Antibiotic and Hemolytic Activity of a β^2/β^3 Peptide Capable of Folding into a 12/10-Helical Secondary Structure

Per I. Arvidsson,[a] Neil S. Ryder,[b] H. Markus Weiss,[c] Gerhard Gross,[c] Olivier Kretz,[c] Ralph Woessner,[c] and Dieter Seebach*[d]

Non-natural peptides capable of evoking a specific biological response are currently receiving immense interest. They most commonly act by mimicking the structure of a naturally occurring biologically active peptide. Several such peptidomimetic oligomers have been described, and their pharmacological properties, for example, protease resistance, lack of immunogenicity, and bioavailability, are usually superior to those of the parent peptide.[1] Oligomers of β-amino acids, that is, β-peptides, are probably the most thoroughly investigated peptidomimetic oligomer.[2] Even short β-peptides, with as few as six residues, have a high propensity to fold into defined secondary structures, similar to those found in Nature. Furthermore, β-peptides with interesting biological properties, for example, antibacterial,[3] antiproliferative,[4] and somatostatin-mimicking,[5] have been described and proven to be stable towards proteolytic[6] and metabolic[7] degradation.

β-Peptides have been especially efficient at mimicking cationic antibacterial peptides.[3, 8] Many organisms, ranging from bacteria to mammals, produce such peptides as part of their innate immunity. Most of these peptides are short, cationic molecules, capable of forming amphiphilic structures (usually α-helices), in which hydrophobic residues are concentrated on one face of the structure while cationic groups are located on the opposite side. Although the precise mode of action of these peptides remains ambiguous, it is generally accepted that the cationic charges direct the peptide to the negatively charged bacterial membrane, and that the hydrophobic residues then interact with the lipid bilayer in a way that causes disruption of membrane integrity, and ultimately cell death. Therapeutic application of cationic peptides, or their biomimetic analogues, as antibacterial agents are promising, as their direct action on the bacterial cell membrane is likely to prevent a rapid development of bacterial resistance.[9]

β-Peptides composed of acyclic β^3-amino acids,[3a,c,d] cyclic *trans*-2-aminocyclohexane- or cyclopentane carboxylic acids,[3b,e] and combinations of cyclic and acyclic residues[3f] have been designed to fold into amphiphilic 2.5_{12}- and 3_{14}-helices and reported to display antimicrobial activity equal to or better than naturally occurring substances. Although promising, the 2.5_{12}- and 3_{14}-helices formed by these molecules are limited in respect to synthetic accessibility and/or in the number of positions available for introduction of functional side-chains. The most studied helical conformation of β-peptides, that is, the 3_{14}-helix, is characterized by 14-membered $i \rightarrow i+3$ N-H···O=C hydrogen-bonded rings and has (*M*)-chirality when prepared from L-amino acids.[10] Upon inspection of an idealized helical-wheel representation of the 3_{14}-helix, Figure 1, one observes that this helix carries side-chains only along three streaks on its surface. The simplicity of this structure restricts the placement of functional groups around the helix; however, it is not anticipated that this is critical for biological activity.

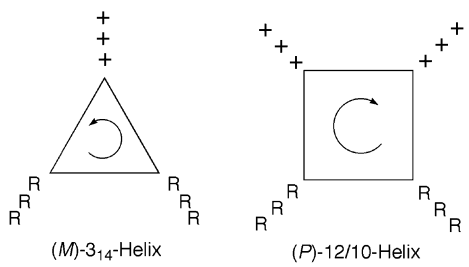

Figure 1. *Schematic helical-wheel-type representation of the β-peptidic 3_{14}-helix and a 12/10-helix. The 12/10-helix offers more possibilities for functionalization, and allows for a more even distribution of the side-chains over the helix surface. + = positively charged side chain, R = side chain of the β-amino acid.*

β-Peptides with alternating β^2- and β^3-amino acid residues, on the other hand, are known to fold into an alternative 12/10-helical conformation of (*P*)-helicity, with alternating 12- and 10-membered hydrogen-bonded rings.[11] The hydrogen bonds in this conformation go from $i \rightarrow i-3$ (12-membered ring) and $i \rightarrow i+1$ (10-membered ring), and the amide C=O group orientation is alternately pointing up and down along the helical axis, thus resulting in a smaller macrodipole of the 12/10-helix or none at all. The α-peptidic 3.6_{13}-helix and the β-peptidic 3_{14}-helix have resulting macrodipoles pointing from the *N* to the *C* terminus (minus end) and from the *C* to the *N* terminus, respectively. As seen in Figure 1, the 12/10 helix offers more possibilities for placement of functionalized side chains than the 3_{14}-helix; thus, this scaffold might offer advantages for biomedical and other applications. The lack of a net dipole moment is also expected to enhance bioavailability of these peptides. In this report we present the first observation of this scaffold as a biomimetic antibacterial oligomer.

The "mixed" β^3/β^2-nonapeptide **1** (Figure 2) was designed to fold into a 12/10-helix with 10/12/10-hydrogen bonded rings, as previous NMR-spectroscopic studies have established for an

[a] *Dr. P. I. Arvidsson*
Uppsala University, Institute of Chemistry
Department of Organic Chemistry
Box 599, 75124 Uppsala (Sweden)

[b] *Dr. N. S. Ryder*
Infectious Diseases Biology
Novartis Institutes for Biomedical Research, Inc.
100 Technology Square, Cambridge, MA 02139 (USA)

[c] *Dr. H. M. Weiss, Dr. G. Gross, Dr. O. Kretz, Dr. R. Woessner*
Novartis Pharma AG
Preclinical Safety or (RW) Exploratory Clinical Development
4002 Basel (Switzerland)

[d] *Prof. Dr. D. Seebach*
Laboratorium für Organische Chemie, Eidgenössische Technische Hochschule
ETH Hönggerberg, 8093 Zürich (Switzerland)
Fax: (+41) 1-632-11-44
E-mail: seebach@org.chem.ethz.ch

 DOI: 10.1002/cbic.200300698 © 2003 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

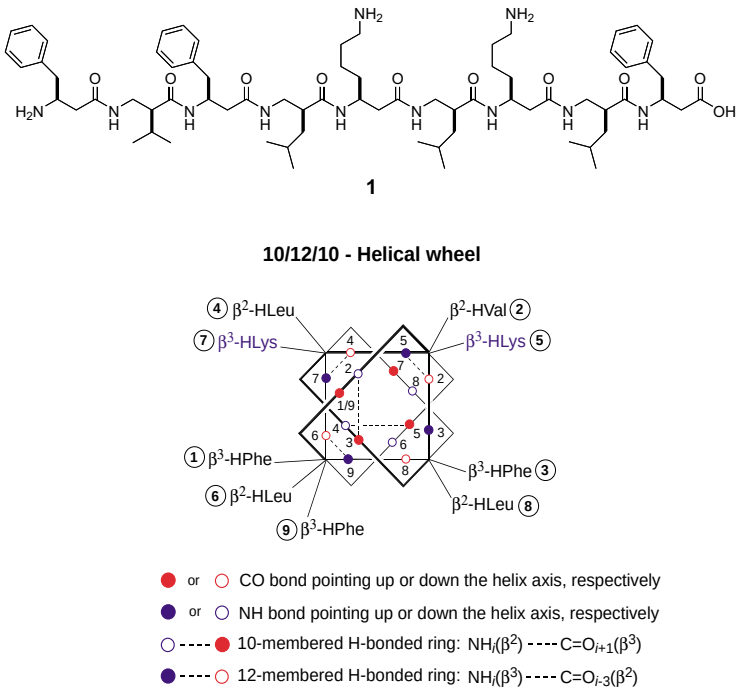

*Figure 2. Molecular formula of the "mixed" β³/β²-nonapeptide **1** and a helical-wheel representation of the 10/12/10-helical conformation this molecule is expected to adopt. The 12/10-helix is characterized by alternating twelve-membered H-bonded rings (from $NH_{(i)}$ of a β³-amino acid residue to the $C{=}O_{(i-3)}$ of a β²-amino acid residue, "rectangular" route) and ten-membered H-bonded rings (from $NH_{(i)}$ of a β²-amino acid residue to $C{=}O_{(i+1)}$ of a β³-amino acid residue, "squaric" route). The amide C=O groups point alternately up and down the helix axis, conferring either a small or no net dipole moment to the helix.*

analogous peptide carrying purely aliphatic side-chains.[11c] Two lysine side-chains were positioned on one face of the helix, thus rendering the 12/10-helical conformation of **1** amphiphilic.

β-Peptide **1** was prepared by solid-phase synthesis on Wang resin as previously described.[12] Preformed Fmoc-protected β²/β³-dipeptide fragments were utilized for the first four couplings in order to avoid epimerization of the β²-amino acid moieties during activation and coupling; the *N*-terminal β³HPhe was introduced as a single amino acid. The peptide was purified by reversed-phase HPLC to a purity of >98% before characterization and biological testing.

The CD-spectra of **1** are shown in Figure 3. The spectrum in methanol is characterized by a very intense absorption around 205 nm, as expected for a "mixed" β-peptide in the 12/10-helical conformation.[11c,d] The intensity of this absorption is drastically reduced in aqueous buffer solution; this suggests that the 12/10-helical conformation of this peptide is far less stable in water than in methanol. Destabilization of β-peptide helices in water has also been noted for the 3_{14}-helical conformation, and several strategies for stabilization have been adopted.[13] It should be kept in mind that the folding may occur upon binding of a peptide to its receptor; this was demonstrated for a β³-peptide that appeared to fold to an amphiphilic 3_{14}-helix only upon docking to a membrane-bound protein.[14]

Although the CD-spectroscopic analysis shows that **1** is only partially folded in aqueous media, it may still display biomimetic function. Studies on both α- and β-peptides have established that a preorganized amphiphilic structure is not a prerequisite for antimicrobial (or other) activity, as no straightforward relationship between helical stability and antimicrobial activity exists.[3f] Thus, the "mixed" β³/β²-peptide **1** was subjected to biological testing to establish whether it could be used to mimic the amphiphilic α-helix of naturally occurring cationic antibiotic peptides.

As seen in Table 1, mixed β-peptide **1** does indeed show good antibacterial activity against a variety of bacterial strains, as determined by the minimum inhibitory concentration (MIC). The effect is highest against the *Staphylococcus* and *Streptococcus* (both Gram positive) strains investigated. Other β-peptides, designed to form 2.5_{12}- and 3_{14}-helices, have proven more active against *Escherichia* (Gram negative) and *Bacillus* (Gram positive) species.[3] Although the currently available data do not allow any conclusions to be drawn, it is interesting to note that the

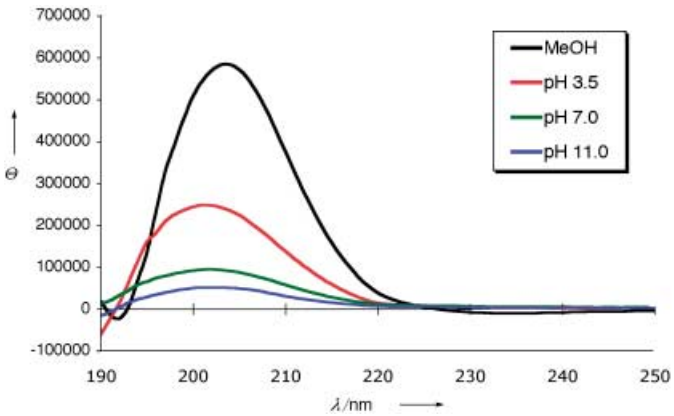

*Figure 3. CD spectra of the mixed β-peptide **1** (Θ in deg cm² dmol⁻¹). The strong absorbance at 205 nm is characteristic of a β-peptidic 12/10-helix. All spectra were recorded at 20.0° with a concentration of 0.2 mM. The peptide was measured as its tris-TFA salt, as obtained after lyophilization, in methanol and in aqueous media (pH 3.5, 0.1 M KOAc buffer; pH 7.0, 0.1 M phosphate buffer; and pH 11.0, 0.1 M NaOH).*

***Table 1.** Antibacterial tests on **1** in vitro.*

Bacterial test strains	IC_{50}[a] [μM]	MIC[b] [μg mL⁻¹]	Selectivity[c]
Enterococcus faecalis	20	32	9
Staphylococcus aureus	5	8	35
Streptococcus pneumoniae	10	16	18
Escherichia coli	20	64	9
Klebsiella pneumoniae	80	>128	2
Pseudomonas aeruginosa	>80	>128	<2

[a] Defined as the lowest β-peptide concentration causing at least 50% reduction of bacterial growth. [b] Defined as the lowest peptide concentration causing *complete* suppression of bacterial growth. The standard drug tetracycline was run in parallel as a positive control. Its MIC values were in the range 0.3–18 μg mL⁻¹, depending on the bacterial strain exposed. [c] Selectivity between bacterial cells and human erythrocytes defined by HC_{50}/IC_{50}. The HC_{50} value (175 μM) for human red blood cells is derived from the fitted curve shown in Figure 4.

 © 2003 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

physicochemical properties of the different helices might give selectivity between bacterial strains.

Selectivity of lytic activity for bacterial over mammalian cells was established by measuring the hemolytic activity of **1** with human red blood cells. As seen in Figure 4, significant lyses of both human and rat red blood cells occurs only at concentrations much higher than the antibacterial MIC values. For

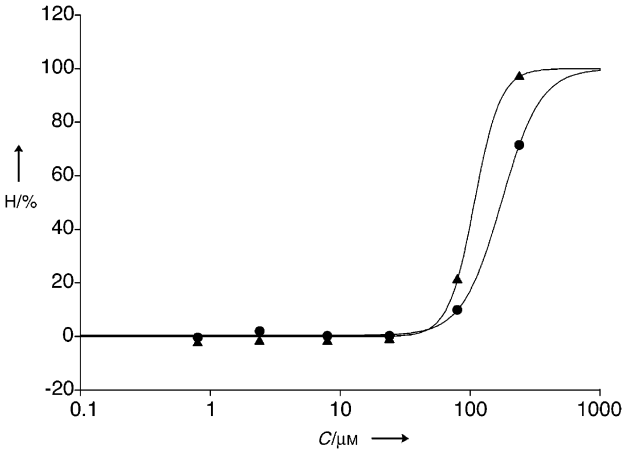

Figure 4. *Activity of β-peptide **1** towards human (L) and rat (s) erythrocytes (H = Hemolysis). Washed blood cells (1%, v/v) were incubated in phosphate-buffered saline (pH 7.4) with different concentrations of the peptide for 1 h at 37 °C. The degree of blood-cell lyses was estimated from the absorption at 405 nm after sedimentation of the intact cells by centrifugation. For calibration, lyses in 1% (w/v) sodium dodecyl sulfate was assumed to correspond to 100% lyses. Data were analyzed by nonlinear least-squares fitting to a four-parameter logistic function by using the program SigmaPlot 8.0, the maximum value was set to 100%. Resulting HC_{50} values were 175 and 110 μM for human and rat, respectively, the corresponding standard errors were 330 and 160 μM.*

comparison, the selectivity as defined by HC_{50}/IC_{50}, is shown in Table 1. It would be interesting and important to establish in future studies how modulation of physicochemical properties of the helices can increase this selectivity in order to provide an acceptable "window".

To summarize, we have designed a "mixed" β-peptide with alternating β^3- and β^2-amino acid residues that can fold into an amphiphilic right-handed 10/12/10-helix. This peptide showed good antibacterial activity against some of the investigated organisms. The antibacterial activity could most likely be increased by replacing the free carboxylic acid group at the *C* terminus with a carboxamide.[3f, 15] Incorporation of more positively charged side chains at positions 2 and 4 is also expected to yield a more potent peptide. The increased modularity of the 12/10-helical surface, as compared to the 3_{14}-helix, is a valuable addition to the collection of non-natural peptidomimetic oligomers.

Acknowledgements

Financial support from Vetenskapsrådet (The Swedish Research Council) and technical assistance from Heidemarie Hügli are gratefully acknowledged.

Keywords: amphiphiles · antibiotics · helical structures · peptides · peptidomimetics

[1] a) D. J. Hill, M. J. Mio, R. B. Prince, T. S. Hughes, J. S. Moore, *Chem. Rev.* **2001**, *101*, 3893; b) J. A. Patch, A. E. Barron, *Curr. Opin. Chem. Biol.* **2002**, *6*, 872; c) G. N. Tew, D. Liu, B. Chen, R. J. Doerksen, J. Kaplan, P. J. Carrol, M. L. Klein, W. F. DeGrado, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 5110.

[2] a) D. Seebach, J. L. Matthews, *Chem. Commun.* **1997**, 2015; b) K. Gademann, T. Hintermann, J. V. Schreiber, *Curr. Med. Chem.* **1999**, *6*, 905; c) P. Cheng, S. H. Gellman, W. F. DeGrado, *Chem. Rev.* **2001**, *101*, 3219.

[3] a) Y. Hamuro, J. P. Schneider, W. F. DeGrado, *J. Am. Chem. Soc.* **1999**, *121*, 12200; b) E. A. Porter, X. Wang, H.-S. Lee, B. Weisblum, S. H. Gellman, *Nature* **2000**, *404*, 565 (erratum: **2000**, *405*, 298); c) P. I. Arvidsson, J. Frackenpohl, N. S. Ryder, B. Liechty, F. Petersen, H. Zimmermann, G. P. Camenisch, R. Woessner, D. Seebach, *ChemBioChem* **2001**, *2*, 771; d) D. Liu, W. F. DeGrado, *J. Am. Chem. Soc.* **2001**, *123*, 7553; e) E. A. Porter, B. Weisblum, S. H. Gellman, *J. Am. Chem. Soc.* **2002**, *124*, 7324; f) T. L. Raguse, E. A. Porter, B. Weisblum, S. H. Gellman, *J. Am. Chem. Soc.* **2002**, *124*, 12774; g) R. F. Epand, N. Umezawa, E. A. Porter, S. Gellman, R. M. Epand, *Eur. J. Biochem.* **2003**, *270*, 1240.

[4] K. Gademann, D. Seebach, *Helv. Chim. Acta* **2001**, *84*, 2924.

[5] a) K. Gademann, M. Ernst, D. Hoyer, D. Seebach, *Angew. Chem.* **1999**, *111*, 1302; *Angew. Chem. Int. Ed.* **1999**, *38*, 1223; b) K. Gademann, T. Kimmerlin, D. Hoyer, D. Seebach, *J. Med. Chem.* **2001**, *44*, 2460; c) D. Seebach, R. Rueping, P. I. Arvidsson, T. Kimmerlin, P. Micuch, C. Noti, D. Langenegger, D. Hoyer, *Helv. Chim. Acta* **2001**, *84*, 3503; d) C. Nunn, M. Rueping, D. Langenegger, E. Schuepbach, T. Kimmerlin, P. Micuch, K. Hurt, D. Seebach, D. Hoyer, *Naunyn-Schmiedeberg's Arch. Pharmacol.* **2003**, *367*, 95.

[6] a) T. Hintermann, D. Seebach, *Chimia* **1997**, *51*, 244; b) D. Seebach, S. Abele, J. V. Schreiber, B. Martinoni, A. K. Nussbaum, H. Schild, H. Schulz, H. Hennecke, R. Woessner, F. Bitsch, *Chimia* **1998**, *52*, 734; c) J. Frackenpohl, P. I. Arvidsson, J. V. Schreiber, D. Seebach, *ChemBioChem* **2001**, *2*, 445.

[7] H. Wiegand, B. Wirz, A. Schweitzer, G. P. Camenisch, M. I. R. Perez, G. Gross, R. Woessner, R. Voges, P. I. Arvidsson, J. Frackenpohl, D. Seebach, *Biopharm. Drug Dispos.* **2002**, *23*, 251.

[8] a) H. G. Boman, *Annu. Rev. Immunol.* **1995**, *13*, 61; b) P. Nicolas, A. Mor, *Annu. Rev. Microbiol.* **1995**, *49*, 277; c) A. Tossi, L. Sandri, A. Giangaspero, *Biopolymers* **2000**, *55*, 4; d) M. B. Strøm, B. E. Haug, M. K. Skar, W. Stensen, T. Stiberg, J. S. Svendsen, *J. Med. Chem.* **2003**, *46*, 1567.

[9] a) R. E. W. Hancock, *Lancet* **1997**, *349*, 418; b) A. R. Koczulla, R. Bals, *Drugs* **2003**, *63*, 389; c) M. R. Yeaman, N. Y. Yount, *Pharmacol. Rev.* **2003**, *55*, 27.

[10] D. Seebach, M. Overhand, F. N. M. Kuehnle, B. Martinoni, L. Oberer, U. Hommel, H. Widmer, *Helv. Chim. Acta* **1996**, *79*, 913.

[11] a) D. Seebach, K. Gademann, J. V. Schreiber, J. L. Matthews, T. Hintermann, B. Jaun, L. Oberer, U. Hommel, H. Widmer, *Helv. Chim. Acta* **1997**, *80*, 2033; b) D. Seebach, S. Abele, K. Gademann, G. Guichard, T. Hintermann, B. Jaun, J. L. Matthews, J. V. Schreiber, L. Oberer, U. Hommel, H. Widmer, *Helv. Chim. Acta* **1998**, *81*, 932; c) M. Rueping, J. V. Schreiber, G. Lelais, B. Jaun, D. Seebach, *Helv. Chim. Acta* **2002**, *85*, 2577; d) A full 750 MHz NMR measurement of **1** has been performed, the NH region displayed good dispersion (suggesting the presence of a preferred conformation); a full analysis, which might lead to a structural assignment, is underway.

[12] P. I. Arvidsson, J. Frackenpohl, D. Seebach, *Helv. Chim. Acta* **2003**, *86*, 1522.

[13] a) M. Rueping, B. Jaun, D. Seebach, *Chem. Commun.* **2000**, 2267; b) P. I. Arvidsson, M. Rueping, D. Seebach, *Chem. Commun.* **2001**, 649; c) T. L. Raguse, J. R. Lai, S. H. Gellman, *J. Am. Chem. Soc.* **2003**, *125*, 5592.

[14] M. Werder, H. Hauser, S. Abele, D. Seebach, *Helv. Chim. Acta* **1999**, *82*, 1774.

[15] a) Z. Q. Li, R. B. Merrifield, I. A. Boman, H. G. Boman, *FEBS Lett.* **1988**, *231*, 299; b) J. H. Cuervo, B. Rodriguez, R. A. Houghten, *Pept. Res.* **1988**, *1*, 81.

Received: June 23, 2003 [Z698]

 © 2003 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim