3-oxazolidinyl)-phosphonic chloride (BOP-Cl) and *N*,*N*-diisopropylethylamine (DIEA), affording the CHDP **4**[14] as shown in Scheme 1. Subsequent nitration of **4** with 98% fuming nitric acid resulted in a (4:1:1) mixture of enniatins **5a**–**c** containing (*R*)-PhLac fragments *mono*-nitrated in 2-, 3- and 4-positions, from which the (*R*)-4-NO_2PhLac derivative **5c**[15] was isolated by preparative HPLC (Scheme 2).

Hydrogenation of the mixture **5a**–**c** in the presence of 20% $Pd(OH)_2$/C in ethanol afforded the amino analogues **6a**–**c**[16] as mixture of enniatin isomers (4-NH_2PhLac/3-NH_2PhLac/2-NH_2PhLac = 4:1:1) in 67% yield, which can be separated from each other by preparative HPLC or in larger amounts (up to 4.0 g) by Craig distribution (ethyl acetate/*n*-heptane/DMF/H_2O = 4:6:5:5). Finally, the *N*-morpholino ring closure forming the (*R*)-4-*N*-MorPhLac enniatin **7**[17] was carried out by reductive alkylation of **6c** with 2,2′-oxy-bis[acetaldehyde], prepared in situ from 2,5-dihydrofuran by ozonolysis, and sodium cyanoborohydride.[18]

The structural assignments of all CHDPs were based on the molecule ion peaks $[M]^+$ in the EI mass spectra and characteristic resonances in the ^{13}C NMR spectra where all fragments could be assigned.

The single crystal X-ray structure of the CHDP **5c** was determined using MoK_α-radiation as X-ray source (see Fig. 1).[19]

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.[20] 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.

The CHDP **4** exhibited a 2:1 mixture of conformers in $CDCl_3$ because of the benzyl side chain. On the other hand, the CHDP **5c** and **6a**–**c**, **7** showed a 3:1 and 2:1 mixture of conformers in $CDCl_3$, the appropriate main

Bn-MeIle[1]-***(R)*-HyCar[2]**-OH (**8**) + H-MeIle[3]-*(R)*-Lac[4]-MeAla[5]-*(R)*-Lac[6]-O-^{t}Bu (**9**)

↓ BOP-Cl, DCM, r. t., [24 h]

Bn-MeIle[1]-***(R)*-HyCar[2]**-MeIle[3]-*(R)*-Lac[4]-MeAla[5]-*(R)*-Lac[6]-O-^{t}Bu (**10**)

↓ gas HCl, 0 °C - r. t., [16 h]

Bn-MeIle[1]-***(R)*-HyCar[2]**-MeIle[3]-*(R)*-Lac[4]-MeAla[5]-*(R)*-Lac[6]-OH (**11**)

↓ H_2, 20% $Pd(OH)_2$-C, EtOH, r. t., [4 h]

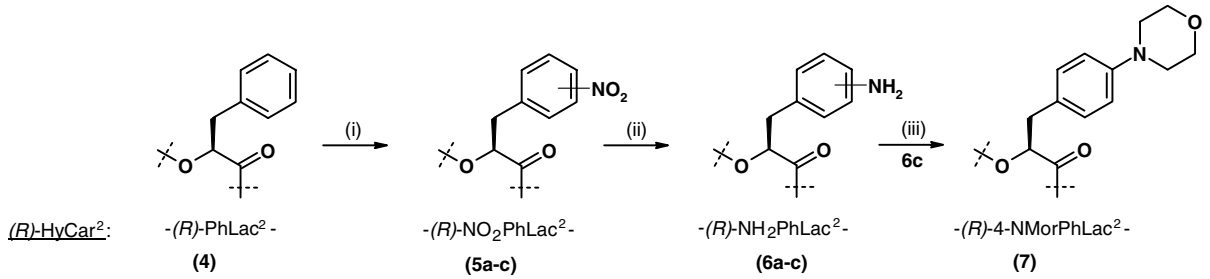
H-MeIle[1]-***(R)*-HyCar[2]**-MeIle[3]-*(R)*-Lac[4]-MeAla[5]-*(R)*-Lac[6]-OH (**12**)

↓ BOP-Cl, DCM, r. t., [24 h]

Cyclo(-MeIle[1]-***(R)*-HyCar[2]**-MeIle[3]-*(R)*-Lac[4]-MeAla[5]-*(R)*-Lac[6]-)

4 *(R)*-HyCar[2] = *(R)*-PhLac[2]

Scheme 1. Synthesis of the CHDP **4** by macrocyclization of deprotected hexadepsipeptide **12**.



Scheme 2. Synthesis of the CHDPs (**5**–**7**) by (*R*)-phenyllactic acid modification in **4**. Reagents and conditions: (i) excess 98% fuming nitric acid, −10 °C, 1 h; (ii) 20% $Pd(OH_2)$/C, H_2, EtOH, 25 °C, 4 h; (iii) 1—2,5-dihydrofuran, ozone gas, MeOH, CH_2Cl_2, −60 °C, 2—$NaBH_3CN$, −50 °C, 10 min.

conformer corresponds to the anthelmintically active enniatin **3** as outlined in Table 1.

Further spectroscopic analysis of the CHDPs **5c**, **6a**–**c** and **7** using a combination of 2D NMR (^{1}H–^{1}H NOESY, ^{1}H–^{1}H-COSY, ^{1}H–^{13}C-HMBC, ^{1}H–^{13}C-HMQC) techniques showed in $CDCl_3$ solution one major conformer with an unsymmetrically folded conformation lacking a *cis*-amide bond like **3**.

CHDP **5c** crystallizes in the chiral space group $P2_1$ together with two molecules of ethyl alcohol from which the crystal was grown. Each solvent molecule was refined into three equal occupied positions. Five intramolecular C–H···O contacts shorter than the sum of the van der Waals radii might be considered to have influence on the conformation of the molecule. About ten intermolecular contacts of the same type and length exist in the crystal lattice which affects the packing of the molecules due to the absence of any strong hydrogen bond donors.

The CHDPs **6a** and **6b** tested in vivo were found to be fully active against the gastrointestinal nematode *H. contortus* in sheep at 0.05 mg kg^{-1} as outlined in Table 1.

Octanol–water partition coefficients (log *P*) were measured by a HPLC method using reverse phase columns, the general principles of which have been described elsewhere.[21] The CHDPs **6a** (log *P* = 3.18) and **6b** (log *P* = 2.35) with (*R*)-2-NH_2PhLac and (*R*)-3-NH_2PhLac in 2-position, containing 2- or 3-amino-benzyl side chains R^2, showed identical activities against *H. contortus* as the parent compound **3** (log *P* = 3.26; in 2-position: (*R*)-Lac) and 2-fold greater activity than **7** (log *P* = 3.58; in 2-position: (*R*)-4-*N*-MorPhLac), respectively. On the other hand, the CHPDs **5c** (in 2-position: (*R*)-4-NO_2PhLac) and **6c** (log *P* = 2.08; in 2-position: (*R*)-4-NH_2PhLac) displayed a 2-fold weaker activity and the CHPD **4** (log *P* = 4.23), with a (*R*)-configurated PhLac in 2-position, displayed up to 5-fold weaker activity against this parasitic nematode compared to the parent compound **3** as well as the CHDPs **6a** and **6b**.

Molecular dynamics simulations of the CHPD **6a**, with simulation times of 100 ps at 300 K each and snapshots taken every 1 ps, were performed in order to evaluate the conformational flexibility of the molecule. As can be seen from representative results in Fig. 2, for CHDP

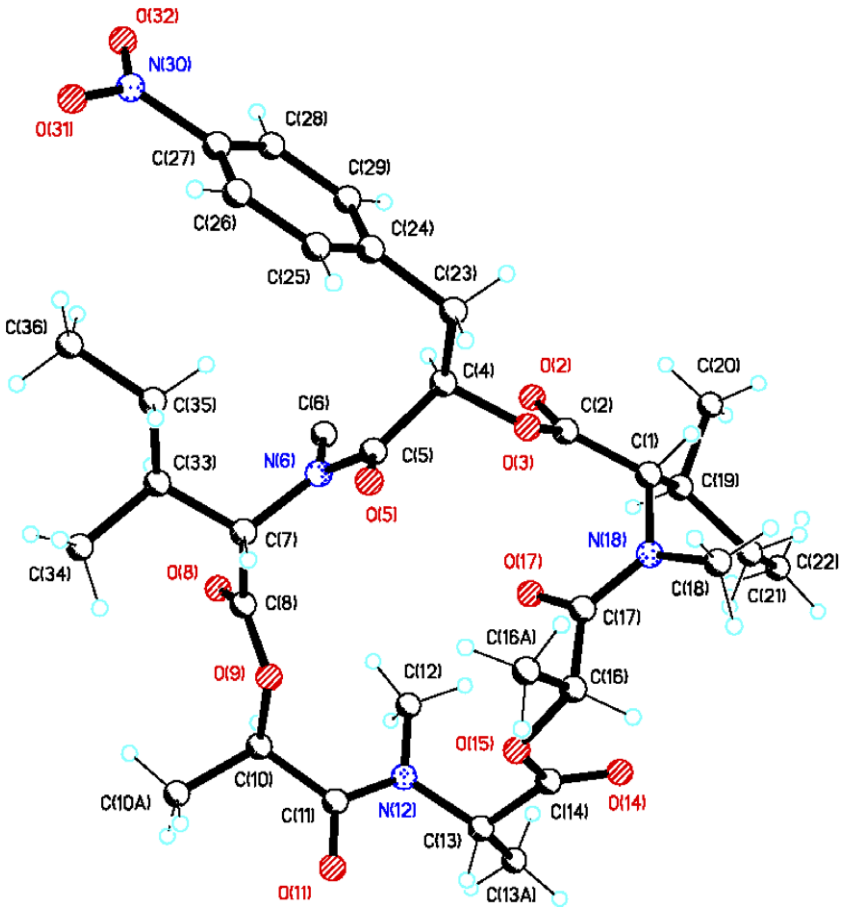


Figure 1. X-ray structure of CHPD **5c** (Ortep Plot 50%).

Table 1. In vivo anthelmintic activities against *Haemonchus contortus* in sheep, ratio of conformers and lipophilicities of the substituted (*R*)-phenyllactic acid containing CHDPs **4**–**7** in comparison with the known CHDP analogue **3**

CHDP	Ratio of conformers[a]	Lipophilicity log *P*[b]	Anthelmintic activity against *H. contortus*
3	3:1	3.26	0.05[c]/3[d]
4	2:1	4.23	0.25/0
5c	3:1	[e]	0.10/1
6a	2:1	3.18	0.05/3
6b	2:1	2.35	0.05/3
6c	2:1	2.08	0.10/1
7	2:1	3.58	0.10/3

[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.

6a the introduction of the 4-amino substituent leads to an internal hydrogen bond to the neighbouring ester-carbonyl oxygen (C═O···H_2N–benzyl) and hence reduces the flexibility of the 4-amino-benzyl group considerably.[11b]

In conclusion, this paper describes the synthesis of novel substituted (*R*)-phenyllactic acid containing CHDPs **6a**, **b** and **7** exhibiting strong in vivo anthelmintic activities against the gastrointestinal nematode *H. contortus* in sheep. The results demonstrate that substituents of the benzyl side chain in 2-position of the CHDP **4** can stabilize better the major conformer with an unsymmetrically folded conformation lacking a *cis*-amide bond. Similar to the CODPs **1** and **2**, incorporation of the 4-*N*-morpholino-benzyl side chain in the 2-position of **3** leads to anthelmintic active CHDPs such as **7**. On the other hand, both the (*R*)-2-NH_2PhLac and (*R*)-3-NH_2PhLac containing CHDPs **6a** and **6b** are as potent as **3**. Therefore, it may be assumed that a similar range of lipophilicity (log *P* = 2.35–3.26; **6a** ≈ **3**) could be more important for the bioavailability of enniatins than their feature 4-*N*-morpholino-benzyl moiety.

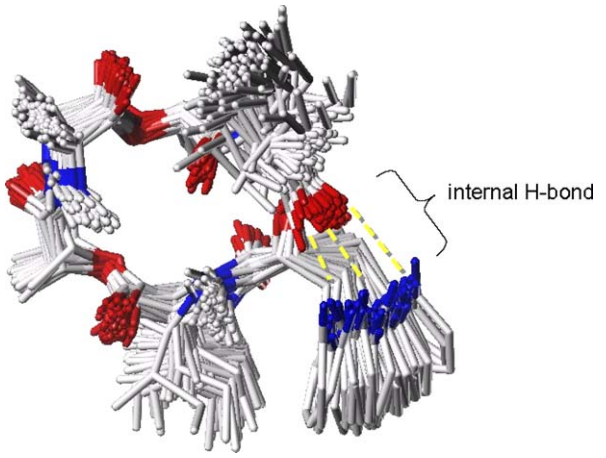


Figure 2. MD-simulation of CHDP **6a**, reduction of flexibility by introduction of an internal H-bond.

Acknowledgments

The authors are very grateful to Rolf Steffes (chemistry), Rolf Suether and Brigitte Stöppler (biology) for skillful technical assistance.

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14. Synthesis of *cyclo*(*N*-methyl-(*S*)-isoleucyl-(*R*)-phenyllactyl-*N*-methyl-(*S*)-isoleucyl-(*R*)-lactyl-*N*-methyl-(*S*)-alanyl-(*R*)-lactyl) **4**. DIEA (0.83 g, 6.43 mmol) and BOP-Cl (0.70 g, 2.78 mmol) are added at 0 °C to a solution of *N*-methyl-(*S*)-isoleucyl-(*R*)-phenyllactyl-*N*-methyl-(*S*)-isoleucyl-(*R*)-lactyl-*N*-methyl-(*S*)-alanyl-(*R*)-lactic acid (1.50 g, 2.31 mmol) in CH_2Cl_2 (DCM) (500 mL) and the mixture is stirred for 24 h at room temperature. Then, a further of DIEA (0.83 g, 6.43 mmol) and BOP-Cl (0.70 g, 2.78 mmol) are added at 0 °C and stirring is continued for 24 h at room temperature. The reaction solution is washed twice with water, and the organic phase is separated off and dried over Na_2SO_4. The filtrate was concentrated in vacuo and the residue was purified by silica gel chromatography (toluene/ethyl acetate, 2:1) to give *cyclo*(*N*-methyl-(*S*)-isoleucyl-(*R*)-phenyllactyl-*N*-methyl-(*S*)-isoleucyl-(*R*)-lactyl-*N*-methyl-(*S*)-alanyl-(*R*)-lactyl) (2.2 g, 65%). ^{13}C NMR (100 MHz, $CDCl_3$) δ 10.3, 10.7, 13.4, 15.5, 15.6, 16.0, 16.9 (CH_3), 24.1, 24.7 (CH_2), 29.9, 30.7 (CH), 32.5, 33.9, 34.2 (NCH_3), 37.3 (CH_2–phenyl), 55.9, 59.5, 61.1 (CH–N), 66.0, 67.5, 70.0 (CH–O), 126.8, 128.4, 129.6, 135.4 (C–phenyl), 168.0, 169.6, 170.3 (CO–O), 168.6, 170.2, 170.5 (CO–N). EI-MS: *m/e* 631 (M^+, 52), 558 (22).
15. *cyclo*(*N*-Methyl-(*S*)-isoleucyl-(*R*)-4-nitro-phenyllactyl-*N*-methyl-(*S*)-isoleucyl-(*R*)-lactyl-*N*-methyl-(*S*)-alanyl-(*R*)-lactyl) **5c**. Mp 122–126 °C. ^{13}C NMR (100 MHz, $CDCl_3$) δ 10.2, 10.5, 15.4, 15.6, 15.6, 15.9, 17.1 (CH_3), 24.2, 24.5 (CH_2), 31.0, 31.5 (CH), 32.2, 34.0, 34.0 (NCH_3), 37.0 (CH_2–phenyl), 56.4, 59.8, 60.3 (CH–N), 65.6, 67.6, 69.4 (CH–O), 123.3, 130.7, 146.9 (C–phenyl), 167.2, 169.8, 170.2 (CO–O), 168.7, 169.8, 170.2 (CO–N). EI-MS: *m/e* 676 (M^+, 28).
16. *cyclo*(*N*-Methyl-(*S*)-isoleucyl-(*R*)-2-amino-phenyllactyl-*N*-methyl-(*S*)-isoleucyl-(*R*)-lactyl-*N*-methyl-(*S*)-alanyl-(*R*)-lactyl) **6a**. ^{13}C NMR (100 MHz, $CDCl_3$) δ 10.2, 10.5, 13.3, 15.5, 15.5, 15.8, 17.1 (CH_3), 23.9, 24.4 (CH_2), 26.8, 30.1

(CH), 30.9, 31.5, 32.0 (NCH_3), 34.0 (CH_2–phenyl), 56.8, 57.9, 60.4 (CH–N), 65.5, 67.5, 68.9 (CH–O), 116.1, 118.5, 119.1, 128.0 131.3, 145.5 (C–phenyl), 168.5, 169.7, 170.3 (CO–O), 168.6, 170.0, 170.8 (CO–N). *cyclo*(*N*-Methyl-(*S*)-isoleucyl-(*R*)-4-amino-phenyllactyl-*N*-methyl-(*S*)-isoleucyl-(*R*)-lactyl-*N*-methyl-(*S*)-alanyl-(*R*)-lactyl) **6b**. ^{13}C NMR (100 MHz, $CDCl_3$) δ 10.3, 10.5, 13.3, 15.3, 15.5, 15.9, 16.7 (CH_3), 24.0, 24.6 (CH_2), 29.8, 30.7 (CH), 32.0, 32.5, 34.0 (NCH_3), 37.3 (CH_2–phenyl), 55.6, 59.5, 61,0 (CH–N), 66.0, 67.3, 69.9 (CH–O), 113.4, 116.1, 119.2, 129.1, 136.1, 146.6 (C–phenyl), 168.2, 169.5, 170.2 (CO–O), 168.6, 170.0, 170.3 (CO–N). *cyclo*(*N*-Methyl-(*S*)-isoleucyl-(*R*)-4-amino-phenyllactyl-*N*-methyl-(*S*)-isoleucyl-(*R*)-lactyl-*N*-methyl-(*S*)-alanyl-(*R*)-lactyl) **6c**. ^{13}C NMR (100 MHz, $CDCl_3$) δ 10.3, 10.7, 15.4, 15.6, 15.6, 16.0, 16.8 (CH_3), 24.2, 24.7 (CH_2), 30.7, 32.2 (CH), 32.6, 33.7, 34.1 (NCH_3), 36.5 (CH_2–phenyl), 55.7, 59.5, 61,2 (CH–N), 66.1, 67.4, 70.1 (CH–O), 115.1, 130.4, 124.9, 145.2 (C–phenyl), 168.4, 169.6, 170.3 (CO–O), 168.6, 170.2, 170.4 (CO–N).

17. *cyclo*(*N*-Methyl-(*S*)-isoleucyl-(*R*)-4-*N*-morpholino-phenyllactyl-*N*-methyl-(*S*)-isoleucyl-(*R*)-lactyl-*N*-methyl-(*S*)-alanyl-(*R*)-lactyl) **7**. ^{13}C NMR (100 MHz, $CDCl_3$) δ 10.5, 10.7, 13.4, 15.5, 15.6, 16.0, 16.9 (CH_3), 29.9, 32.2 (CH_2), 32.6, 34.2 (CH), 30.8, 32.6, 34.2 (NCH_3), 36.4 (CH_2–phenyl), 49.4 (CH_2–N), 55.5, 59.9, 61,1 (CH–N), 66.8 (CH_2–O), 66.0, 67.5, 70.0 (CH–O), 115.7, 130.4, 126.2, 150.2 (C–phenyl), 168.2, 168.6, 169.6 (CO–N), 170.2, 170.3, 170.5 (CO–O). EI-MS: *m/e* 716 (M^+, 100).
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19. Crystal data: $C_{33}H_{48}N_4O_{11}$, $M_r = 768.89$; monoclinic; space group $P2_1$, $a = 9.714(2)$ Å, $b = 15.244(3)$ Å, $c = 14.279(2)$ Å, $\beta = 109.68(2)$, $V = 1990.9(6)$ Å^3, $Z = 2$, $\rho_{calcd} = 1.283$ mg/m^3, $m = 0.10$ mm^{-1}. Data collection: Measurements were performed on a Siemens P4 four circle diffractometer using MoK$^{\alpha}$ radiation at a temperature of −80 °C. Data were collected up to $\Theta = 22.49°$. 6274 data were collected of which 5162 are unique reflections ($R_{int} = 0.0273$). Structure solution and refinement Shelxtl package. 3742 $F_o > 4\sigma(F_o)$, 596 refined parameters, $R_1 = 0.097$, $wR_2 = 0.2655$, goodness of fit on $F^2 = 1.298$, maximum residual electron density 0.39 e Å^3. Crystallographic data (excluding structure factors) for the structure in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication number *CCDC 286511*. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1 EZ, UK (fax: +44 1223 336033 or e-mail: deposit@ccdc.cam.ac.uk).
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