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The Biological Stability of β-Peptides: No Interactions between α- and β-Peptidic Structures?

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Abstract. The use of peptides in pharmaceutical applications is always hindered by the rapid degradation of the peptides by enzymes. Recent work on β-peptides, peptides constructed entirely from β-amino acids, has shown that even the short-chain oligomers (6 or 7 residues) exhibit remarkably stable secondary structures. However, before certain applications of β-peptides can be considered, an information about their possible interactions or reactions with enzymes must be acquired.

Ames tests (all negative) have now been performed on a selection of β-amino acids, which would be the result of the enzymatic degradation of a β-peptide. Furthermore, digestion experiments using a variety of peptidases and β-peptides have now shown that β-peptides, in sharp contrast to their α-peptide counterparts, undergo no degradation.

secondary structures [5–10]. However, before potential applications of this new class of unnatural peptide analogues can be considered, it is essential to find out if and how they interact with enzymes built of α-amino acids. We have, therefore, performed *Ames* tests on some β-amino acids and investigated the stability of oligomers, formed from up to seven β-amino acids, towards peptidases.

Introduction

Aspartic acid is the only proteinogenic amino acid containing a β-amino-acid structural unit. However, some simple β-amino acids are found in the metabolism of mammals [1], and there are numerous highly active compounds, which contain complex β-amino-acid building blocks as part of their structure, found in plants (*e.g.* taxol [2], in microorganisms (*e.g.* the enediyne chromophore of C-1027 [3], and especially in marine organisms (often macrocyclic peptidic compounds [4]).

Short-chain oligomers built only from β-amino acids (β-peptides) have recently been shown to form surprisingly stable

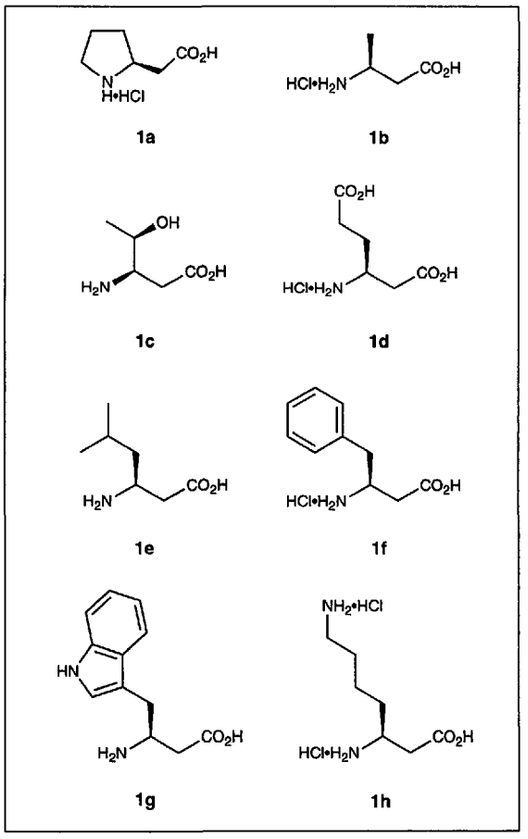

Fig. 1. *The* β*-amino acids* **1a–h** *investigated in the* Ames *tests*

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Results

Using our standard method which involves the application of *Arndt-Eistert* methodology [11], we have now synthesised (in some cases, for the first time) the eight β-amino acids **1a–h**, derived from proline, alanine, threonine, glutamic acid, leucine, phenylalanine, tryptophan, and lysine, respectively (*Fig. 1*). Studies of their mutagenic properties (*Ames* test [12] with *Salmonella typhimurium* and rat liver activation, see *Experimental*) have revealed that none of these amino acids are mutagenic under standard conditions [13]. In other words, if the β-peptides constructed from these building blocks were to undergo enzymatic cleavage, the resulting compounds would, according to these tests, not be toxic.

We selected the β-tetra-(**2a–d**), β-hexa-(**3**, **4**), and β-heptapeptides (**5**, **6**) for the experiments with peptidases (*Fig. 2*). With the exception of **6**, the syntheses of these compounds have been previously described [5][6][8]. They contain the β-amino-acid building blocks β^3-HAla, β^3-HVal, β^3-HLeu, and β^3-HLys (in **2a–d**, **3**, **5**, **6**), the α-substituted β^2-HAla, β^2-HVal, and β^2-HLeu (in **4**), the diastereoisomers of β-HAla(αMe) (in **2a**, **b**). The peptides **2c**, **2d**, and **5** also contain the α-amino-acids Ala and Aib (for the definition of the β-amino-acid abbreviations, see [5][7]).

The following commercially available peptidases were employed: pepsin (**P**; from gastric mucosa), chymotrypsin (**CT**), trypsin (**T**), elastase (**E**), carboxypeptidase A (**CPA**) (all from the pancreas), and leucine aminopeptidase (**LAP**) (from the kidney). Whereas **P** is an aspartic peptidase, **CT**, **T**, and **E** are serinases, and **CPA** and **LAP** are zinc-dependent proteases. These peptide-cleaving enzymes differ further in that **P**, **CT**, **T**, and **E** are endopeptidases, while **CPA** and **LAP** are exopeptidases (C-terminal and N-terminal, respectively) [14]. The activity of each enzyme was first determined using standard substrates, and this led to the selection of specific concentrations that resulted in total cleavage within 30 min. Solutions, which contained *ca.* 0.3 mg/ml (0.15 mg/ml in the case of **3** due to its low solubility) of the β-peptides **2–6**, were treated with the enzymes (*Table*).

Occasionally, samples were taken and analysed by reversed-phase HPLC (*Nucleosil C8* column). The result is easy to describe: there was no evidence of any cleavage of the β-peptides over a period up to two days. The tetrapeptide **2c**, which contains an N-terminal α-amino acid, was found to have sustained 10% cleavage after treatment with **LAP** for two days.

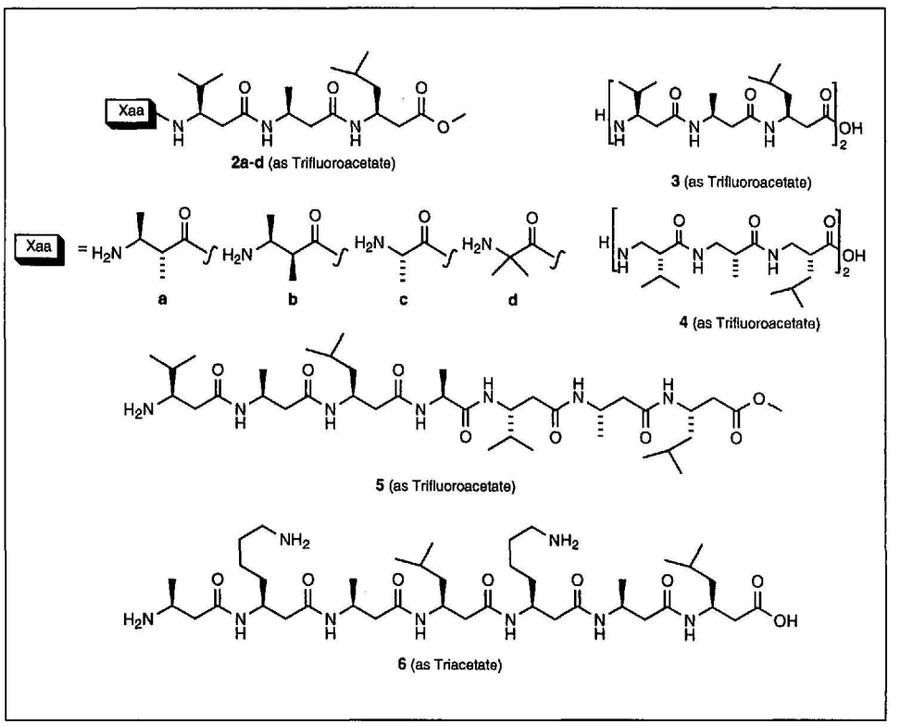

Fig. 2. *The* β*-peptides* **2–6** *used for the enzymatic cleavage experiments*

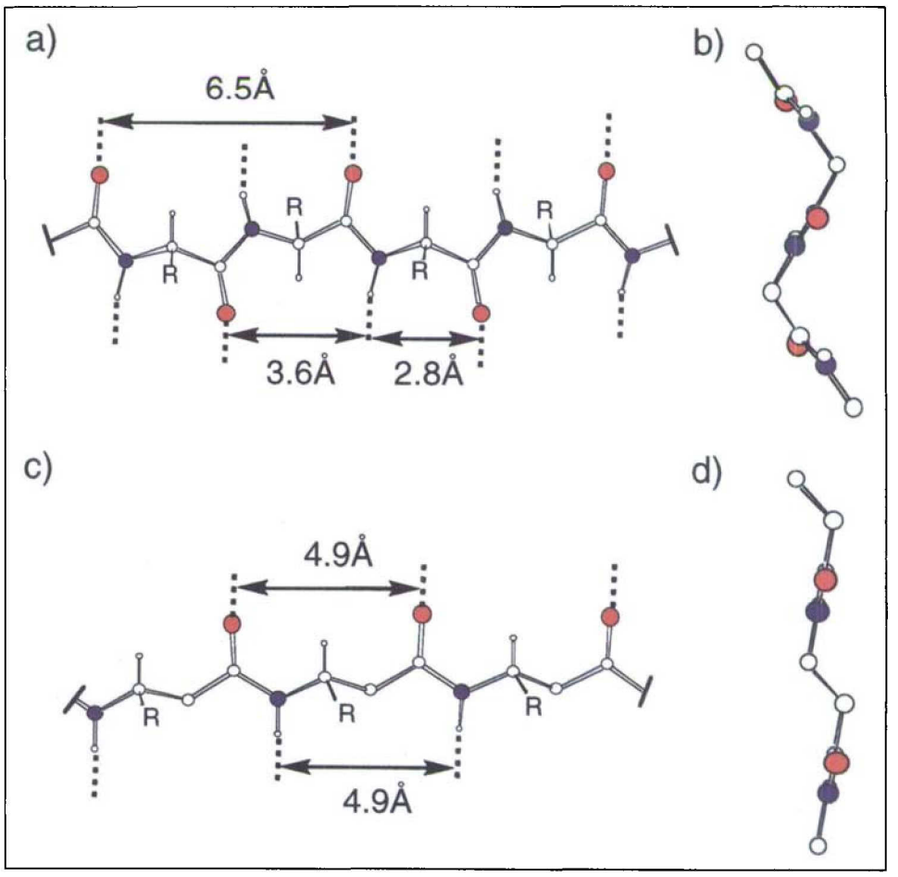

Fig. 3. *Comparison of the extended conformations of an* α*- and a* β*-peptide:* a) *side view;* b) *projection along the backbone of the extended conformation of an* α*-peptide* [15]; c) *side view;* d) *projection along the backbone of the extended conformation of a* β*-peptide* [5]. Clearly, the distances between the C=O and N–H groups involved in H-bond formation are different. Moreover, in the α-peptide, both the CO and NH groups point alternately up and down, and the R groups alternately forwards and backwards. In the β-peptide, all C=O are aligned in the same direction and this is also true for all the N–H and all the R groups.

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Table. *The Results of the Enzymatic Cleavage Experiments on the* β*-Peptides* **2–6**. With the exception of **2c**, which contains an N-terminal L-alanine and which was cleaved (10%) by **LAP**, all the β-peptides are stable at 25° for 2 d. Abbreviations for enzymes are explained in the text. For more detailed specifications see *Experimental*.

β-Peptide	Tested with
2a	LAP
2b	LAP
2c	LAP
2d	LAP
3	P, CT, T, E, CPA, LAP
4	P, CT, T, E, CPA, LAP
5	P, CT, E
6	T

With the standard substrates, the enzymes still exhibited at least 75% of their initial activity after this time.

Discussion

Clearly, any possible interactions between our β-peptides and the α-peptidic enzymes are insufficient for cleavage of the β-peptide. This is not astonishing, if the basis of 'recognition' of a substrate by a peptidase is considered – this being the formation of H-bonds and the three-dimensional environment found within the active site [14]. A subsequent comparison of the extended conformations that are required for the formation of pleated sheets (*Fig. 3*) and the helical secondary structures (*Fig. 4*) of α-peptides [15] and β-peptides [5–10] leads to the following conclusion:

1) The nature of the pairing of the NH and CO groups for H-bonds is different in each case.

2) The spacing between the side chains in both the extended and helical conformations are different.

3) The dipoles of the helices point in different directions with respect to the appropriate helix axis.

In other words, although both kinds of peptide have the possibility of adopting pleated sheet or helical conformations, these secondary structures are, in fact, very different in nature.

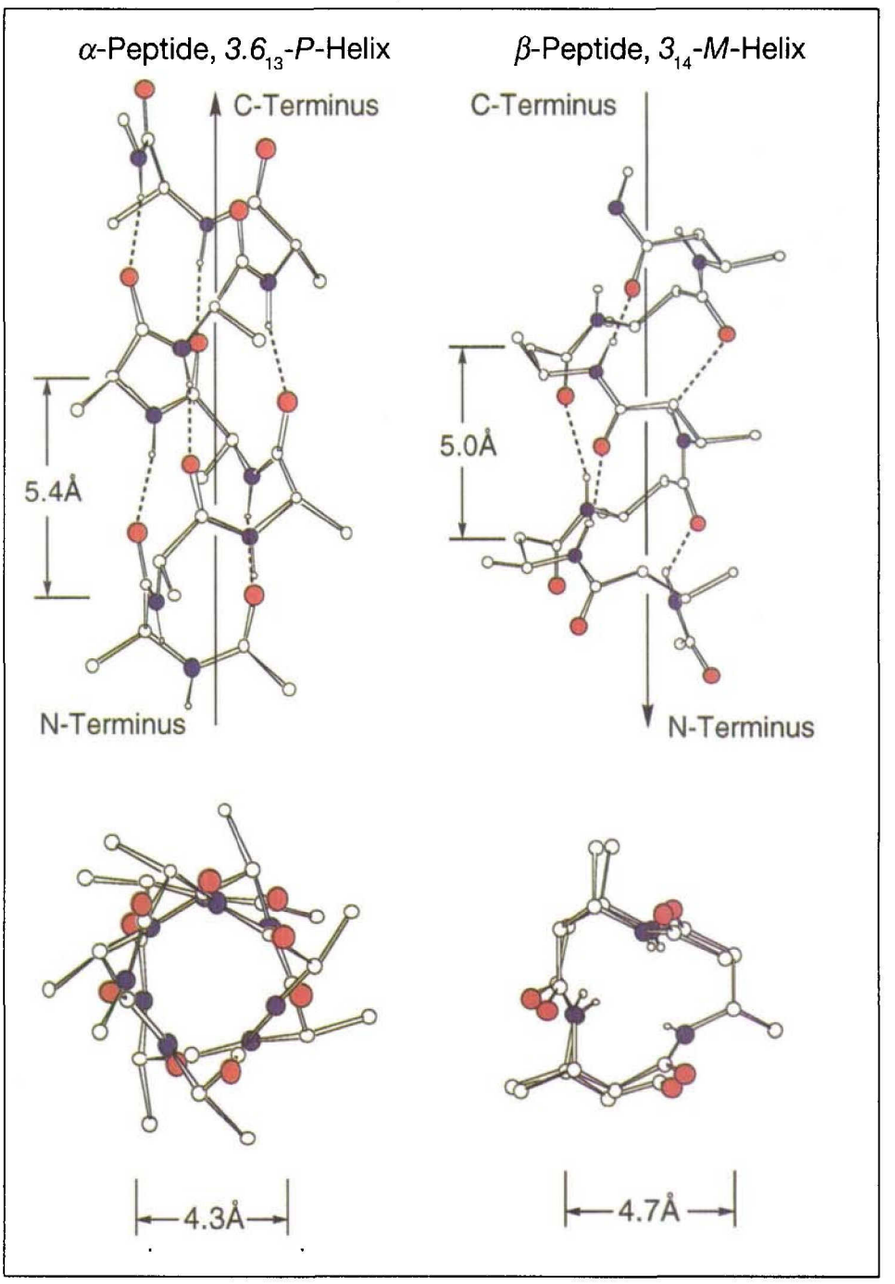

Fig. 4. *Helices formed by peptides of* L-α- (left, [15]) *and* L-β-*amino acids* (right, [5][6]). For the α-peptide, the 3.6_{13} or α-helix ((*P*)-helicity with 3.6 α-amino acids for a pitch of 5.4 Å) is shown. β-Peptides (from homologated L-α-amino acids) form 3_{14} helices (peptide nomenclature) or 3_1 helices (crystallographic nomenclature) with (*M*)-helicity (3 β-amino acids for a pitch of 5.0 Å). From the side view (upper), the opposite dipoles are evident. From the projections along the axes (lower), the differing diameters, profiles, and arrangements of the side chains in both helices are evident.

The potential uses of peptides with known secondary structures which are not degraded by enzymes and are constructed from nontoxic building blocks are immense.

Following the *in vitro* results described herein, there can now only be a sense of expectation about the likely outcome of *in vivo* tests on the bioavailability and toxicity of β-peptides and on the structure of complexes – if they exist at all!? – of β-peptidic antigens with α-peptidic antibodies.

Experimental

Ames *Tests*

Ames tests were performed under standard conditions [13]: Addition (50 μl/plate) of S9-liver homogenate from male rats pretreated with *Aroclor 1254*. The compounds **1a–h** were tested on the *Salmonella typhimurium* strain TA 100. In addition, **1a–c**, **1e–f**, and **1h** were tested on the strain TA 98.

Peptidase Experiments

The enzyme concentrations of the stock solns. were selected such that the standard substrates

(same concentration as the tested substrates) were totally cleaved after a maximum of 30 min. The enzymatic activity was checked spectrophotometrically immediately prior to the experiments.

General Procedure: 0.2 ml of a stock soln. of the substrate in H_2O (5 mg/ml) and 0.1 ml of the stock soln. of the enzyme were added to 2.7 ml of the particular buffer soln. The mixture was incubated at 25° for 2 d and analysed by RP-HPLC from time to time.

HPLC-Analysis: RP-HPLC Analysis was performed on a *Macherey-Nagel C8* column (*Nucleosil 100-5 C_8* (250 × 4 mm)) with a precolumn (*Nucleosil 100-5 C_8* (11 × 4 mm)). The samples were directly injected from the reaction mixture. Detection by measurement of the UV absorption at 218 nm. A mixture of CH_3CN/H_2O (0.1% trifluoroacetic acid; TFA) was used (25:75 to 15:85) as eluent, such that the retention time fell somewhere between 20 and 30 min.

Enzymes: *Pepsin* (EC 3.4.23.1) from porcine gastric mucosa (*Boehringer*, Mannheim). Buffer soln.: 1.74M AcOH pH 2.1. Stock soln. in 1.74M AcOH. Standard substrate: H-Val-Ala-Leu-Val-Ala-Leu-OH. *Chymotrypsin* (EC 3.4.21.1) from bovine pancreas (*Fluka*). Buffer soln.: 0.1M Tris buffer pH 8.0 (0.1M $CaCl_2$). Stock soln. in buffer. Standard substrate: *N*-Benzoyl-L-tyrosine-OEt. *Trypsin* (EC 3.4.21.4) from porcine pancreas (*Novo Industri*). Buffer soln.: 0.05M Tris buffer pH 8.2 (0.02M $CaCl_2$). Stock soln. in 1 mM HCl. Standard substrate: DL-Benzoyl-arginine-4-nitroanilide hydrochloride. *Elastase* (EC 3.4.21.36) from hog pancreas (*Fluka*). Buffer soln.: 0.1M Tris buffer pH 8.0. Stock soln. in 1 mM AcOH. Standard substrate: Suc-Ala-Ala-Ala-4-nitroanilide. Carboxypeptidase A (EC 3.4.17.1) from bovine pancreas (*Fluka*). Buffer soln.: 0.05M Tris buffer pH 7.5 (0.45M KCl). Stock soln. in buffer. Standard substrate: Carbobenzoxy-glycyl-L-phenylalanine. *Leucine-Aminopeptidase* (EC 3.4.11.1) from porcine kidney (*Sigma*). Buffer soln.: 0.05M Tris buffer pH 8.2. Stock soln. in 0.1 M Tris buffer (2.9M $(NH_4)_2SO_4$, 5 mM $MgCl_2$). Standard substrate: L-Leucine-4-nitroanilide.

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[1] O.W. Griffith, *Ann. Rev. Biochem.* **1986**, *55*, 855.

[2] K.C. Nicolaou, W.-M. Dai, R.K. Guy, *Angew. Chem.* **1994**, *106*, 38; *ibid. Int. Ed.* **1994**, *33*, 45.

[3] K.-I. Yoshida, Y. Minami, R. Azuma, M. Saeki, T. Otani, *Tetrahedron Lett.* **1993**, *43*, 2637.

[4] G. Cardillo, C. Tomasini, *Chem. Soc. Rev.* **1996**, 117.

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

[6] D. Seebach, P.E. Ciceri, M. Overhand, B. Jaun, D. Rigo, L. Oberer, U. Hommel, R. Amstutz, H. Widmer, *Helv. Chim. Acta* **1996**, *79*, 2043.

[7] T. Hintermann, D. Seebach, *Synlett*, in press.

[8] D. Seebach, J.L. Matthews, A. Meden, T. Wessels, C. Baerlocher, L.B. McCusker, *Helv. Chim. Acta* **1997**, *80*, 173.

[9] D.H. Appella, L.A. Christianson, I.L. Karle, D.R. Powell, S.H. Gellman, *J. Am. Chem. Soc.* **1996**, *118*, 13071.

[10] B.L. Iverson, *Nature (London)* **1997**, *385*, 113.

[11] J.L. Matthews, C. Braun, C. Guibourdenche, M. Overhand, D. Seebach, in 'Enantioselective Synthesis of β-Amino Acids', Chapt. 5, Ed. E. Juaristi, Wiley – VCH, Weinheim and New York, 1997, p. 105–126.

[12] B.N. Ames, L.S. Gold, *Angew. Chem.* **1990**, *102*, 1233; *ibid. Int. Ed.* **1990**, *29*, 1197, and refs. cited therein.

[13] D.M. Maron, B.N. Ames, *Mutation Research* **1983**, *113*, 173.

[14] L. Stryer, 'Biochemistry', 4th edn., Freeman, New York, 1995.

[15] G. Quinkert, E. Egert, C. Griesinger, 'Aspekte der Organischen Chemie/Struktur', Verlag Helvetica Chimica Acta, Basel, and VCH, Weinheim, 1995.