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Sequencing of β-Peptides by Mass Spectrometry

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Abstract. Seven β-peptides containing up to 18 β^2-, β^3- and $\beta^{2,3}$-amino acids have been subjected to ESI-tandem mass spectrometry (low-energy fragmentation, positive ions). From the fragment ions formed from the free β-peptides, as well as from the corresponding methyl esters (+14 U) and *N*-acetyl derivatives (+42 U), the known sequences of β-amino acids could be confirmed unambiguously with the program *Sherpa*. Thus, the commonly used MS-sequencing procedure for α-peptides can be adopted for β-peptides without modification. However, there are pronounced differences in the fragmentation patterns of the two types of peptides: the β-peptides disclose their relationship to *Mannich* bases in the mass-spectrometric experiment by the elimination of ammonia from the *N*-terminus (→ RCH=CH–CO–NH–R') and the occurence of retro-*Mannich* cleavage (*cf.* formation of HN=CHR + CH_3CO–NH–R' from β-amino-acid residues).

1. Introduction

Attempts to determine the sequence of β-peptides with an *Edman*-type sequencer under standard conditions have failed so far [2]. Apparently, the cyclization in the first step of the *Edman* degradation [3] leading to the thiazolinone **I** is more favorable than the cyclization which yields the six-membered ring analogue **II**, although heterocycles of this type, *e.g.*, **III**, are known to form readily in other cases (*Fig. 1*) [4]. In view of the growing interest in β-peptides [5], which are now being synthesized with increasing chain lengths, it is of utmost importance to have a quick, routine method for their sequencing, which would also be applicable to on-bead analysis of samples from solid-phase or combinatorial syntheses.

A method which is used most successfully for sequencing 'normal' α-peptides is the analysis of the fragments produced by collision-induced dissociation by tandem mass spectroscopy [6]. *A priori*, it was not obvious that this method could be applied to β-peptides, since their building blocks have an additional bond which might be broken (*cf.* **IV** and **V** in *Fig. 1*), and the increased number of fragmentation modes could lead to complications in the interpretation of the spectra.

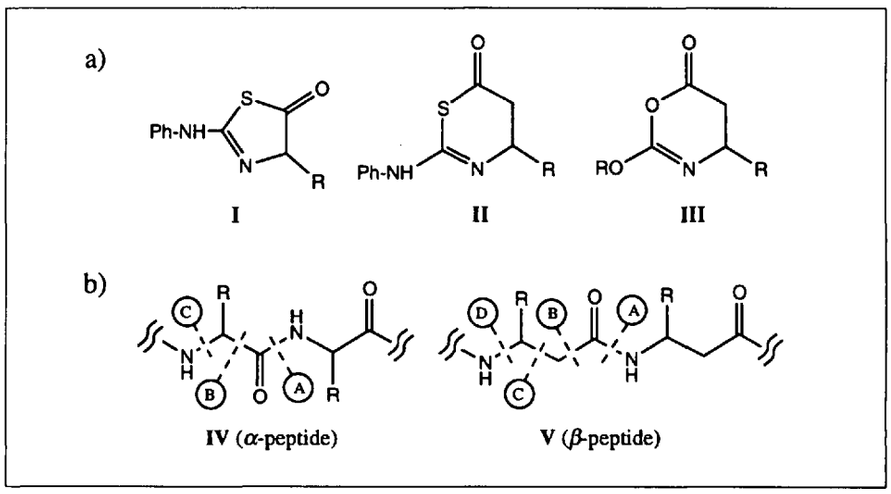

Fig. 1. *a) Heterocycles which are* (**I**) *or which should be* (**II**) *formed by* Edman *degradation of α- and β-peptides, respectively.* Heterocycle **III** formed from the ketene intermediate in the *Arndt-Eistert* homologation of carbonyl-protected α-amino acids. *b)* Possible fragmentations A, B, C of α- (**IV**) and A, B, C, D of β-peptides (**V**).

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For the present study, we employed several β-peptides of various structural types and sizes containing β^2-, β^3-, and $\beta^{2,3}$-amino-acid residues, functionalized side chains, and terminal or other protecting groups (*Fig. 2*, **1–7**) [7–10].

2. Sequencing of β-Peptides by Mass Spectrometry

For the sequencing experiments, we used the β-hexa- (**1**), β-hepta- (**2–5**), β-pentadeca- (**6**) and β-octadecapeptides (**7**). With the exception of **7** [7], the syntheses with full experimental detail of these compounds have been described previously by us [8–9], or are part of a publication to appear soon [10]. The β-peptides **1–7** contain the β-amino-acid building blocks β-HGly, (*R*)- and (*S*)-β^3-HAla, (*S*)-β^2-HAla, (*R*)-β^3-HVal, (*R*)-β^2-HVal, (*S*)-β^3-HLeu, (*R*)-β^2-HLeu, (*R*)- and (*S*)-β^3-HPhe, (*S*)-β^3-HLys, (*R*)-β^3-HThr, β-HAsp, H-(2*R*,3*S*)-$\beta^{2,3}$-HAla(α-Me)-OH, H-(2*R*,3*S*)-$\beta^{2,3}$-HVal(α-Me)-OH, H-(2*R*,3*S*)-$\beta^{2,3}$-HLeu(α-Me)-OH and (*S*)-β^2-HHop (for β-amino-acid abbrevations, see [9][11–13]). The molecular formulae of these β-amino acids, together with their symbols and masses, are listed in the *Table*.

The β-peptides **1–5** were esterified (methyl esters) at the *C*- and acetylated at the *N*-terminus and at the Lys side-chain amino groups (for experimental details, see *Sect. 4*). The β-pentadecamer **6** and the β-octadecamer **7** were esterified and *N*-acetylated only. After derivatization, the β-peptides and their derivatives were purified by analytical HPLC. Collected fractions containing the pure β-peptides or their derivatives were analyzed directly

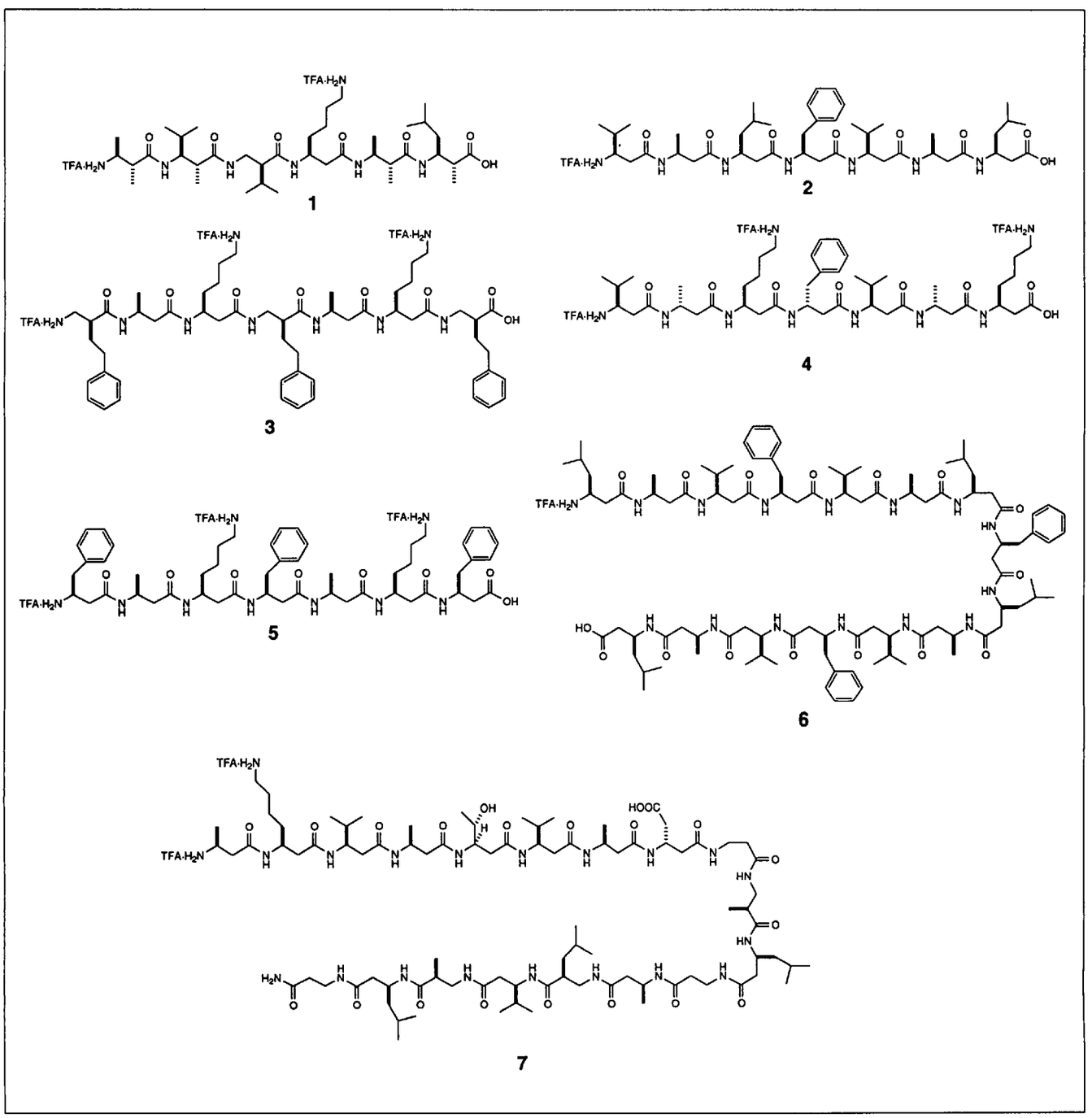

Fig. 2. *β-Peptides* **1–7**, *the primary structure of which was determined by mass-spectrometric sequencing* (TFA = trifluoroacetic acid)

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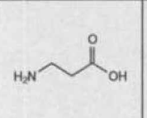
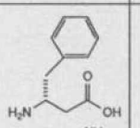
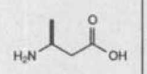
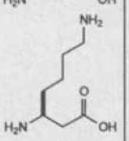
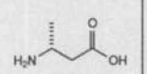
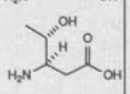
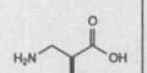
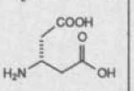
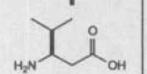
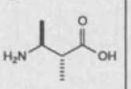
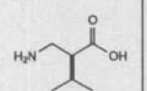
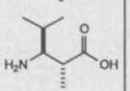
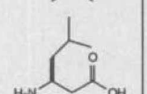
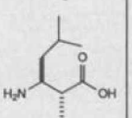
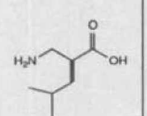
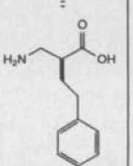
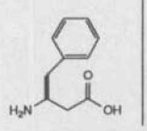
by electrospray ionization (ESI)-MS and ESI-MS/MS. The ester of β-peptide **6** and the acetyl derivatives of **4**, **5**, and **7** are too hydrophobic to be separated by reversed-phase chromatography. They were only desaltet over *POROS R2* column material prior to analysis. The β-peptides **3**, **6**, and **7** were analyzed without further purification. We have 19 spectra recorded, and they could all be fully interpreted to confirm the known primary structures of the β-peptides **1**–**7**. In this paper, the sequencing of two representative β-peptides, the β-hexa- and the β-heptapeptide **1** and **2**, respectively, is described in detail [14].

The solution of the β-peptides **1** and **2** and of their *N*-acetyl and ester derivatives derived from HPLC separation were analyzed by electrospray ionization. The spectra obtained are shown in *Figs. 4* and *5*. The nomenclature for presentation of the fragmentation pattern was adopted from the system used for α-peptides, which was originally proposed by *Roepstorff* and *Fohlman* [15] and later modified by *Biemann* [16], (see *Fig. 3*). Fragmentation patterns of the β-peptides and their esterified and acetylated derivatives were simulated using the program *Sherpa* [17]. The calculated masses of all possible y- and b-ions were compared with the experimental data. In *Fig. 4* and *5*, the cleavage patterns of the β-peptides **1** and **2**, of their methyl esters, and of the acetyl derivatives are shown, together with their interpreted MS/MS-spectra.

We observed the following β-peptide backbone cleavage modes: *i*) cleavage of the CO–NH amide bonds; *ii*) loss of NH_3 (17 U) from ions containing a free β-

Table. β-Amino Acids with Their Abbreviations and Masses Used as Building Blocks for the Investigated β-Peptides. For the abbreviations of the various β-amino acids, see [9][11–13].

β-amino acid	abbreviation	mass	β-amino acid	abbreviation	mass
	H-β-HGly-OH	89.09		H-(*R*)-β^3-HPhe-OH	179.22
	H-(*S*)-β^3-HAla-OH	103.12		H-(*S*)-β^3-HLys-OH	160.21
	H-(*R*)-β^3-HAla-OH	103.12		H-(*R*)-β^3-HThr-OH	133.15
	H-(*S*)-β^2-HAla-OH	103.12		H-β^3-HAsp-OH[a]	147.13
	H-(*R*)-β^3-HVal-OH	131.17		H-(2*R*,3*S*)-β^{2,3}-HAla(α-Me)-OH	117.15
	H-(*S*)-β^2-HVal-OH	131.17		H-(2*R*,3*S*)-β^{2,3}-HVal(α-Me)-OH	145.20
	H-(*S*)-β^3-HLeu-OH	145.20		H-(2*R*,3*S*)-β^{2,3}-HLeu(α-Me)-OH	159.23
	H-(*S*)-β^2-HLeu-OH	145.20		H-(*S*)-β^2-HHop-OH	193.24
	H-(*S*)-β^3-HPhe-OH	179.22			

[a]) This amino acid itself is achiral, when incorporated in a β-peptide, its stereogenic center has (*R*)- or (*S*)-configuration; **7** contains the (*S*)-enantiomer [7].

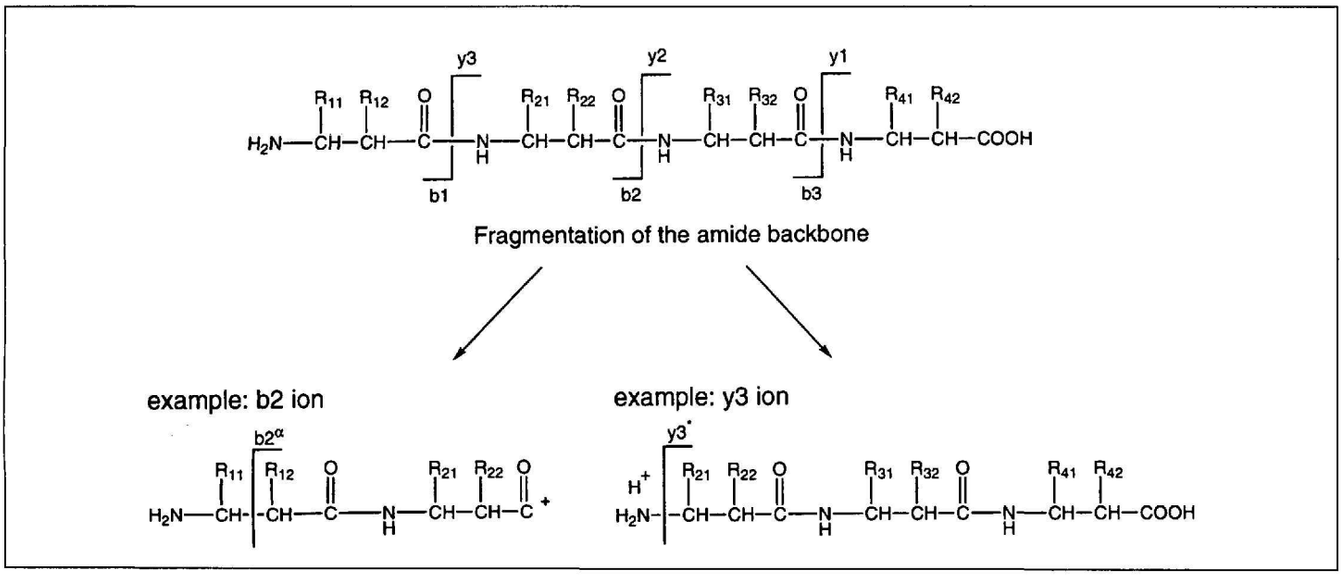
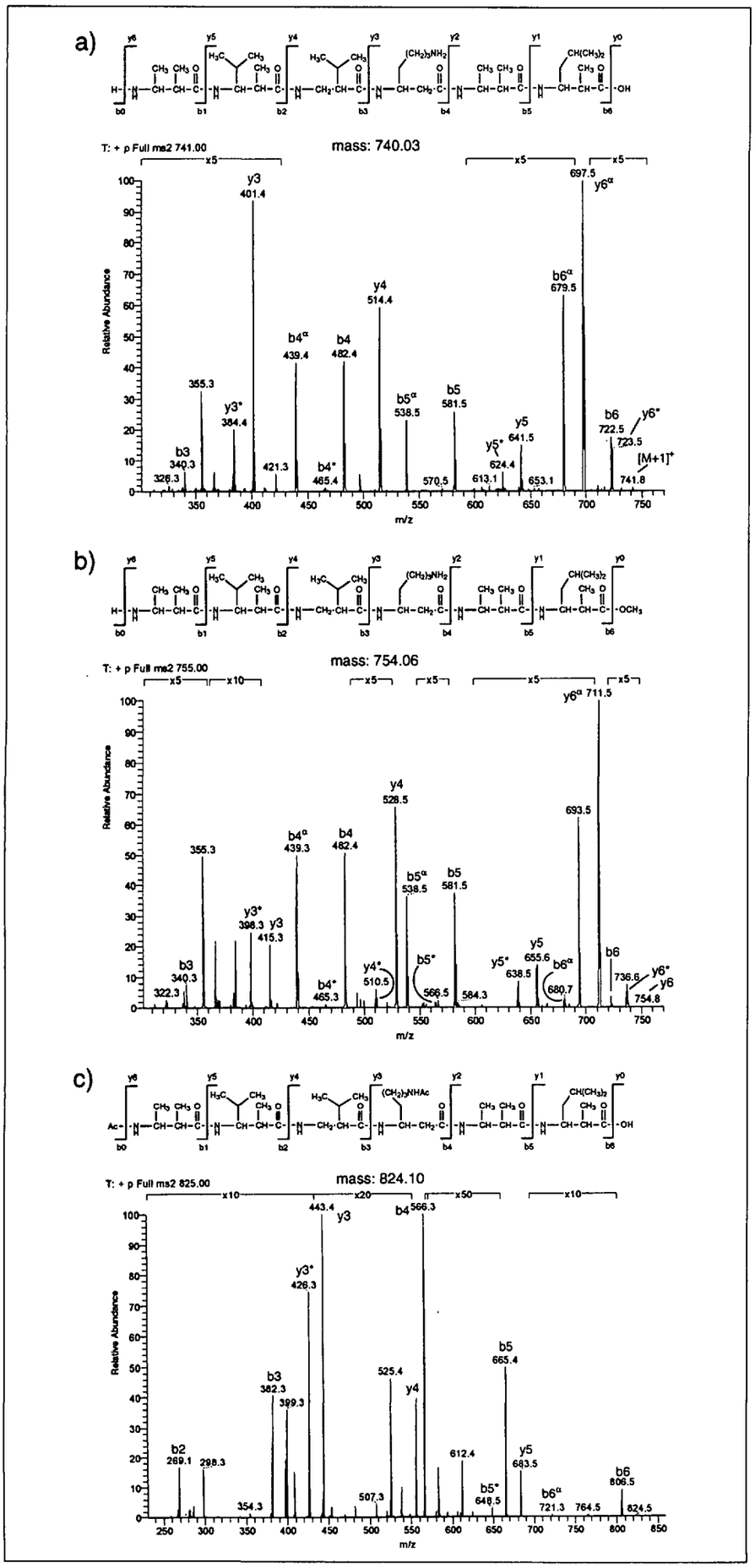


Fig. 3. *Fragmentation nomenclature for β-peptides.* The loss of ammonia (17 U) is marked with a star (*). Fragment ions, formed by cleavage of the C(2)–C(3) bond in β-amino-acid residues, are indicated by the superscript alpha ($^{\alpha}$).



amino group, with formation of ions with an *N*-terminal enoyl moiety (R–CH=CH–CO–NH–R', *cf.* the elimination of R_2NH from a *Mannich* base [18]); *iii*) cleavage of the C(2)–C(3) bond in the *N*-terminal β-amino-acid residues, with formation of CH_3CO–NH–R' and RCH_2–CO–NH–R' fragment ions (from β^3- and β^2- or $\beta^{2,3}$-amino acid residues, respectively; a kind of retro-*Mannich* reaction with loss of HN=CHR [19]).

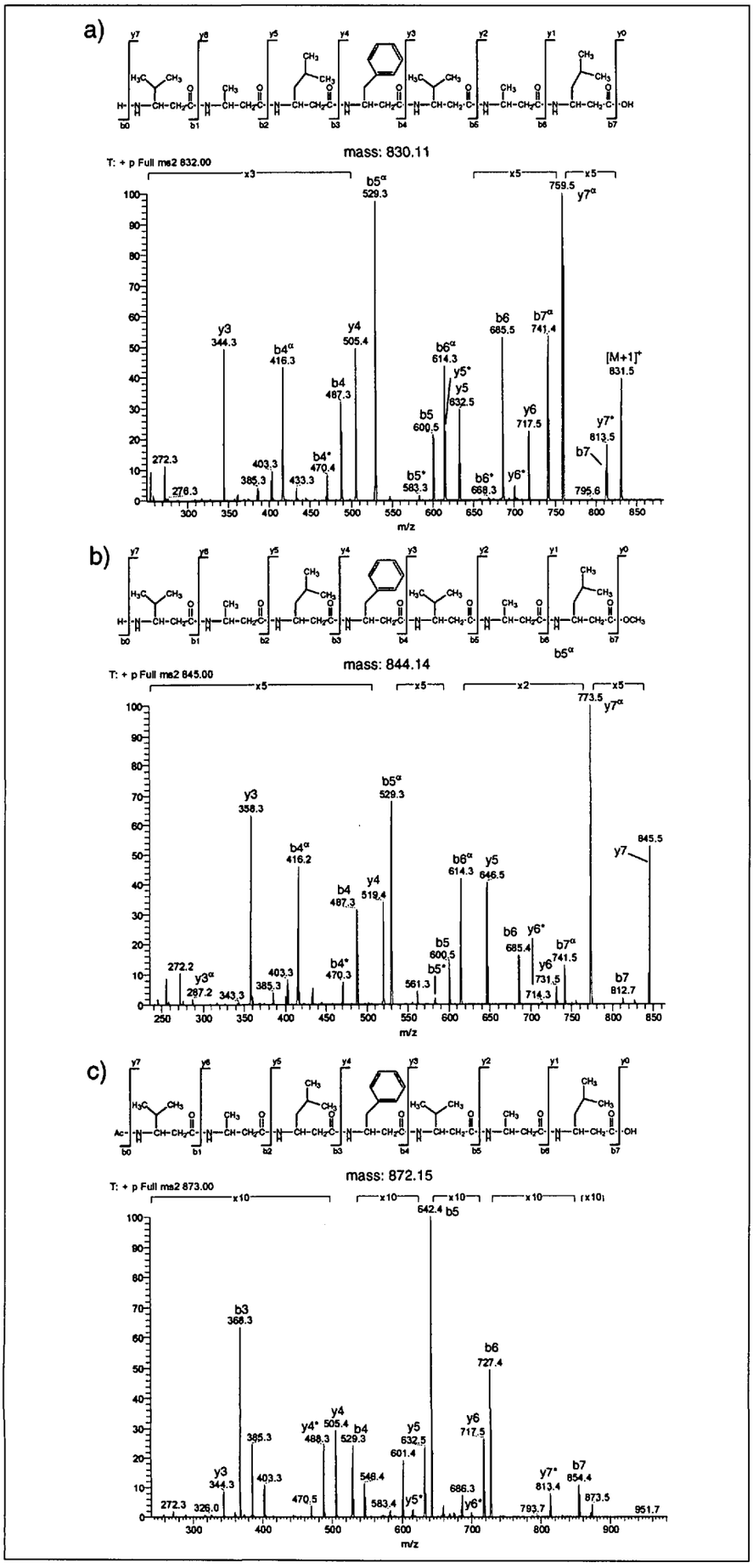
The masses of the methyl ester y-ions are increased by 14 U, and the masses of the acetylated b-ions are increased by 42 U per introduced acetyl group, as compared to the corresponding ions resulting from the free β-peptides.

As expected (*Sect. 1*), a much larger number of peaks is observed in the spectra of β-peptides, as compared to the α-peptidic counterparts. However, most of the fragment ions can be assigned and the sequence determined (see the systematic procedure described in the caption of *Fig. 4*). In this way, even the sequence of the largest β-peptide **7**, which contains 18 β-amino-acid residues, could be derived from the mass spectrum [14]. It must be realized, however, that under the low-energy fragmentation conditions used, it is not possible to distinguish β^2- and β^3-amino-acid residues in the sequencing procedure. Also, it is obvious that the configuration of the amino-acid moiety in the β-peptide chain cannot be determined by MS analysis.

3. Discussion and Conclusion

The mass-spectrometric sequencing of β-peptides, described here, is yet another methodology which can be adopted, essentially without adjustments, from the field of α-peptides. We have previously shown that other standard techniques of α-peptide chemistry can also be directly applied to β-peptides: the protecting groups

Fig. 4. *The MS/MS spectra of the free (a), the esterified (b), and the acetylated (c) β-peptide* **1**. The ions can be assigned to b- and y-ion series by comparing the three spectra. The y- and b-ions can be distinguished since all yy-ions shift by 14 U after esterification and all b-ions by 42 U after acetylation, respectively. The sequence of the β-peptide can be obtained from the mass differences between the ions of the same series since they correspond to the residue mass of the amino acid that occupies that position (*cf.* the masses listed in the *Table*).



(Cbz, Boc, Fmoc), reagents (EDC/Et_3N or BOP/HOBt) and conditions ($CHCl_3$/DMF) for coupling, the protecting groups for side-chain functionalities, RP-HPLC purification, the resins (*Wang*, *o*-Cl-trityl) for manual (and automated [20]) solid-phase synthesis, as well as molecular-dynamics programs for calculations of solution structures (*GROMOS 96*). Thus, a host of knowledge and experience available in the field of peptide chemistry can be used to make and study the unnatural β-peptides, which are otherwise so fundamentally different from their counterparts, the α-peptides [5].

4. Experimental

Derivatization of 1–7

a) *Esterification*: A standard soln. of 3M methanolic HCl was prepared by adding (dropwise with stirring) 800 µl of acetyl chloride (*Fluka*) to 1 ml of dry MeOH (*Fluka*). After stirring for 5 min, the β-peptides **1** (162 µg), **2** (144 µg), **3** (70 µg), **4** (224 µg), **5** (163 µg), and **6** (223 µg) were added each to 400 µl of the reagent. After standing for 1 1/2 h at r.t., the solvent was evaporated under reduced pressure. The crude product was either purified by RP-HPLC or desalted over *POROS R2* reversed-phase packing material. The *POROS* material was packed into the tip of a pulled glass capillary. The diameter of the tip is usually less than 10 µm so that the reversed-phase beads pack easily. The β-peptides were loaded in aq. AcOH (5% *v/v*), washed with the same solvent with a few column volumes and eluted into 3 column volumes of $MeOH/H_2O/AcOH$ 60:39:1. *b*) *Acetylation*: The acetylation reagent was prepared by adding 500 µl Ac_2O (*Fluka*) to 1.5 ml MeOH (*Fluka*). Each of the β-peptides **1** (122 µg), **2** (195 µg), **3** (100 µg), **4** (330 µg), **5** (106 µg), and **7** (80 µg) was treated with 200 µl of the reagent, with 5 µl of aq. 1M $NaHCO_3$ soln., and with 76 µl of H_2O. After standing for

Fig. 5. *The MS/MS spectra of the free (a), the esterified (b), and the acetylated (c) β-peptide* **2**. For the systematic procedure to obtain the primary structure of the β-peptide, see the caption of *Fig. 4*.

1 1/2 h at r.t., the solvent was evaporated under reduced pressure. The crude product was either purified by HPLC or desalted over *POROS R2* column material.

HPLC Purification

RP-HPLC Purification was performed on a *Macherey-Nagel* C_{18}-column (*Nucleosil 100-5* C_{18} (250 × 4 nm)), with an analytical *Knauer* HPLC system (pump type *64*, *EuroChrom 2000*, integration package, degasser, UV detector with variable-wavelength monitor). The free β-peptides **1**, **4**, and **5**, the methyl esters of **1** and **3–5**, and the acetyl derivatives of **1** and **3** were separated using gradient *1* of solvent *A* (0.1% CF_3COOH in H_2O) and *B* (MeCN). The free β-peptide **2**, the methyl ester of **2**, and the acetyl derivative of **2** were purified using gradient *2* of solvent *A* and *B*. Gradient *1*: 0% *B* for 5 min, within 35 min to 40 % *B*, within 5 min to 75% *B*, within 5 min to 80% *B* and up to 95% *B* in another 1 min. Gradient *2*: 0% *B* for 5 min, within 35 min to 40 % *B*, within 5 min to 75% *B*, within 5 min to 80% *B*, and up to 100% *B* in another 1 min. Detection by measurement of the UV absorption at 220 nm. For all separations, the flow rate was 1 ml/min.

ESI-MS/MS Analysis

Electrospray-ionization mass spectrometry was carried out on a *Finnigan LCQ* ion-trap instrument equipped with a modified ion-spray source (*New Objectives*). The carrier solvent was MeOH/H_2O/AcOH 60:39:1, and the flow rate was 0.7 µl/min. The soln. derived from the HPLC separations of the free β-peptides **1**, **2**, **4**, and **5**, the methyl esters of **1–5** and the acetyl derivatives of **1–3** were directly injected into the spectrometer. The free β-peptides **3**, **6**, and **7** were first dissolved in the carrier solvent, and the methyl ester of **6**, as well as the acetyl derivatives of **4–7** were subjected as solns. derived from desalting over *POROS R2* column material. The spray voltage was 3.5 kV. Ion isolation was performed with a mass window of 3.0 V. MS/MS-Spectra were accumulated using a relative collision energy of 30–40% in the profile data mode. 7–20 Scans were averaged to yield the spectra (see *Figs. 4*, *5*, and [14]).

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[18] So far, we have observed such eliminations in one case only (treatment of β-peptides with *t*-BuLi!). (T. Hintermann, C. Mathes, D. Seebach, *Eur. J. Org. Chem.* **1998**, 2379). On the other hand, we were able to sublimate a *N*-Boc-protected β-hexapeptide methyl ester at 180°/10^{-5} Torr, without decomposition (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).
[19] Loss of CO (28 U) was not observed under the conditions used by us. It is of course much less likely for an R–NH–CHR'–CH_2–$CO^{\oplus}$ ion to lose CO from a β-peptide than for an R–NH–CHR'–$CO^{\oplus}$ ion from an α-peptide (retro-*Koch* reaction).
[20] See, *e.g.*, R.E. Marti, K.H. Bleicher, K.W. Bair, *Tetrahedron Letters* **1997**, *38*, 6145.