Pergamon

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Bioorganic & Medicinal Chemistry Letters 13 (2003) 3285–3288

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Synthesis and Anthelmintic Activity of Cyclohexadepsipeptides with (*S*,*S*,*S*,*R*,*S*,*R*)-Configuration

Peter Jeschke,[a,*] Jordi Benet-Buchholz,[b] Achim Harder,[c] Winfried Etzel,[a] Michael Schindler[a] and Gerhard Thielking[a]

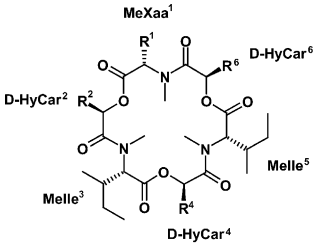
[a]*Bayer CropScience AG, Research Monheim, Global Chemistry Insecticides, Building Q18, D-51368 Leverkusen, Germany*
[b]*Bayer Industry Services, Analytics X-ray Laboratory, Building Q18, D-51368 Leverkusen, Germany*
[c]*Bayer HealthCare, Animal Health Business Group, Research & Development, Parasiticides, Alfred-Nobel-Str 50, D-40789 Monheim, Germany*

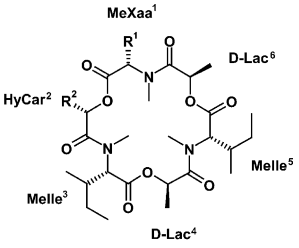
Received 31 March 2003; revised 10 June 2003; accepted 17 June 2003

Abstract—The (*S*,*S*,*S*,*R*,*S*,*R*)-configurated cyclohexadepsipeptides (CHDPs) represent novel enniatin derivatives with strong in vivo activity against the parasitic nematode *Haemonchus contortus* Rudolphi in sheep. 2D NMR spectroscopic analysis revealed for the major conformation the asymmetric conformer, containing a *cis*-amide bond between C_α protons of neighbouring 2-hydroxy-(*S*)-carboxylic acid and *N*-methyl-(*S*)-amino acid. The absolute configuration of the novel CHDPs was determined by X-ray crystallography. A correlation between the major conformer and its anthelmintic activity was found. Here, we report on a simple total synthetic pathway for this particular type of CHDPs.

Helminths, including parasitic nematodes, cause significant health problems in both humans and animals. Gastrointestinal nematodes like *Haemonchus contortus* Rudolphi occur worldwide and parasitize the abomasus of cattle and sheep.[1] Development of novel anthelmintically drugs is an urgent matter because many nematode worms have become resistant towards traditional anthelmintics including benzimidazole derivatives and macrocyclic lactones.[2] The 24-membered cyclodepsipeptides represent the most promising substance class within the small group of anthelmintics newly developed in recent years.[3] 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. To obtain further insights also into the anthelmintic efficacy of the structurally related 18-membered cyclohexadepsipeptides (CHDPs), the so called enniatins, we became interested in the evaluation of the enniatin A **1** structure with regard to its efficacy against the above mentioned nematode *H. contortus* in sheep.

Enniatin A **1** belongs to the class of naturally occurring *N*-methylated CHDPs and consists of three *N*-methylated-(*S*)-isoleucines (MeIle), in positions 1, 3 and 5, and three residues of 2-hydroxy-(*R*)-isovaleric acid (*R*-HyIv), in positions 2, 4 and 6 of the molecule, that is, arranged in an alternating fashion to give an 18-membered cyclic skeleton.[4] Its antiparasitic activity against *Nippostrongylus brasiliensis* and *Trichinella spiralis* at a concentration of 5 μg/mL in vitro was described recently.[5] In order to optimize the anthelmintic activity of **1**, numerous CHDPs containing (*R*)-lactic acid (*R*-Lac) have been synthesized and tested for anthelmintic activity.[6]





1 R^1= *s*Bu; R^2, R^4, R^6 = *i*Pr (Enniatin A)
2 R^1= *s*Bu; R^2, R^4, R^6 = Me
3 R^1 = *s*Bu; R^2 = H; R^4, R^6 = Me
4 R^1, R^2, R^4, R^6 = Me

5 R^1 = *s*Bu; R^2 = *n*Bu
6 R^1, R^2 = Me

*Corresponding author. Tel.: +49-214-30-52155; fax: +49-214-30-46410; e-mail: peter.jeschke@bayercropscience.com

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doi:10.1016/S0960-894X(03)00688-7

Among the different CHDPs with three residues of *(R)*-Lac, in positions 2, 4 and 6 of the molecule, analogue **2** was the most potent. The CHDP **2** showed in vivo biological activity against the gastrointestinal nematode *H. contortus* in sheep at an intravenous dose rate of 0.5 mg kg^{-1}. Furthermore, the exchange of only one (*R*)-Lac for the non chiral 2-hydroxy-acetic acid (HyAc) in the 2-position led to the CHDP **3**, which was found to be twice as potent against *H. contortus* in sheep as the parent enniatin **2**. However, replacement of only one MeIle in the 3-position of **2** by *N*-methyl-(*S*)-alanine (MeAla), as exemplified by **4**, resulted in 10-fold higher activity than **2** against *H. contortus* in sheep.

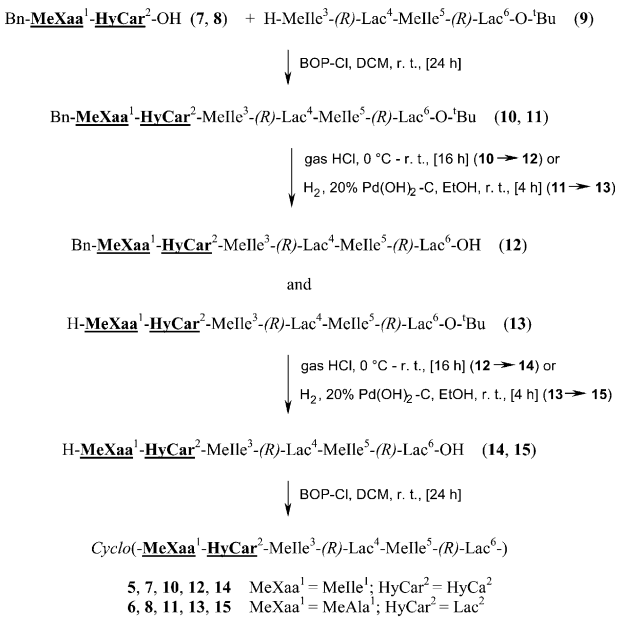
Recently, a correlation between the nature of the CHDP major conformers and their anthelmintic activities was described.[7] It was found that all CHPDs with strong in vivo activity exist in $CDCl_3$ solution as one major conformer either with one *cis*-amide bond or with an unsymmetrically folded conformation and with no *cis*-amide bond like **3** and **4**, whereas CHPDs with C_3-symmetry such as **1** and **2** show only weak activity (oral or intramuscular route) against gastrointestinal nematodes in sheep. On the other hand, the anthelmintic activity of the closely related (*S*,–,*S*,*R*,*S*,*R*)-configurated DCHP **3** suggests that the stereochemistry in the 2-position is not of primary importance for high binding affinity. In order to better understand the effect of stereochemistry, for example (*R*)-configuration, on the form of the macrocycle in connection with its biological activity we have now focussed our attention on the 2-position of the CHDPs **2** and **4**. Such (*S*,*S*,*S*,*R*,*S*,*R*)-configuration may alter the biological profile, as is already described for antimicrobial synthetic enniatin B analogues.[8] Alternation of configuration in individual residues of enniatin B causes loss of activity, showing the importance of steric factors for the activity.[9]

As part of our ongoing efforts to find novel anthelmintic drugs, we started to investigate DCHPs containing in the 2-position (*S*)-HyCar such as 2-hydroxy-(*S*)-capronic acid (HyCa) and (*S*)-lactic acid, respectively.

Chemistry

The method for preparing both CHDPs involved formation of the depsipeptide hexamers (**10**–**15**)[10] from three dimeric fragments by a [2+4]-fragment condensation reaction, for example by using the N-terminal protected didepsipeptides (**7**, **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.[11]

The macrocyclization was accomplished by ring closure of N- and O-terminal deprotected hexadepsipeptides (**14**, **15**) under high dilution conditions using the phosphonium coupling reagent bis(2-oxo-3-oxazolidinyl)-phosphonic chloride (BOP-Cl) and *N*,*N*-diisopropylethyl-amine (DIEA), affording the CHDPs **5** and **6** as shown in Scheme 1.[12] The structural assigments of



Scheme 1. Synthesis of the CHDPs by macrocyclization of deprotected hexadepsipeptides.

both 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 absolute stereochemistry of the stereogenic center in the 2-position of the 2-hydroxy-carboxylic acids was proved directly for the CHDP **5** by single crystal X-ray structure determination using Cu_{K_α}-radiation as X-ray source (see Fig. 1).[13] The absolute configuration for CHDP **5** was confirmed as *S*(C1);*R*(C4);*S*(C7);*S*(C10);*S*(C13);*R*(C16);*S*(C19);*S*(C23);*S*(C27) (based on X-ray structure).

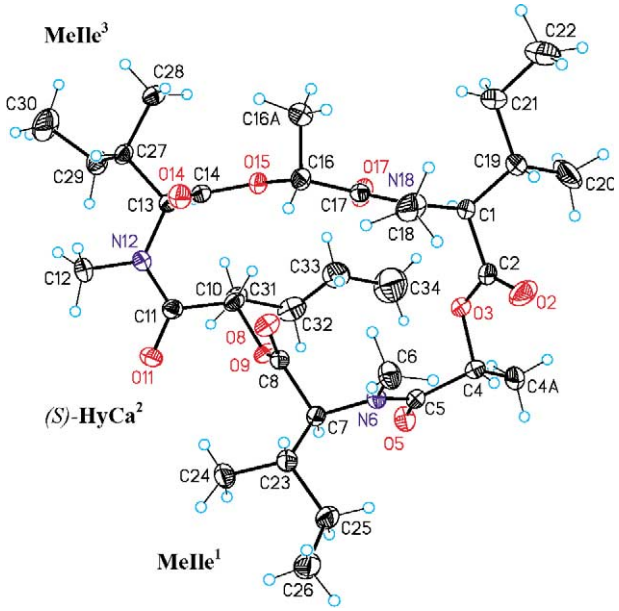


Figure 1. X-ray structure of CHPD **5** (Ortep Plot 50%).

Biological Screening

Sheep (*Ovis aries* L, Merino or Schwarzkopf breed, 25–35 kg body weight) were infected experimentally with 5000 *H. contortus* Rudolphi L_3 and treated with the test substance after the end of the prepotency 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 count. For the purpose of counting eggs, freshly obtained faeces from experimental animals were prepared using the McMaster method as modified by Wetzel.[14] 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: $3 \geq 95\%$, $2 = 75$–95%, $1 = 50$–75% and $0 = \leq 50\%$ egg reduction.

Results and Discussion

In contrast to the more stabilized compound **6**, the CHDP **5** exhibited a 6:3:1 mixture of conformers in $CDCl_3$ because of the *n*-butyl side chain. Further spectroscopic analysis of both CHDPs, 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 a *cis*-amide bond between MeIle^1 and the (*S*)-configurated HyCa^2 and MeIle^3 in 2-position for the main conformer of **5** and between the (*S*)-configurated Lac^2 and MeIle^3 in 2-position for **6**, respectively. The observation of intensive NOE cross peaks between C_α protons of neighbouring MeIle^1/HyCa^2 (δ 4.04/5.33) or MeAla^1/Lac^2 (δ 3.85/5.61) units in the NOESY spectra strongly supports the presence of this *cis*-amide bond geometry in **5** and **6**, respectively.

CHDP **5** crystallizes free of solvents in the chiral space group $P2_12_12_1$ showing a folded packing with C_1-symmetry (see Fig. 1). In the crystal packing there are no relevant intramolecular and intermolecular hydrogen bond. The shortest intermolecular contacts are O11···H4′ (uncorrected 2.472 Å) and O2···H22B′ (uncorrected 2.479 Å). The *n*-butyl residue at the R^2-position is oriented towards the top of the molecule centre, being disordered in two different positions with a ratio of 70:30. The amide fragments O5–C5–N6–C6 and O17–C17–N18–C18 are showing a *trans* bond geometry having weak hydrogen contacts O5···H7–C7 (uncorrected O5···H7 distance: 2.111 Å) and O17···H1–C1 (uncorrected O5···H7 distance: 2.239 Å), respectively. The amide fragment O11–C11–N12–C12, next to the *n*-butyl residue, is in a *cis* conformation in accordance with the NMR results and is oriented to the exterior of the molecule.

The CHDPs **5** and **6** tested in vivo were found to be fully anthelmintically active against the gastrointestinal nematode *H. contortus* in sheep at 0.10 mg kg^{-1} as outlined in Table 1.

Octanol–water partition coefficients (logP) were measured by an HPLC method using reverse phase columns, the general principles of which have been described elsewhere.[15] The CHDP **5** (logP = 5.14) with (*S*)-configuration in the 2-position, containing a *n*-butyl side chain as side chain R^2 showed approximately 5-fold greater activity against *H. contortus* in sheep than the parent compound **2** [logP = 4.20; in the 2-position: (*R*)-configuration] and 2.5-fold greater activity than **3** (logP = 4.00; in the 2-position: achiral), respectively. On the other hand, the CHPD **6** (logP = 3.10) with a (*S*)-configurated lactic acid in the 2-position displayed a 2-fold weaker activity against the parasitic nematode than the parent compound **4** (logP = 3.26).

Table 1. In vivo anthelmintic activities against *H. contortus* in sheep and lipophilicities of the (*S,S,S,R,S,R*)-configurated CHDPs in comparison with known CHDP analogues

CHDP no.	Configuration of HyCar in 2-position	Lipophilicity LogP[a]	Anthelmintic activity in sheep *H. contortus*
2	*R*	4.20	0.50[b]/3[c]
3	—	4.00	0.25/3
4	*R*	3.26	0.05/3
5	*S*	5.14	0.10/3
6	*S*	3.10	0.10/3

[a]LogP value from HPLC (pH 2.3).
[b]Dose in mg test substance kg^{-1} body weight.
[c]0 = ≤50% egg reduction; 1 = 50–75% egg reduction; 2 = 75–95% egg reduction; 3 = ≥95% egg reduction.

Starting with the minimized X-ray structure of **5** (Fig. 1) molecular dynamics simulation (0.5 ns simulation period) was carried out in order to describe its conformational variability. In addition, the obtainable conformation space was scanned by using simulated annealing techniques. In this computational approach, the molecule was artificially heated and then cooled down several times. Thereby, the molecule could easily cross even higher rotational barriers.

Reprensentative results of the MD simulation are shown in Figure 2. A region of increased flexibility is observed in the upper didepsipeptide sequence [-(*R*)-Lac^2-MeIle^1-*O*-] of the molecule, containing mostly nonpolar bulky substituents incapable of forming specific interactions. However, the tetradepsipeptide fragment [-(*S*)-HyCa^2-MeIle^3-(*R*)-Lac^4-MeIle^5-], which contains the *cis*-amide bond is clearly less flexible.

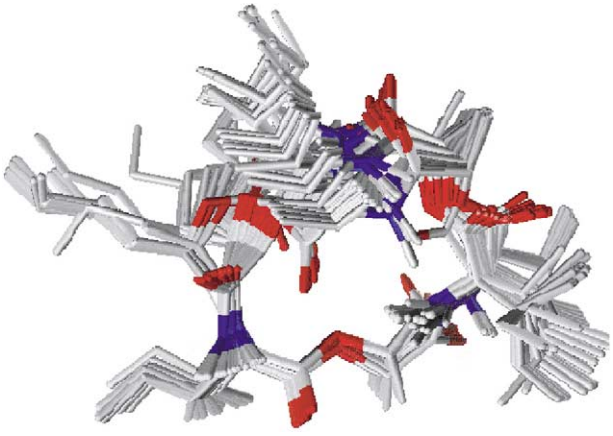


Figure 2. MD simulation of CHDP **5**, rigid and flexible regions.

In conclusion, this paper describes the synthesis of two novel (*S,S,S,R,S,R*)-configurated CHDPs **5** and **6** having strong in vivo anthelmintic activity against the gastrointestinal nematode *H. contortus* in sheep. The results reflect that the stereochemistry in the 2-position of CHDPs is not so important for their high binding affinity. On the other hand, the (*S*)-configurated HyCar[2] can influence the rigidity of the whole 18-membered ring system markedly by forming a *cis*-amide bond. Therefore, it may be assumed that the identified inflexible region of the major conformer might mimic the active conformation of these CHDPs, which could be useful for the rational design of more simplified molecules.

Acknowledgements

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

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10. All compounds gave satisfactory spectral and/or accurate mass data. Characteristical EI-mass data of the synthetic depsipeptide precursors are given below. **7**. *m/e*: 350 (M^+, 3%); **8**. *m/e*: 265 (M^+, 1%); **9**. *m/e*: 472 (M^+, 2%); **10**. *m/e*: 805 (M^+, 2%); **11**. *m/e*: 719 (M^+, 2%); **12**. *m/e*: 747 (M^+, 34%); **13**. *m/e*: 629 (M^+, 3%), 573 ($M^+ - H_2C{=}CMe_2$, 4%); **14**. *m/e*: 658 (M^+, 1%); **15**. *m/e*: 573 (M^+, 1%).
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12. *Cyclo*(*N*-methyl-(*S*)-isoleucyl-(*S*)-capronyl-*N*-methyl-(*S*)-isoleucyl-(*R*)-lactyl-*N*-methyl-(*S*)-isoleucyl-(*R*)-lactyl) **5**. ^{13}C NMR (100 MHz, $CDCl_3$) δ 10.2, 10.3, 11.1, 13.8, 15.0, 15.4, 15.6, 16.4, 17.0 (CH_3), 22.4, 23.9, 25.3, 25.6, 27.0, 31.1 (CH_2), 32.5, 33.4, 34.8 (NCH_3), 28.7, 30.5, 31.0, 31.1 (CH), 59.8, 60.6, 63.6 (CH–N), 66.4, 67.6, 71.4 (CH–O), 170.0, 170.1, 170.2 (CO–O), 169,0, 169.2, 169.2 (CO–N). EI–MS: *m/e (%)* 639 (M^+, 14), 566 (3), 423 (2), 296 (14), 182 (49), 100 (100). Synthesis of *cyclo*(*N*-methyl-(*S*)-alanyl-(*S*)-lactyl-*N*-methyl-(*S*)-isoleucyl-(*R*)-lactyl-*N*-methyl-(*S*)-isoleucyl-(*R*)-lactyl) **6**. DIEA (0.62 g, 4.8 mmol) and BOP-Cl (0.59 g, 2.3 mmol) are added at 0 °C to a solution of *N*-methyl-(*S*)-alanyl-(*S*)-lactyl-*N*-methyl-(*S*)-isoleucyl-(*R*)-lactyl-*N*-methyl-(*S*)-isoleucyl-(*R*)-lactic acid (1.1 g, 1.9 mmol) in CH_2Cl_2 (DCM) (1100 mL) and the mixture is stirred for 24 h at room temperature. The, a further of DIEA (0.62 g, 4.8 mmol) and BOP-Cl (0.59 g, 2.3 mmol) are added at 0 °C and stirring is continued for 24 h at room tempearature. 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 purified by silica gel chromatography (toluene/ethyl acetate, 2:1) to give *cyclo*(*N*-methyl-(*S*)-alanyl-(*R*)-lactyl-*N*-methyl-(*S*)-isoleucyl-(*R*)-lactyl-*N*-methyl-(*S*)-isoleucyl-(*R*)-lactyl) (0.4 g, 38%). ^{13}C NMR (100 MHz, $CDCl_3$) δ 10.0, 10.0, 15.1, 15.3, 15.7, 16.6, 17.6 (CH_3), 24.2, 25.1 (CH_2), 28.1, 30.3, 30.5 (NCH_3), 33.7, 33.7 (CH), 54.3, 58.3, 63.7 (CH–N), 66.4, 68.5, 73.7 (CH–O), 168.7, 169.0, 169.2 (CO–O), 169.0, 170.2, 171.8 (CO–N). EI–MS: *m/e (%)* 555 (M^+, 42), 182 (100).
13. **Crystal data**: $C_{33}H_{57}N_3O_9$, $M_r = 639.82$; orthorhombic; space group $P2_12_12_1$, $a = 10.7479(2)$ Å, $b = 16.8860(3)$ Å, $c = 20.5733(4)$ Å, $V = 3733.83(12)$ Å^3, $Z = 4$, $\rho_{cal} = 1.138$ Mg/m^3, $\mu = 0.671$ mm^{-1}. *Data Collection:* Measurements were made on a Bruker-Nonius diffractometer equipped with a Proteum CCD area detector, a FR591 rotating anode with Cu_{K_α} radiation, Montel mirrors as monochromator and a Kryoflex low temperature device ($T = 90$ K). The measurements were made in the range 4.30 to 70.80°. 35,270 reflections were collected of which 6871 are unique ($R_{int} = 0.0403$). Full-sphere data collection ω and φ scans. Programs used: Data collection Proteum V. 1.37 (Bruker-Nonius 2002), data reduction Saint Plus Version 1.6 (Bruker-Nonius 2002) and absorption correction SADABS V. 2.03 (2002). **Structure solution and refinement:** SHELXTL Version 6.10 (Sheldrick, 2000); 6745 Fo >4sig(Fo), 512 refined parameters, $R_1 = 0.0433$, $wR2 = 0.1219$, Goodness of fit on $F^2 = 1.041$, Flack parameter −0.05(0.14), maximum residual electron density 0.933 (−0.332) 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 206966. 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; e-mail: deposit@ccdc.cam.ac.uk).
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