152. β-Loop, γ-Loop, and Helical Peptide Conformations in Cyclopeptides Containing a Steroidal Pseudo-Amino Acid

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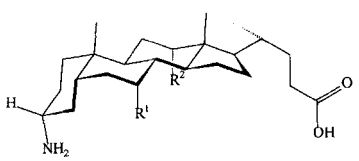
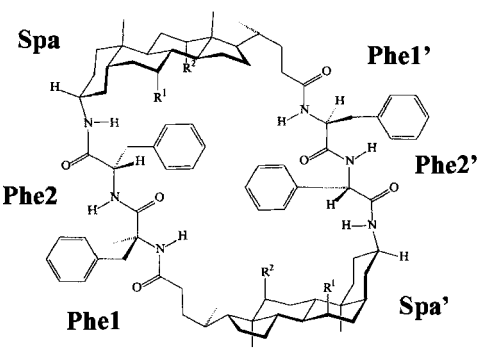
The steroidal pseudo-amino acids 3α-amino-5β-cholan-24-oic acid (**2a**), 12α-acetoxy-3α-amino-5β-cholan-24-oic acid (**2b**), and 7α,12α-diacetoxy-3α-amino-5β-cholan-24-oic acid (**2c**) are used as rigid spacers in the backbone of the cyclic peptides cyclo(–**2a**–Phe–Phe–)$_2$ (**1a**), cyclo(–**2b**–Phe–Phe–)$_2$ (**1b**), and cyclo(–**2c**–Phe–Phe–)$_2$ (**1c**). A homogeneous βI-loop conformation is found in the peptide chains of **1a** and **1b**, while **1c** exists as a mixture of α-helical and γ-loop conformations. The structure and homogeneity of the conformations are established by several NMR techniques and are supported by molecular-dynamics calculations. The peptide conformations depend on the distance and attraction of the two large and lipophilic steroidal parts of the cyclic molecules.

Introduction. – Many processes in living structures, as the immunological response or the signal functions of hormones, call for an understanding of the relationship 'peptide conformation – biological action'. Hereby, the chemist's aim is to produce conformational homogeneous molecules. Specific peptide conformations may be obtained by several techniques; *e.g.*, unnatural peptide isosters are used [1], the peptide backbone is rigidified by cyclization [2], or rigid spacer molecules are introduced into the chain [3]. The most important conformational motifs which appear in small cyclopeptides are β- and γ-loops. This paper reports the peptide conformations in the macrocycles **1a**–**1c** where the occurrence of β- and γ-turn structures is influenced by a subtle change in the molecular structure of the steroid.

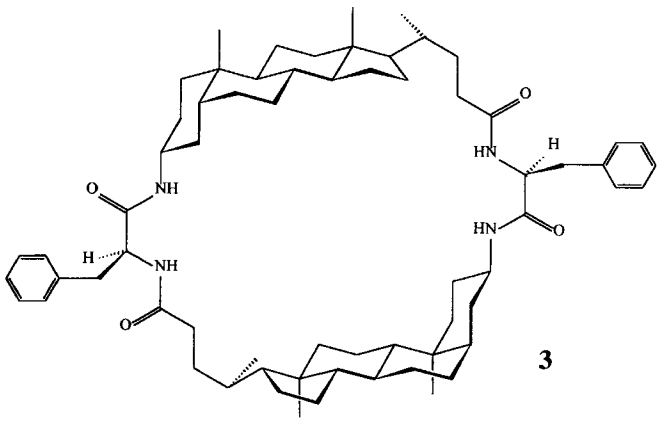


2a R^1=R^2=H
b R^1=H, R^2=MeCOO
c R^1=R^2=MeCOO



1a R^1=R^2=H
b R^1=H, R^2=MeCOO
c R^1=R^2=MeCOO

We have already reported the synthesis of the macrocycle **3** where the steroidal pseudo-amino acid (Spa) **2a** and L-phenylalanine formed the cyclo-tetrapeptide **3** [4]. The NMR-spectroscopic analysis revealed a structure with a fixed conformation of the peptide chain. The next step is to find out whether the conformational homogeneity is maintained in macrocycles with longer peptide chains and to analyze the influence of the steroidal part on the peptide conformation.



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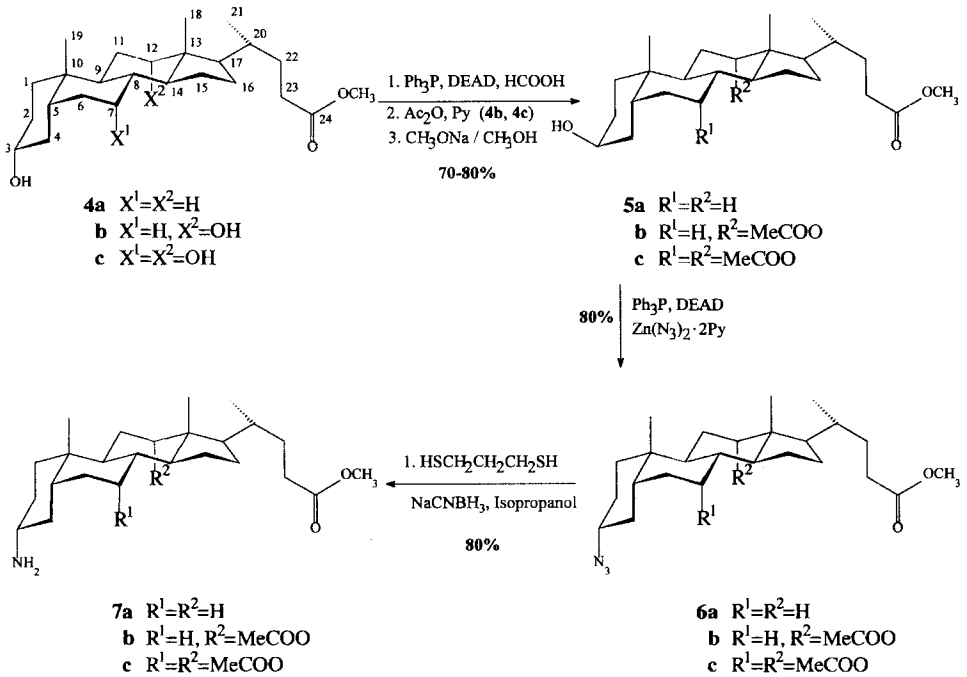
The synthesis and conformational study of three such extended cycles, *i.e.*, of compounds cyclo(–**2a**–Phe–Phe–)$_2$ (**1a**), cyclo(–**2b**–Phe–Phe–)$_2$ (**1b**), and cyclo(–**2c**–Phe–Phe–)$_2$ (**1c**), are reported below.

Synthesis. – The preparation of the steroidal spacer 3α-amino-5β-cholan-24-oic acid methyl ester (**7a**) from **4a** *via* **5a** and **6a** was already described [4]. The 12α-acetoxy-3α-amino-5β-cholan-24-oic acid methyl ester (**7b**) and 7α,12α-diacetoxy-3α-amino-5β-cholan-24-oic acid methyl ester (**7c**) were obtained from 7-deoxycholic (**4b**) and cholic acid methyl ester (**4c**), respectively, by two *Mitsunobu* [5] reactions *via* **5b,c** and **6b,c** (*Scheme 1*) conserving the configuration at the C(3)-atom (see also the solid-state structure of **6c** in the *Exper. Part* (*Fig. 5*)). The azides **6a–c** were reduced with Na[B(CN)H$_3$] and propane-1,3-dithiol in propan-2-ol to give the corresponding amines **7a–c**.

The cyclic peptides **1a–c** were then obtained by classical liquid peptide synthesis (*Scheme 2*). The linear *N*-[(*tert*-butoxy)carbonyl]-protected dipeptide (Boc-Phe-Phe) was condensed with the steroidal pseudo-amino acid methyl esters (Spa-OMe) **7a–c** using diethyl phosphorocyanidate [6] to yield the Boc-protected tripeptide methyl esters **8a–c**, respectively. The methyl esters **8a–c** were hydrolyzed and converted to the pentafluorophenol esters **9a–c**. After removal of the Boc group, the activated esters reacted in a dimerization process to the cyclo-hexapeptides **1a–c** [7].

^{1}H-NMR Studies of the Conformations of Peptides 1a–c. – *General Remarks.* The macrocycle **3** (= cyclo(–**2a**–Phe–)$_2$) has been found to be fairly rigid [4]. Extending the peptide chains, as in **1a–c** (= cyclo(–2–Phe–Phe–)$_2$), gives access to various conformations typical for small cyclopeptides, *e.g.*, β- or γ-loops. A careful molecular-dynamics (MD) search of the accessible conformations of **1a** and **1c** (see below) reveals

Scheme 1. *Synthesis of the Steroidal Pseudo-amino Acid Methyl Esters* (SpaOMe) **7a–c**



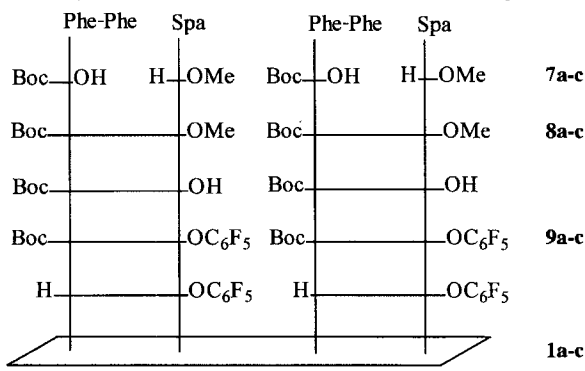
4a $X^1=X^2=H$
b $X^1=H, X^2=OH$
c $X^1=X^2=OH$

5a $R^1=R^2=H$
b $R^1=H, R^2=MeCOO$
c $R^1=R^2=MeCOO$

7a $R^1=R^2=H$
b $R^1=H, R^2=MeCOO$
c $R^1=R^2=MeCOO$

6a $R^1=R^2=H$
b $R^1=H, R^2=MeCOO$
c $R^1=R^2=MeCOO$

Scheme 2. *Scheme of the Synthesis of the Cyclopeptides* **1a** (Spa = **2a**), **1b** (Spa = **2b**), *and* **1c** (Spa = **2c**)



four basic conformations for the peptide part of the molecules (*Fig. 1*): two γ-loop structures **A** and **B**, a β-loop geometry **C**, and a twisted conformation **D** with the dihedral angles of an α_R-helix. The following discussion of the experimental results is based on these four local structures. It is expected that the successive introduction of acetoxy groups in the spacer influences the adopted conformation.

The study of the conformation and its homogeneity in solution is best made by NMR. As pointed out by *Kessler* [8], the combination of several NMR techniques provides a reliable picture of the presence and location of secondary-structure elements.

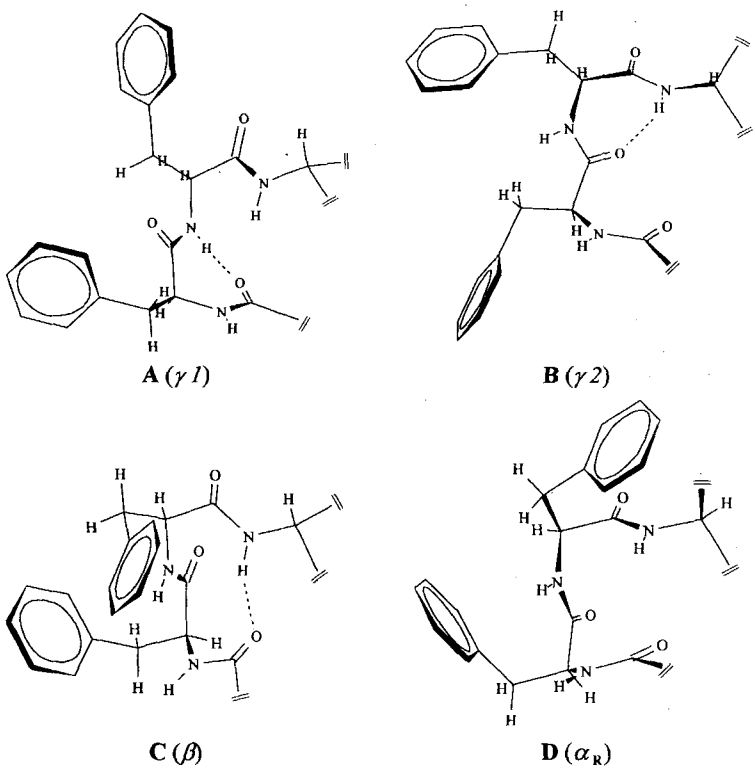


Fig. 1. *Possible conformations of the peptide chains: γ^i-loop* **A** *between NH (Phe2) and CO(Spa'), γ^i-loop* **B** *between NH(Spa) and CO(Phe1), β-loop* **C** *between NH(Spa) and CO(Spa'), and α-helical conformation* **D**

Data Extracted from the 1D-NMR Spectra **1a–c**. The assignments in the ^{1}H-NMR spectra of the cycles **1a–c** (*Fig. 2*) were made using 2D-TOCSY and ROESY spectra. A ROESY spectrum of **1a** is reproduced in *Fig. 3*. The ^{1}H-NMR spectra reflect the C_2 symmetry of **1a–c**. There is a high similarity between the spectra of **1a** and **1b**, whereas the spectrum of **1c** differs.

Table 1 contains the vicinal coupling constants that define the dihedral angles (ϕ) together with the temperature dependence of the chemical shift of the NH groups that indicate conformational homogeneity. The coupling constants ^3J(NH,H–C(α)) of Phe provide the following information: **1a** and **1b** may exist as β-turn structures, whereas **1c** cannot be addressed to a definite structure. As expected, the coupling information is not sufficient to differentiate between several conformational possibilities.

Additional information is contained in the temperature dependence of the chemical shift of the NH protons (*Table 1*). A large temperature gradient $\Delta\delta$(NH)/ΔT is observed for NH(Phe2) of **1c** in $CDCl_3$. This indicates that at least two different conformations are populated in the weakly coordinating solvent. One conformation has to be favored by enthalpy, the other conformation by entropy. The pattern of intramolecular H-bonding should be different in both structures to explain the enhanced gradient of the chemical shift (see *Table 1*). In contrast, the small temperature gradients observed in **1a**

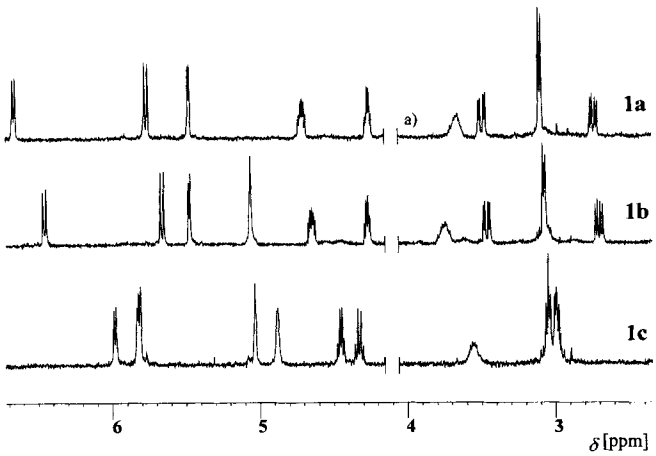


Fig. 2. *^{1}H-NMR Spectrum of the steroidal cyclopeptides* **1a**–**1c** *at 30°*. a) For clarity, the signals of the solvent (AcOEt) are not represented.

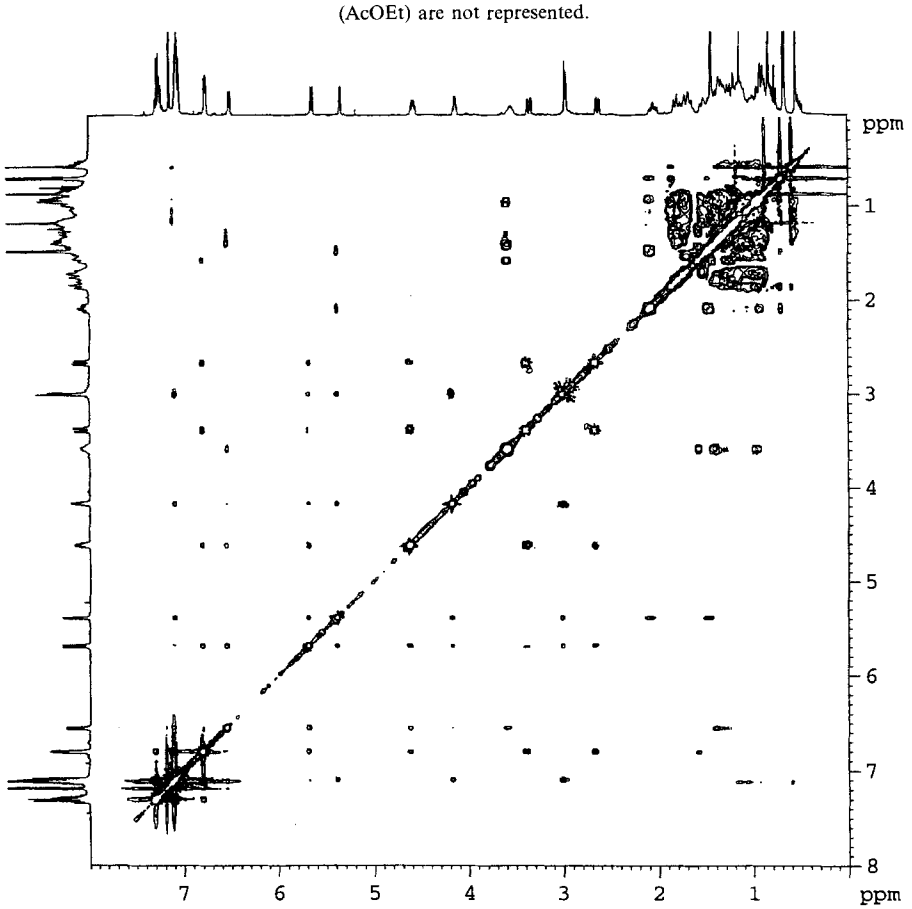


Fig. 3. *2D-ROESY Spectrum of* **1a** *in $CDCl_3$ at 30°*. Mixing time 250 ms.

Table 1. *Vicinal Coupling Constants* $^3J(NH,H-C(\alpha))$ [Hz], *Derived Dihedral Angles* ϕ, *Chemical Shifts* $\delta(NH)$ *and Temperature Dependence of the Chemical Shift of the Amide Protons* $\Delta\delta(NH)/\Delta T$ [ppm/K · 10^3] *in* $CDCl_3$

		Spa	Phe2	Phe1
1a	^3J(NH,H–C(α))	7.03	9.04	4.01
	ϕ [a])	28–42, 78–92	48–72, –142 to –152	11–18, 91–98
		–78 to –82, –158 to –164	–88 to –98	–60 to –64, –176 to –180
	δ(NH) [b])	6.61	5.75	5.45
	$\Delta\delta$(NH)/ΔT	–1.75	+0.63	–1.09
1b	^3J(NH,H–C(α))	8.52	9.04	4.02
	ϕ [a])	40–80, –84 to –92	48–72, –142 to –152	11–18, 91–98
		–148 to –156	–88 to –98	–60 to –64, –176 to –180
	δ(NH) [b])	6.42	5.64	5.44
	$\Delta\delta$(NH)/ΔT	–1.03	–1.10	–1.66
1c	^3J(NH,H–C(α))	8.04	7.02	5.52
	ϕ [a])	36–84, –81 to –89	28–42, 78–92	20–29, 91–100
		–151 to –159,	–76 to –82, –158 to –164	–58 to –62, –168 to –172
	δ(NH) [b])	5.81	5.75	5.93
	$\Delta\delta$(NH)/ΔT	+1.03	–5.55	–2.52
Expected in a γ^i-loop 1 [c]) (see **A**)	ϕ [a])			–85
	NH in H-bond	no	no	yes
Expected in a γ^i-loop 2 [d]) (see **B**)	ϕ [a])			–85
	NH in H-bond	yes	no	no
Expected in a β-loop [e]) (see **C**)	ϕ [a])		–90	–60
	NH in H-bond	yes	no	no
Expected in a α-helix (see **D**)	ϕ [a])		–60	–60
	NH in H-bond	no	no	no

[a]) The *Bystrow-Karplus* equation [9] is used for the estimation of the torsion angles. Two values are noted for ϕ due to the form of the *Karplus* curve. [b]) At 30°. [c]) γ-Loop between NH (Phe2) and CO (Spa'). [d]) γ-Loop between NH (Spa) and CO (Phe1). [e]) β-Loop between NH (Spa) and CO (Spa').

and **1b** are more difficult to explain. NH Protons which are involved in intramolecular H-bonding or are not H-bonded but fixed in a well-defined conformational position should have small temperature gradients in $CDCl_3$. The chemical shift of the NH protons may be an additional indicator for H-bonding. The NH signals of the steroidal pseudo-amino acids (Spa) in **1a** and **1b** are located at 6.6 and 6.4 ppm, respectively, well separated from the NH signals of the Phe residues. This indicates H-bonding of the NH(Spa) protons (see *Table 1*). The NH(Spa) proton can build a H-bond to the CO of Phe2, forming a γ-loop, or can contact the carbonyl O-atom of the Spa′ unit forming a β-turn (see the structures **B** and **C** in *Fig. 1*). The β-turn conformation **C** has been already postulated from the coupling constants (see above). In summary, **1a** and **1b** posses a conformationally rigid β-turn structure while **1c** is more flexible. Additional data from NOE measurements are necessary to define the structures more precisely (see below).

Information from ROE Spectra. As emphasized by *Landis et al.* [10], for relatively rigid, compact molecules, the weight of NOE spectra in the conformational evaluation is strongly increased by the occurrence of many topologically nonlocal NOE cross-peaks which severely restrict the conformational possibilities. A brief inspection of the NOE-derived internuclear distances for **1a** and **1b** with distances in the four possible geometries **A**–**D** (*Table 2*) shows that both compounds should either exist as the helical structure **D** or the β-loop geometry **C**.

Table 2. *Comparison of Nuclear Distances* [Å] *between Protons of Amino Acids Involved in an* α*-Helical Conformation* (α)[a]) *in* γ^i*-Turn Conformations* ($\gamma 1$, $\gamma 2$)[b]) *and in a* βI*-Turn*[a]) *with Distances in* **1a**, **1b**, *and* **1c**, *Estimated from ROESY Experiments*

α[a]) (see **D**)	$\gamma 1$[b]) (see **A**)	$\gamma 2$[b]) (see **B**)	βI[a]) (see **C**)	**1a**	**1b**	**1c**	Proton pair[c])
2.7	2.89	2.88	2.7	2.75	2.81	2.84	α(Phe1)-NH(Phe1)
2.7	2.95	2.88	2.8	2.97	2.98	2.77	α(Phe2)-NH(Phe2)
2.2–2.9	3.03, 2.47	2.4, 2.5	2.2–2.9	[d])	[d])	[d])	α(Phe1)-β(Phe1)
2.2–2.9	3.05, 2.45	2.48, 3.03	2.2–2.9	2.38,[d])	2.4,[d])	[d])	α(Ph2)-β(Ph2)
2.0–3.4	3.56, 2.39	4.01, 3.44	2.0–3.5	2.6	2.67	2.75	β(Phe1)-NH(Phe1)
2.0–3.4	3.89, 2.84	3.57, 2.41	2.0–3.5	2.86, 3.0	2.85, 3.09	2.39[e])	β(Ph2)-NH(Phe2)
3.5	2.37	2.46	3.4	3.18	2.96	2.37	α(Phe1)-NH(Ph2)
3.5	3.57	2.13	3.2	3.07	2.81	2.3	α(Phe2)-NH(Spa)
2.8	3.77	4.35	2.6	2.66	2.72	3.46	NH(Phe1)-NH(Ph2)
2.8	2.01	4.25	2.4	2.32	2.47	> 3.5	NH(Phe2)-NH(Spa)
2.5–3.8	4.27, 4.49	3.79, 2.36	2.9–4.1	2.97	3.27	[e])	β(Phe1)-NH(Ph2)
2.5–3.8	2.25–3.43	4.47, 3.85	3.6–4.4	> 3.5	> 3.5	2.88	β(Ph2)-NH(Spa)
4.2	4.26	5.4	3.6	3.41	> 3.5	> 3.5	α(Phe1)-NH(Spa)

[a]) α-Helical and βI distances for [11]. [b]) Distances calculated for two different γ-loop conformations in **1a** as a model (see the legend to *Table 1* and *Fig. 1* for the definition of $\gamma 1$ and $\gamma 2$). [c]) The short form α means the proton attached to C(α), and β means the protons attached to C(β) of the amino acid. [d]) Overlap with coupling cross-peaks. [e]) Possible overlap between NOE peaks.

It is difficult to distinguish between the conformations **D** and **C** based on the crude estimation of distances by the NOE data. Nearly all distances proposed for **D** and **C** (entry α and βI in *Table 2*) are similar and differ by less than 0.5 Å. Such differences are comparable to the error limits of the NOE measurement. However, the weak NOE

between H–C(α)(Phe1) and NH(Spa) (last entry in *Table 2*) can only be explained by the βI-loop structure **C** and not by the α-helical conformation **D**. These arguments, together with the location of the chemical shift of the NH(Spa) proton at low field (see above), underline that **1a** and **1b** exist most likely as the β-loop structure **C**.

The situation is more complicated for **1c** the list of NOE-derived distances for **1c** (*Table 2*) cannot be explained by the existence of only one structure. The best agreement would be achieved if the γ-loop geometry **B** and the helical structure **D** are coexistent. The temperature-dependent chemical shifts and the coupling constants of **1c** were also indicators for an equilibrium of conformations (see above). Free-energy calculations of such an equilibrium may give more details about the coexisting species.

Theoretical Results. – *A Molecular-Dynamics Study.* Convincing experimental conclusions were derived so far for compounds **1a** and **1b**: all NMR data support a relatively rigid conformation in the peptide backbone, presumably a β-loop conformation (see *Fig. 1*, **C**). A molecular-dynamics study was performed to provide answers to the following questions: *1*) What are the conformations of the minima of low energy? *2*) Does the conformational entropy of the minima differ due to different flexibility of the structures? *3*) Do the calculations support the experimentally derived homogeneous β-loop structure of **1a** and the heterogeneous equilibrium of conformations of **1c**? We used the Amber force field within the Hyper-Chem program package for the MD calculations.

The following procedure was applied to **1a** and **1c**. Two starting geometries with differently twisted steroid arrangements were used for 20-ps MD simulations at 1000 K. Coordinates were collected at intervals of 0.2 ps giving a total of 2 × 100 new geometries. After energy minimization of each structure to a gradient of 1 kcal/Å mol, the structures were relaxed for 1 ps at 300 K. The final conformations were minimized again to a gradient of 0.01 kcal/Å mol. Eight of the obtained local minima with completely different conformations were used as starting geometries for a similar MD procedure[1]). The 8 × 100 obtained minimized conformations showed three different conformations within 5 kcal/mol of the global minimum for **1c** and three conformations within 10 kcal/mol for **1a**. The peptide chains of the three minima of **1a** resemble the structures **A**, **B**, and **C** already shown in *Fig. 1*, whereas the peptide chains of **1c** adopt the conformations **A**, **B**, and **D**. (It should be noted that the geometric difference between 'helical' structure **D** and the β-loop structure **C** is small; the structures differ by *ca.* 30 deg in ϕ and ψ at Phe2 (see *Table 1*). Hence, the differentiation between **D** and **C** may be artificial. For example, MM + as force field converts **1c** from **D** to **C** upon energy minimization.)

The bond angles, torsional angles, and improper dihedrals were collected during a 100 ps MD simulation at 50 K for each of the located minima (**A**–**D**) to evaluate the differences in their conformational entropy [12]. The results of this thermodynamic calculation are summarized in *Table 3*. Enthalpy and entropy values obtained by the quasi-harmonic calculation (according to *Karplus* [12]) are a crude approximation of the real factors influencing the thermodynamic quantities. Only the conformational contributions to the entropy are considered, and even these values are based on a force-field

[1]) The sterane backbone was fixed in space to allow a more serious screening of the conformational space of the mobile peptide chains.

Table 3. *Conformational Entropy Differences* (S_i) [12] *Relative to* **C**, *Enthalpies* (H_i) *at 50 K, and Calculated Occupancies* (molar ratios, x_i) *for the Conformations of Low Energy of* **1a** *and* **1c**

	i	S_i [cal/molK]	H_i [kcal/mol]	x_i (*T* 233 K)	x_i (*T* 313 K)
1a	γ1 (see **A**)	11.7	64.6	0	0
	γ2 (see **B**)	2.1	61.8	0	0
	β (see **C**)	0	56.5	1	1
	α (see **D**	–	–	–	–
1c	γ1 (see **A**)	−8.8	63.0	0	0
	γ2 (see **B**)	5.0	57.8	0.38	0.57
	β (see **C**)	–	–	–	–
	α (see **D**)	0	56.4	0.62	0.43

approximation without a solvent. Nevertheless, it is interesting to calculate the relative populations of the conformers and to check the agreement with the experimental data.

A brief inspection of *Table 3* shows that compound **1a** should exist exclusively as the β-loop structure **C** for enthalpic reasons. The structure is also favored strongly by the NOE measurements (see above). The proposed conformational homogeneity of **1a** results obviously in small temperature gradients of the NH chemical shifts of **1a** (see *Table 1*). However, the situation is more complicated for **1c**. The calculated enthalpic differences between the conformers are small and entropic compensation leads to populations of the conformations γ2 (see **B**) and α (see **D**) which strongly depend on temperature (see *Table 3*). This is consistent with the temperature dependence of the NMR spectrum of **1c**. The larger NH gradients of **1c** (*Table 1*) indicate the presence of at least two interconverting conformers, and the NOE data can be explained with an equilibrium between the conformers **B** and **D**.

The resulting final structures **1a(C)**, **1c(B)**, and **1c(D)** are grouped together in *Fig. 4*. The acetoxy groups in **1c(B)** and **1c(C)** expand the distance between the cholanic spacers. This is mainly due to the local conformation of the acetoxy groups where the Me groups are located perpendicular to the surface of the steroid. The solid-state structure of a corresponding open-chain compound **6c** shows exactly the same conformation (see *Fig. 5* in the *Exper. Part*).

In summary, the experimentally determined structures of **1a** and **1c** in solution are well supported by the theoretical MD search. This congruence is even more delightful as experimentally derived constraints are not used at any step of the calculation [13].

Conclusion. – The cholanic amino acids Spa are rigid spacers which keep the N- and C-terminal residues of attached peptides at distance. It is, therefore, possible that cyclopeptides like **1a**–**1c** develop a cavity suitable for the encapsulation of organic guests. The present study establishes by a combination of NMR techniques and MD calculations that a homogeneous β-loop conformation is found in the peptide parts of **1a** and **1b**. The structural information for **1c** is less convincing. A reasonable agreement between the experimental data and the theoretical predictions is found when an equilibrium between two different conformations is assumed for **1c** (see *Fig. 4*).

All structural data, chemical shifts of the NH protons, coupling constants for the moieties of dihedral angle ϕ, NOE-derived distances, and the structure of the global

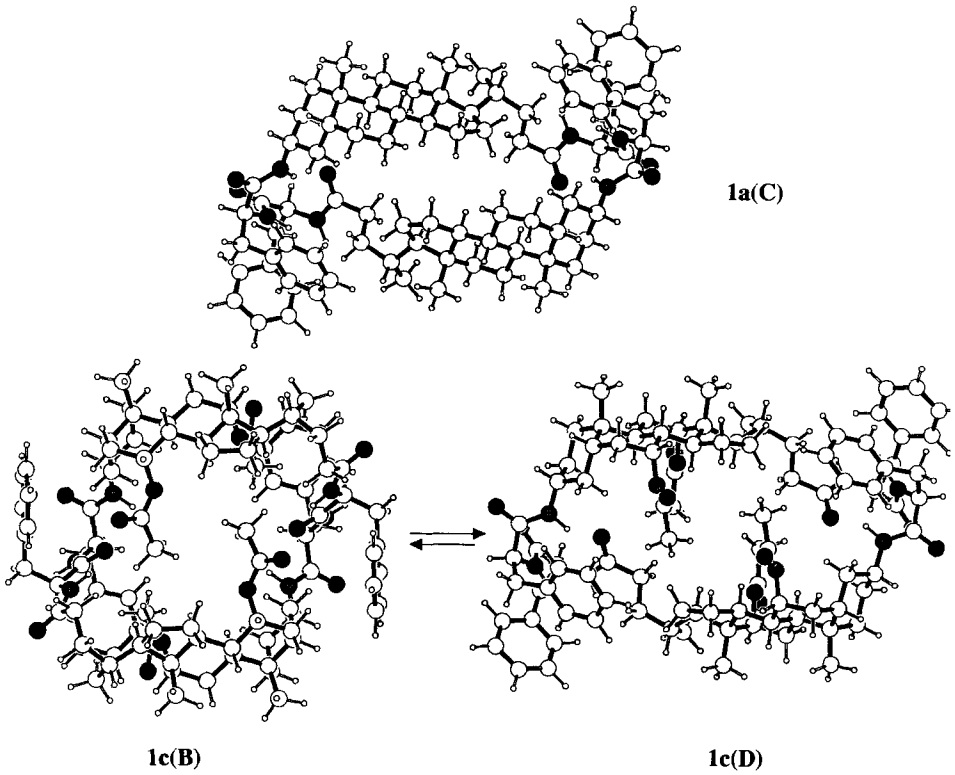


Fig. 4. *Structure of* **1a** (top) *containing two β-turns* (conformation **C**) *and proposed equilibrium of structures of* **1c** (conformations **B** and **D**). Atom code: white, C and H; black, O; net, N. The conformation **B** contains two γ-turns, the peptide parts in **D** have helical conformations. The structures were obtained from an exhaustive MD procedure using the Amber force field (see text and *Table 3*).

minimum found in the conformational search using a MD protocol point clearly to **C** as the only populated conformation of **1a**. The two steroid parts of **1a** are here in *van der Waals* contact. The approach of the steroids is only possible by the rigid turn geometry in the peptide part of the molecule; so it may be concluded that the geometry of the peptide chain is defined by the attraction of the steroids.

In contrast, compound **1c** contains four additional acetoxy groups at the contact surface of the steroids. Consequently, the sterane groups move a part and the peptide chains develop two different conformations **B** and **D** to bridge the larger distance.

In the past, definitive conformations have been induced in peptides by cyclization, or by the introduction of rigidifying units as unnatural peptide isosters [14]. The present cyclic structure **1** has the additional feature, that the peptide conformation depends on the distance or attraction of two large lipophilic molecular parts in the cycle. Thus, the natural way, influencing peptide conformations indirectly by long-range attractions, is modelled.

It may be interesting to regulate the still unknown functions of steroidal peptides by this mechanism.

Experimental Part

General. The org. solvents were dried before use, and the reactions were carried out under Ar. The dipeptide Boc-Phe-Phe-OMe [15] was prepared from L-phenylalanine using propyl-phosphonic acid cyclic anhydride as a coupling reagent [16]. Melting points: not corrected. ^{1}H-NMR Spectra: *Bruker-DRX* (400 MHz) instrument; chemical shifts in δ (ppm) rel. to Me_3Si. FAB Mass spectra: *Fison-Instrument-AutoSpec* and *MAT-8200* spectrometer, NBA = 3-nitrobenzyl alcohol; in *m/z* (rel. %).

2D-NMR Studies and Distance Calculations. COSY, TOCSY, and ROESY ^{1}H-NMR spectra were obtained at 400 MHz on degassed samples of **1a–c**. The ratios s_{ij} (= I_{ij}/I_{ii}) of cross- to diagonal-peak volume integrals were divided by $\sin^2\alpha_j$ to correct for off-resonance effects according to [17] (α_j is the off-resonance angle of spin j to the lock field). The corrected ratios were finally used to obtain distances r_{ij} by calibration with a reference according to $r_{ij}/r_{ref} = (s_{ref}/s_{ij})^{1/6}$. For **1a** and **1b**, we used the distance between geminal diastereotopic protons $r(H_a\text{–C}(\beta\text{(Phe)})/H_b\text{–C}(\beta\text{)(Phe)}) = 1.79$ Å and in **1c** the distances between the conformationally fixed protons $r(H_{ax}\text{–C(3)(Spa)}/H_{eq}\text{–C(4)(Spa)}) = 2.48$ Å and $r(H_{ax}\text{–C(1)(Spa)}/H_{ax}\text{–C(3)(Spa)}) = 2.53$ Å as r_{ref}. The distances r_{ij} (NOE) in *Table 2* are averages over r_{ij} and r_{ji} if the volume integrals of both cross-peaks are accessible. Being aware of the methods severe limitations (isolated spin pair approximation, neglect of scalar coupling, referencing to one 'virtual' distance), one dare say that the correlation of the derived distances r_{ij} (NOE) with X-ray and theoretically calculated distances r_{ij} are basically very good (see *Table 2*).

General Procedure I (GPI): Mitsunobu *Reaction with Formic Acid as a Nucleophilic Agent* [5]. To a soln. of methyl cholanoate **4b**, or **4c** (5 mmol) and triphenylphosphine (10 mmol) in THF (60 ml), HCOOH (10 mmol) was slowly added. After dropwise addition of a soln. of diethyl azodicarboxylate (= diethyl diazenedicarboxylate, DEAD; 10 mmol) in THF (10 ml), stirring was continued for 1 day at r.t. Then the solvent was evaporated and the residue treated with pyridine (55 mmol) and Ac_2O (340 mmol). After refluxing for 2 h, the mixture was evaporated and the residue purified by chromatography (silica gel, hexane/AcOEt 9:1). The acetylation product was dissolved in MeOH (120 ml) and treated for 2 h with 10% NaOMe/MeOH (80 ml) at r.t. After neutralization of the chilled mixture with conc. HCl soln. followed by dilution with H_2O and partial evaporation of MeOH the 3β-hydroxy methyl ester crystallized.

Methyl 12α-Acetoxy-3β-hydroxy-5β-cholan-24-oate (**5b**). According to *GP I* from methyl 3α,12α-dihydroxy-5β-cholan-24-oate (12.3 mmol): 3.4 g (70%) of **5b** (94% pure). ^{1}H-NMR ($CDCl_3$): 7.04 (br., OH–C(3)); 5.05 (*s*, H_β–C(12)); 4.08 (*s*, H_α–C(3)); 3.61 (*s*, Me); 2.36–2.27 (*m*, 1 H, CH_2(23)); 2.22–2.13 (*m*, 1 H, CH_2(23)); 2.05 (*s*, MeCOO–C(12)); 0.90 (*s*, Me(19)); 0.77 (*d*, Me(21)); 0.70 (*s*, Me(18)).

Methyl 7α,12α-Diacetoxy-3β-hydroxy-5β-cholan-24-oate (**5c**). According to *GP I*, from methyl 3α,7α,12α-trihydroxy-5β-cholan-24-oate (11 mmol): 4.0 g (71%) of **5c**. White solid. M.p. 138–139°. ^{1}H-NMR ($CDCl_3$): 5.07 (*s*, H_β–C(12)); 4.90 (*s*, H_β–C(7)); 4.05 (*s*, H_β–C(3)); 3.64 (*s*, MeO); 2.36–2.28 (*m*, 1 H, CH_2(23)); 2.22–2.15 (*m*, 1 H, CH_2(23)); 2.08 (*s*, MeCOO–C(7)); 2.04 (*s*, MeCOO–C(12)); 0.93 (*s*, Me(19)); 0.80 (*d*, Me(21)); 0.72 (*s*, Me(18)).

General Procedure II (GP II): Mitsunobu *Reaction with Azide as Nucleophilic Agent* [5]. To a soln. of 3β-hydroxy methyl ester **5b** or **5c** (10 mmol) and triphenylphosphine (20 mmol) in toluol (75 ml) powdered zinc azide bis-pyridine complex [18] (7.5 mmol) was slowly added. After stirring for 1 h (→ good suspension), DEAD (10 mmol) was added very slowly (8 drops/min). After 3 days of stirring, the solvent was evaporated. Rough chromatography (silica gel, hexane/AcOEt 9:1) yielding a purity of 80% was followed by fractional crystallization from hexane/AcOEt.

Methyl 12α-Acetoxy-3α-azido-5β-cholan-24-oate (**6b**). According to *GP II*, (3 d at 80°), from **5b** (8 mmol). Crystallization from hexane/AcOEt 9:1 afforded **6b** (2.6 g, 68.5%). Slightly yellow solid. ^{1}H-NMR ($CDCl_3$): 5.05 (*s*, H_β–C(12)); 3.64 (*s*, MeO); 3.25 (*tt*, H_β–C(3)); 2.36–2.28 (*m*, 1 H, CH_2(23)); 2.21–2.14 (*m*, 1 H, CH_2(23)); 2.07 (*s*, MeCOO–C(12)); 0.89 (*s*, Me(19)); 0.78 (*d*, Me(21)); 0.70 (*s*, Me(18)).

Methyl 7α12α-Diacetoxy-3α-azido-5β-cholan-24-oate (**6c**). According to *GP II*, from **5b** (7 mmol). Crystallization from hexane/AcOEt 9:1 afforded **6c** (2.8 g, 75%). Colorless prisms. M.p. 98–102°. ^{1}H-NMR ($CDCl_3$): 5.07 (*s*, H_β–C(12)); 4.92 (*s*, H_β–C(7)); 3.64 (*s*, MeO); 3.12 (*tt*, H_β–C(3)); 2.37–2.29 (*m*, 1 H, CH_2(23)); 2.23–2.15 (*m*, 1 H, CH_2(23)); 2.10 (*s*, MeCOO–C(7)); 2.07 (*s*, MeCOO–C(12)); 0.91 (*s*, Me(19)); 0.80 (*d*, Me(21)); 0.71 (*s*, Me(18)).

Crystal-Structure Determination of **6c** (*Fig. 5*). $C_{29}H_{45}N_3O_6$, $M_r = 531.68$; $\mu = 0.084$ mm^{-1}, $F(000) = 576$, $d_x = 1.207$ g · cm^{-3}, monoclinic, $P2(1)$, $Z = 2$, $a = 12.851(7)$, $b = 8.332(4)$, $c = 14.701(7)$ Å, $V = 1462.4(13)$ Å^3; $1.71 < \theta < 22.76°$; colorless prism, $0.06 \times 0.22 \times 0.32$ mm. Cell dimensions and intensities were measured at 173(2) K on a *Siemens-P4* 4-circle diffractometer (λ 0.71069 Å); index ranges: $0 \le h \le 13$, $0 \le k \le 9$, $-15 \le l \le 14$; 2088 reflections collectes, 2011 independent reflections; refinement method, full-matrix least-

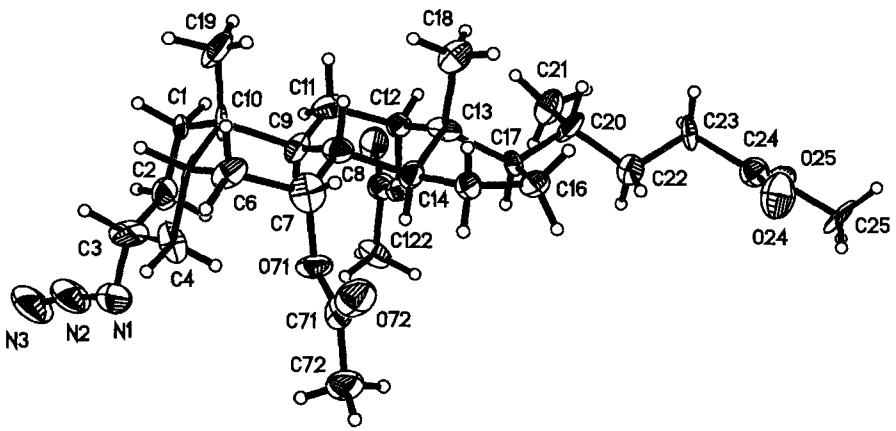


Fig. 5. *X-Ray-determined solid-state structure of* **6c**

squares on F^2; data/restraints/parameters, 2009/1/334; goodness-of-fit on F^2, 1.032; final R indices ($I > 2\sigma(I)$), $R1 = 0.0889$, $wR2 = 0.1771$; R indices (all data), $R1 = 0.2297$, $wR2 = 0.2694$; the final difference electron density map showed a maximum of + 0.305 and a minimum of −0.377 eÅ^{-3}. Crystallographic data have been deposited with the *Cambridge Crystallographic Data Center*, University Chemical Laboratory, 12 Union Road, Cambridge CB2 1EZ, England, as supplementary publication No. CCDC-10/59.

General Procedure III (GP III): Azide Reduction. To a soln. of azide **6b** or **6c** (10 mmol) in dry i-PrOH (35 ml), sodium cyano/trihydroborate (10 mmol) was added and stirred for 1 h (→ good suspension). Then propane-1,3-dithiol (5 mmol) was added. After 36 h, the mixture was filtered, the solvent evaporated, and the amino derivative precipitated from CH_2Cl_2/Et_2O as a HCl salt. The salt was dried *in vacuo*.

Methyl 12α-Acetoxy-3α-amino-5β-cholan-24-oate (**7b**). According to *GP III*, (36 h at r.t.), from **6b** (5.5 mmol): **7b** · HCl (1.88 g, 70.9%). White solid. M.p. 163–166°. ^{1}H-NMR ($CDCl_3$): 7.83 (br., NH_3^+–C(3)); 5.03 (*s*, H_β–C(12)); 3.64 (*s*, MeO); 3.08 (*m*, H_β–C(3)); 2.35–2.29 (*m*, 1 H, CH_2(23)); 2.24–2.15 (*m*, 1 H, CH_2(23)); 2.16 (*s*, MeCOO–C(12)); 0.92 (*s*, Me(19)); 0.80 (*d*, Me(21)); 0.70 (*s*, Me(18)). HR-MS: 448.339400 ($C_{27}H_{46}N_1O_4^+$; calc. 448.342684).

Methyl 7α,12α-Diacetoxy-3α-amino-5β-cholan-24-oate (**7c**). According to *GP III*, (36 h at r.t.), from **6c** (4.5 mmol): **7c** · HCl (1.85 g, 75%). White solid. M.p. 235° (subl.). ^{1}H-NMR ($CDCl_3$/DMSO 3:1): 8.28 (br., NH_3^+–C(3)); 5.05 (*m*, H_β–C(12)); 4.88 (*m*, H_β–C(7)); 3.66 (*s*, MeO); 2.91 (*m*, H_β–C(3)); 2.42–2.33 (*m*, 1 H, CH_2(23)); 2.28–2.20 (*m*, 1 H, CH_2(23)); 2.19 (*s*, MeCOO–C(12)); 2.15 (*s*, MeCOO–C(7)); 0.97 (*s*, Me(19)); 0.86 (*d*, Me(21)); 0.62 (*s*, Me(18)). HR-MS: 506.348200 ($C_{29}H_{48}N_1O_6^+$; calc. 506.348164).

General Procedure IV (GP IV): Peptide Coupling [6]. To a soln. of the amino methyl ester **7a**, **7b**, or **7c** (1 mmol) and Boc–Phe–Phe–OH (1 mmol) in dry DMF (12 ml) at −15°, Et_3N (1.9 ml) and diethyl phosphorocyanidate (0.5 ml) was added. The mixture was stirred at r.t. for 2 days. Precipitated material was removed by filtration, and H_2O was added to the limpid soln. until the precipation of the steroidal peptide **8a**, **8b**, or **8c** was complete. The peptide was dried *in vacuo* and taken up in AcOEt (30 ml). The AcOEt soln. was washed with 5% $NaHSO_4$ soln., sat. $NaHCO_3$ soln., and brine, dried (Na_2SO_4), and evaporated.

*Methyl 3α-{{N-[(*tert*-Butoxy)carbonyl]-*L*-phenylalanyl-*L*-phenylalanyl}-amino}-5β-cholan-24-oate* (Boc–Phe–Phe–**2a**–OMe; **8a**). According to *GP IV*, from **2a**-OMe · HCl (= **7a** · HCl; 0.4 mmol): **8a** (257 mg, 82.0%). Colorless foam. ^{1}H-NMR ($CDCl_3$): 6.50 (*d*, 1 H, NH(Phe)); 5.99 (*d*, 1 H, NH(Phe)); 4.82 (*d*, NH(**2a**)); 4.56 (*q*, H–C(α)(Phe)); 4.27 (*q*, H–C(α)(Phe)); 3.64 (*m*, H_β–C(3)); 3.65 (*s*, COOMe); 3.19, 2.97 (*dd*, 2 H, CH_2(β)(Phe)); 3.07, 2.81 (*dd*, 2 H, CH_2(β)(Phe)); 2.42–2.34 (*m*, 1 H, CH_2(23)); 2.28–2.20 (*m*, 1 H, CH_2(23)); 1.34 (*s*, *t*-Bu); 0.90 (*s*, Me(19)); 0.89 (*d*, Me(21)); 0.62 (*s*, Me(18)). HR-MS: 806.509800 ($C_{48}H_{69}N_3O_6Na_1^+$; calc. 806.508408).

*Methyl 12α-Acetoxy-3α-{{N-[(*tert*-Butoxy)carbonyl]-*L*-phenylalanyl-*L*-phenylalanyl}amino}-5β-cholan-24-oate* (Boc–Phe–Phe–**2b**-OMe; **8b**). According to *GP IV*, from **2b**-OMe · HCl (= **7b** · HCl; 0.3 mmol): **8b** (203 mg, 80.5%). Colorless foam. ^{1}H-NMR ($CDCl_3$): 6.49 (*d*, 1 H, NH(Phe)); 5.80 (*d*, 1 H, NH(Phe)); 5.07 (*s*, H_β–C(12)); 4.82 (*d*, NH(**2b**)); 4.53 (*q*, H–C(α)(Phe)); 4.23 (*q*, H–C(α)(Phe)); 3.66 (*m*, H_β–C(3)); 3.64

(*s*, COOMe); 3.18, 2.98 (*dd*, 2 H, $CH_2(\beta)$(Phe)); 3.06, 2.81 (*dd*, 2 H, $CH_2(\beta)$(Phe)); 2.40–2.32 (*m*, 1 H, CH_2(23)); 2.27–2.18 (*m*, 1 H, CH_2(23)); 1.33 (*s*, *t*-Bu); 0.87 (*s*, Me(19)); 0.80 (*d*, Me(21)); 0.70 (*s*, Me(18)). HR-MS: 864.516400 ($C_{50}H_{71}N_3O_8Na_1^+$; calc. 864.513887).

*Methyl 7α,12α-Diacetoxy-3α-{{N-[(*tert*-Butoxy)carbonyl]-*L*-phenylalanyl-*L*-phenylalanyl}amino}-5β-cholan-24-oate* (Boc–Phe–Phe–**2c**–OMe; **8c**). According to *GP IV*, from **2c**-OMe · HCl (= **7c** · HCl; 1 mmol): **8c** (744 mg, 82.7%). Colourless foam. ^{1}H-NMR ($CDCl_3$): 6.60 (*d*, 1 H, NH(Phe)); 5.34 (*d*, 1 H, NH(Phe)); 4.98 (*s*, H_β–C(12)); 4.87 (*s*, H_β–C(7)); 4.82 (*d*, NH(**2c**)); 4.45 (*q*, H–C(α)(Phe)); 4.32 (*q*, H–C(α)(Phe)); 3.65 (*s*, COOMe); 3.42 (*m*, H_β–C(3)); 3.12–2.98 (*m*, $CH_2(\beta)$(Phe)); 3.02, 2.84 (*dd*, $CH_2(\beta)$(Phe)); 2.39–2.28 (*m*, 1 H, CH_2(23)); 2.24–2.16 (*m*, 1 H, CH_2(23)); 1.36 (*s*, *t*-Bu); 0.89 (*s*, Me(19)); 0.81 (*d*, Me(21)); 0.71 (*s*, Me(18)). HR-MS: 922.514700 ($C_{52}H_{73}N_3O_{10}Na_1^+$, calc. 922.519366).

General Procedure V (GP V): Cyclization Reaction [7]. To a soln. of Boc–Phe–Phe–Spa–OMe (**8a**, **8b**, or **8c**, 1 mmol) in EtOH (10 ml), 1N aq. NaOH (2.5 ml) was added and the mixture stirred for 5 h. After evaporation, the residue was taken up in H_2O and brought to pH 1–2 with dil. HCl soln. The free acid was extracted with AcOEt (3 × 10 ml) and the combined org. phase washed with 5% $NaHSO_4$ soln., sat. $NaHCO_3$ soln., and brine, dried ($MgSO_4$), and evaporated. Without further purification, the residue (90% pure by NMR) was taken up in CH_2Cl_2 (10 ml), and pentafluorophenol (1.5 mmol) was added at r.t., followed by dicyclohexylcarbodiimide (1.25 mmol) in CH_2Cl_2 (5 ml) at −20°. The mixture was stirred overnight at r.t., and the precipitated urea was filtered. An equal volume of CF_3COOH was added at 0° and the mixture evaporated after 1 h. The residue was taken up in CH_2Cl_2 (250 ml), and 4-(dimethylamino)pyridine (DMAP; 2 mmol) and Na_2HPO_4 (4 mmol) were added. After 5 days of stirring at r.t., the mixture was filtered and the filtrate evaporated. The product was purified by fractional crystallization from AcOEt.

*Cyclo(–***2a**–*Phe–Phe–)*$_2$ (**1a**). According to *GP V*, from **8a** (0.305 mmol). Crystallization from AcOEt (8 ml) afforded in the first fraction **1a**/DMAP 1:1. The slightly yellow solid was taken up in CH_2Cl_2 (15 ml) and washed with 5% $NaHSO_4$ soln. and brine. The org. phase was dried ($MgSO_4$) and evaporated. Recrystallization from CH_2Cl_2/AcOEt afforded pure **1a** (45 mg, 22.5%). Colorless prisms. M.p. 205–208°. ^{1}H-NMR ($CDCl_3$): 7.40–7.15 (*m*, 20 arom. H(Phe1, Phe2)(2)); 6.86 (*d*, 4 arom. H_o(Phe2)); 6.61 (*d*, 2 H, NH(Spa,Spa′)); 5.75 (*d*, 2 H, NH(Phe2, PHe2′)); 5.45 (*d*, 2 H, NH(Phe1, Phe1′)); 4.68 (*m*, 2 H, H–C(α)(Phe2, Phe2′)); 4.23 (*q*, 2 H, H–C(α)(Phe1, Phe1′)); 3.63 (*m*, 2H, H_β–C(3)(Spa,Spa′)); 3.45, 2.73 (*dd*, 4 H, $CH_2(\beta)$(Phe2, Phe2′)); 3.06 (*d*, 4 H, $CH_2(\beta)$(Phe1, Phe1′)); 2.147 (*m*, 2 H, H–C(23)(Spa,Spa′)); 0.94 (*s*, 6 H, Me(19)(Spa,Spa′)); 0.77 (*d*, 6 H, Me(21)(Spa,Spa′)); 0.65 (*s*, 6 H, Me(18)(Spa,Spa′)). DCI-MS (NH_3, neg.): 1302 (98, M^-), 1284 (52, $[M - H_2O]^-$), 1258 (54), 1241 (100), 1183 (32), 1155 (30), 1137 (80), 884 (37.5), 867 (55). FAB-MS (NBA, pos.): 1303 (28, M^+), 1212 (1), 800 (1.5), 652 (3.5, M^{++}), 505 (5), 307 (12).

*Cyclo(–***2b**–*Phe–Phe–)*$_2$ (**1b**). According to *GP V*, from **8b** (0.225 mmol). Fractional crystallization from AcOEt (5 ml) afforded in the 2nd fraction pure **1b** (31 mg, 18.8%). White solid. M.p. 299–302°. ^{1}H-NMR ($CDCl_3$): 7.40–7.14 (*m*, 20 arom. H(Phe1, Phe2); 6.85 (*d*, 4 arom. H_o(Phe2)); 6.42 (*d*, 2 H, NH(Spa,Spa′)); 5.64 (*d*, 2 H, NH(Phe2, Phe2′)); 5.44 (*d*, 2 H, NH(Phe1, Phe1′)); 5.05 (*s*, 2 H, H_β–C(12)(Spa, Spa′)); 4.64 (*m*, 2 H, H–C(α)(Phe2, Phe2′)); 4.26 (*q*, 2 H, H–C(α)(Phe1, Phe1′)); 3.74 (*m*, H_β–C(3)(Spa, Spa′)); 3.45–2.70 (*dd*, 4 H, $CH_2(\beta)$(Phe2, Phe2′)); 3.05 (*d*, 4 H, $CH_2(\beta)$(Phe1, Phe1′)); 1.90 (*s*, 6 H, MeCOO–C(12)(Spa, Spa′)); 0.91 (*s*, 6 H, Me(19)(Spa, Spa′)); 0.74 (*s*, 6 H, Me(18)(Spa, Spa′)); 0.64 (*d*, 6 H, Me(21)(Spa, Spa′)). DCI-MS (NH_3, pos.): 1436 (22, $[M + NH_3]^+$), 1420 (110, MH^+), 1401 (24), 1360 (79), 1301 (20), 1172 (18.5), 1125 (98), 1006 (19). FAB-MS (NBA, neg.): 1571 (4, $[M + NBA]^-$), 1418 (21, M^-), 1124 (3.5), 850 (6), 780 (4), 339 (19), 199 (25.5), 168 (40), 153 (100, NBA).

*Cyclo(–***2c**–*Phe–Phe–)*$_2$ (**1c**). According to *GP V*, from **8c** (0.45 mmol). Crystallization from AcOEt (5 ml) afforded in the 2nd fraction pure **1c** (40 mg, 11.3%). White solid. M.p. 275–278°. ^{1}H-NMR ($CDCl_3$): 7.31–7.14 (*m*, 20 arom. H(Phe1, Phe2)); 7.02 (*d*, 4 arom. H_o(Phe2)); 5.93 (*d*, 2 H, NH(Phe1, Phe1′)); 5.81 (*d*, 2 H, NH(Spa, Spa′)); 5.75 (*d*, 2 H, NH(Phe2, Phe2′)); 5.02 (*s*, 2 H, H_β–C(12)(Spa, Spa′)); 4.87 (*s*, 2 H, H_β–C(7)(Spa, Spa′)); 4.43 (*q*, 2 H, H–C(α)(Phe2, Phe2′)); 4.32 (*q*, 2 H, H–C(α)(Phe1, Phe1′)); 3.50 (*m*, 2 H, H_β–C(3)(Spa, Spa′)); 3.02 (*m*, 8 H, $CH_2(\beta)$(Phe1, Phe1′, Phe2, Phe2′)); 2.05 (*s*, 6 H, MeCOO–C(12)(Spa, Spa′)); 1.86 (*s*, 6 H, MeCOO–C(7)(Spa, Spa′)); 0.90 (*s*, 6 H, Me(19)(Spa, Spa′)); 0.71 (*s*, 6 H, Me(18)(Spa, Spa′)); 0.67 (*d*, 6 H, Me(21)(Spa, Spa′)). FAB-MS (NBA, pos.): 1558 (4, $[M + Na]^+$), 1535 (4, M^+) 1296 (3.5), 814 (1), 648 (1.5), 549 (4.5) 307 (7), 259 (11.5), 176 (20), 153 (93, NBA), 120 (100).

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