Amino Acids (1996) 11: 425–434

Amino
Acids

Peptide modification by incorporation of α-trifluoromethyl substituted amino acids

Review Article

B. Koksch[1], **N. Sewald**[2], **K. Burger**[2], and **H.-D. Jakubke**[1]

[1]Department of Biochemistry and [2]Department of Organic Chemistry, University of Leipzig, Leipzig, Federal Republic of Germany

Accepted March 8, 1996

Summary. Metabolic stabilization of pharmacologically active peptides can be achieved by incorporation of sterically hindered non-natural amino acids, e.g. $C^{\alpha,\alpha}$-disubstituted amino acids. α-Trifluoromethyl substituted amino acids, a subclass of $C^{\alpha,\alpha}$-disubstituted amino acids, also fulfil this requirement while featuring additional properties based on the electronic influence of the fluorine substituents.

This review summarizes the results concerning the stability of peptides containing α-TFM amino acids towards proteolysis by α-chymotrypsin. Furthermore, configurational effects of α-TFMAla on the proteolytic stability of peptides are explained using empirical force field calculations. The influence of α-TFMAla incorporation on the secondary structure of selected tripeptide amides is compared to the effects exerted by its fluorine-free analogue, aminoisobutyric acid.

Finally, results on metabolic stabilization and biological activity of modified thyrotropin releasing hormone are interpreted.

Keywords: α-Trifluoromethyl substituted amino acids – α-Chymotrypsin – Proteolytic stability – $C^{\alpha,\alpha}$-Disubstituted glycines – TRH

1 Introduction

Some major disadvantages for the application of peptides as pharmaceutics are their low bioavailability, their sensitivity to enzymatic degradation, and their low selectivity because of the considerable conformational flexibility leading to interactions with different receptors (Hölzemann, 1991; Miller et al., 1994; Gillmor and Cohen, 1993). Therefore, investigation of the biological properties and three-dimensional structure of peptides rich in the conformationally restricted $C^{\alpha,\alpha}$-disubstituted amino acids is of current

interest. Certain $C^{\alpha,\alpha}$-dialkylated amino acids have been shown to impart well defined and predictable conformations to the peptide backbone (Rizo and Gierasch, 1992; Valle et al. 1991; Bindra and Kuki, 1994; Altmann et al., 1992). These amino acids, especially α-methylalanine (α-aminoisobutyric acid; Aib) are present in naturally occurring peptide antibiotics (Matha et al., 1992) conferring on them a stable helical secondary structure and thereby, ion transporting properties. Furthermore, peptides containing these residues tend to dramatically slow down degradation processes (Toniolo et al., 1991).

α-Trifluoromethyl substituted amino acids (α-TFM amino acids) constitute a special class of $C^{\alpha,\alpha}$-disubstituted amino acids due to the unique electronic properties of fluorine substituents. Therefore, they are interesting building blocks for peptide synthesis (Sewald and Burger, 1995). A trifluoromethyl group in α-position of an amino acid exerts considerable polarization effects on the neighbouring substituents. This structural alteration influences the hydrolytic stability of peptides containing TFM amino acids resulting in retarded degradation by peptidases (Burger et al., 1993) and, consequently, in prolonged intrinsic activity. The often postulated quasi-isosterism between a methyl and a trifluoromethyl group is still controversial (Seebach, 1990). The steric requirement of a trifluoromethyl group seems to be closer to that of an isopropyl than a methyl group. Hence, upon incorporation of α-TFM amino acids, severe conformational restrictions are exerted on the peptide chain. Furthermore, due to the high electron density, the trifluoromethyl group is capable of participating in hydrogen bonding as a proton acceptor. In contrast to an α-methyl group this property enables α-TFM substituted peptides to interact additionally with enzyme or receptor subsites.

2 Proteolytic stability of α-TFM amino acid derivatives

2.1 Enzymatic hydrolysis of N-protected α-TFM amino acid esters

Proteases like subtilisin, α-chymotrypsin or papain accept α-TFM amino acid esters only to a very limited extent (Burger et al., 1993). Both the hydrolysis rate and the turnover decrease in the order Z-(α-TFM)Gly-OMe > Z-(α-TFM)Ala-OMe > Z-(α-TFM)Leu-OMe. Z-(α-TFM)Phe-OMe is not turned over at all (Fig. 1). These data exclude the application of proteases in the resolution of enantiomeric α-TFM amino acid derivatives except in the case of Z-(α-TFM)Gly-OMe (Koksch et al., manuscript in preparation).

2.2 Protease catalyzed peptide synthesis using α-TFM amino acid esters as substrates

Dipeptide esters with an N-terminal TFM amino acid are accepted as substrates by proteolytic enzymes (Fig. 1). For example, H-(α-TFM)Phg-Phe-OMe is hydrolyzed by α-chymotrypsin or subtilisin within short reaction times

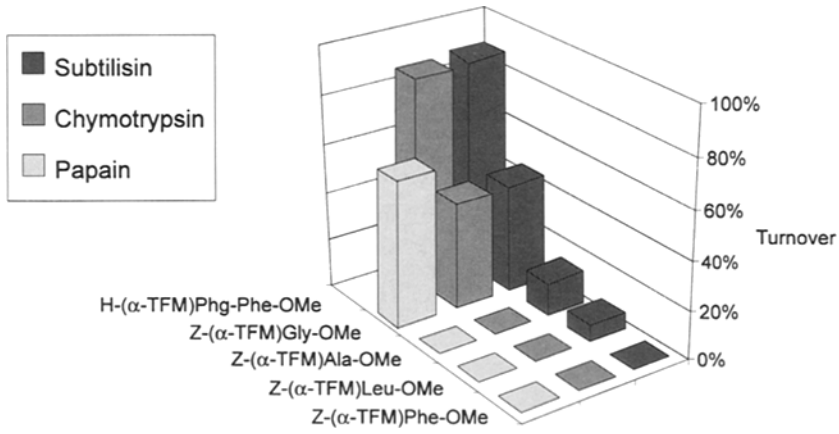

Fig. 1. Enzymatic hydrolysis of N-protected α-TFM amino acid esters

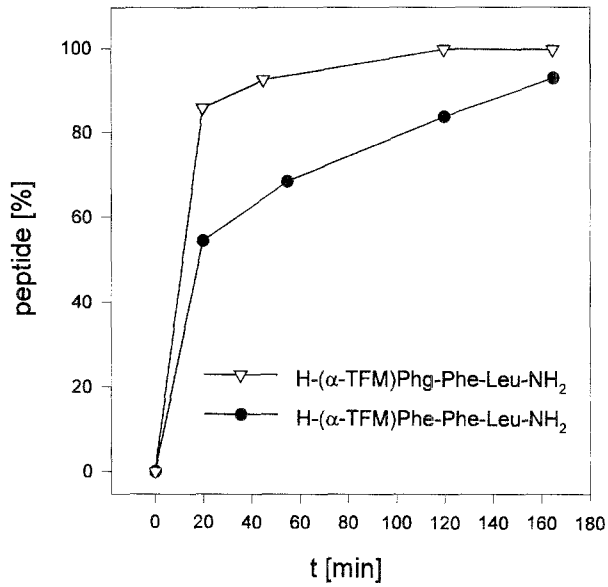

Fig. 2. Enzymatic peptide synthesis using N-unprotected α-TFM amino acid esters as substrates

to give the unprotected dipeptide. H-(α-TFM)Phg-Phe-OMe and H-(α-TFM)Phe-Phe-OMe are converted by α-chymotrypsin in the presence of H-Leu-NH_2 to give the tripeptides H-(α-TFM)Phg-Phe-Leu-NH_2 and H-(α-TFM)Phe-Phe-Leu-NH_2, respectively (Fig. 2) (Koksch, 1995). Stereoselective aminolysis of H-(R)-(α-TFM)Phe-(S)-Tyr-OMe by peptide amides can be achieved using cryoenzymatic reaction conditions (Jakubke, 1994; Gerisch, 1996). Thus, several tachykinin analogs containing α-TFM amino acids have been synthesized by enzymatic fragment condensation (Koksch, 1995).

Consequently, the interaction between substrate and enzyme can be influenced by incorporation of an α-TFM amino acid into peptides, depending on both the enzyme and the position of the substitution.

3 Proteolytic stability of α-TFM amino acid substituted model peptides

The serine protease α-chymotrypsin is one of the best investigated proteases with respect to its catalytic mechanism, its substrate specificity, and its three-dimensional structure (Fersht, 1985). Therefore, it represents an ideal model protease for the investigation of the proteolytic stability of peptides chemically modified by incorporation of non-natural amino acids.

A series of peptides was designed and synthesized by solution methods in order to compare α-TFM substituted peptides with Aib substituted and unsubstituted peptides, respectively (Fig. 3). These model peptides all contain an aromatic amino acid at P_1 position (P-nomenclature according to Schechter and Berger, 1967) with respect to the known sequence specificity of α-chymotrypsin (Schellenberger et al., 1990). Furthermore, these peptides are substituted by an α-TFM amino acid or Aib, respectively, in various positions relative to the preferred cleavage site of the protease used (in the range P_3-P'_2). Qualitative determination of the hydrolysis rates directly reveals the influence of an α-TFM amino acid substitution in a specific position of the peptide on its proteolytic stability. Comparison with peptides containing the fluorine-free disubstituted amino acid Aib (α-aminoisobutyric acid) allows to separate electronic from steric effects (Koksch et al., 1996a).

The unsubstituted model peptides as reference standards are proteolyzed rapidly. In contrast, all α-methyl and α-TFM containing peptides show considerable proteolytic stability, depending on the position of the substituted amino acid in the peptide. At P_1-position substituted peptides remain essentially unaffected by the protease. Substitutions at P_2 result in a significantly increased proteolytic stability towards enzymatic hydrolysis compared to the unsubstituted reference. Even for peptides substituted at P_3 a diminished rate of degradation is observed. Substitutions at P'_2 position show nearly the same effect on the proteolytic behaviour of peptides as substitutions at P_2. There is

cleavage site ↓

	P_3	P_2	P_1	P_1'	P_2'	
Z	**(α-TFM)Ala**	Ala	Phe	Leu	NH_2	
Z	**Aib**	Ala	Phe	Leu	NH_2	
	Z	**(α-TFMAla)**	Phe	Leu	NH_2	
	Z	**Aib**	Phe	Leu	NH_2	
		Z	**(α-TFM)Phe**	Leu	NH_2	
		Z	**(α-CH_3)Phe**	Leu	NH_2	
		Z	Phe	**(α-TFM)Ala**	Ala	NH_2
		Z	Phe	**Aib**	Ala	NH_2
		Z	Phe	Leu	**(α-TFM)Ala**	NH_2
		Z	Phe	Leu	**Aib**	NH_2

Fig. 3. Sequences of the α-TFM and α-methyl substituted model peptides

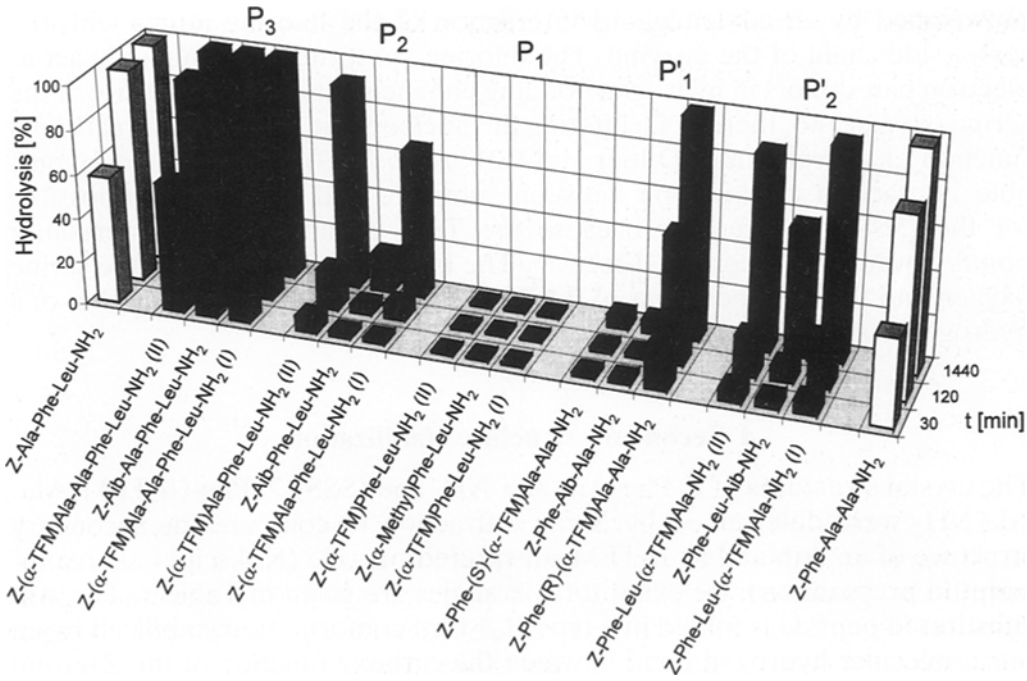

Fig. 4. Results of the proteolysis studies

a significant difference between α-methyl and α-TFM substituted peptides in the sense that the former show a stronger retardation of proteolysis in all positions examined (Fig. 4).

Comparison of the proteolysis data of all α-TFM substituted peptides reveals that not only the position of the substitution but also the absolute configuration of the α-TFM amino acid significantly influences the proteolytic stability of peptides. The strongest influence of the configuration is detected for the diastereomeric model peptides containing an α-TFM amino acid in P'_1-position. While the (SRS)-diastereomer is hydrolyzed rapidly, the (SSS)-diastereomer shows an extraordinary proteolytic stability similar to that of the Aib substituted peptide. This surprising effect can not be explained by the steric constraints of the TFM group (Seebach, 1990). The steric bulk of a TFM group seems to be close to that of an isopropyl group. Moreover, the hydrolysis rate of the (SRS)-configured TFM substituted diastereomer compares to that of the alanine containing reference peptide. The different proteolytic stability of the diastereomers is based on a specific interaction between the substrate and the enzyme induced by the high electron density of the TFM group.

A qualitative explanation for this phenomenon is given by empirical force field calculations (CHARMm 22 force field, Quanta) using the α-chymotrypsin/phenyl boronic acid complex taken from the Brookhaven Protein Data Bank (Tulinsky and Blevins, 1987). Analysis of the energy minimized complex between α-chymotrypsin and the (SRS)-diastereomer clarifies that the steric constraints exhibited by the α-TFM group can be

outweighed by an advantageous interaction of the fluorine atoms with the serine side chain of the enzyme. The fluorine substituents presumably act as electron pair donors in hydrogen bonding enhancing the nucleophilicity of the serine oxygen and, thereby facilitating the nucleophilic attack on the carbonyl function of the substrate (Dutler and Bizzozero, 1989). In contrast, a favourable interaction of this type between substrate and enzyme is impossible for the (SSS)-diastereomer because the TFM group in this diastereomer points towards the opposite direction. The large distance between the serine oxygen and the fluorine atoms of the TFM group prevents the formation of a hydrogen bond.

4 Secondary structure stabilization

The crystal structures of Z-Phe-Aib-Ala-NH_2 and (SSS)-Z-Phe-(α-TFM)Ala-Ala-NH_2 were determined by X-ray diffraction to compare the secondary structure of an Aib and an α-TFM substituted peptide (Koksch et al., manuscript in preparation). Relevant torsion angles are given in Table 1. The Aib substituted peptide is folded in a type II β-turn conformation stabilized by an intramolecular hydrogen bond between the carboxy function of the Z-group and the amino function of Ala. The backbone torsion angles of the TFM substituted peptide show a remarkable similarity to that of the Aib substituted peptide. The values are comparable to those found for an ideal β II-turn. Therefore, the incorporation of an (α-TFM)Ala residue into a bioactive peptide might result in a significant stabilization of a type II β-turn. Further investigations on the solution conformation of α-TFM substituted peptides are in progress.

Table 1. Relevant torsion angles from X-ray structure analysis of the two model peptides Z-Phe-Aib-Ala-NH_2 and (SSS)-Z-Phe-(α-TFM)Ala-Ala-NH_2 in comparison to that of an ideal β-turn

Angle	Ideal β-turn	Z-Phe-Aib-Ala-NH_2	(SSS)-Z-Phe-(α-TFM)Ala-Ala-NH_2
ϕ^{i+1}	−60	−50.8	−59.2
ψ^{i+1}	120	132.5	133.0
ϕ^{i+2}	80	65.7	71.3
ψ^{i+2}	0	16.6	13.7

5 TRH modification

We started to incorporate α-TFM amino acids into functional relevant positions of biological active peptides to investigate the steric, electronic, and hydrophobic effects of a trifluoromethyl group on proteolytic stability and biological activity (Koksch et al., 1996b).

A peptide structurally as simple as TRH (thyrotropin releasing hormone: pGlu-His-Pro-NH_2) (Fig. 5), with high biological activity as well as pronounced specificity, represents a model of choice for the synthesis of ana-

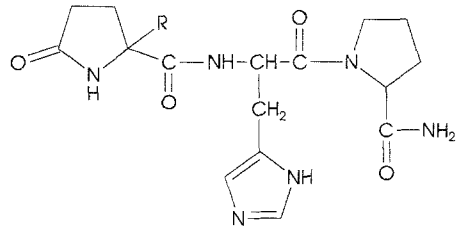

Fig. 5. Structure of TRH; R = H: native peptide, R = TFM: [(α-TFM)pGlu[1]]-TRH

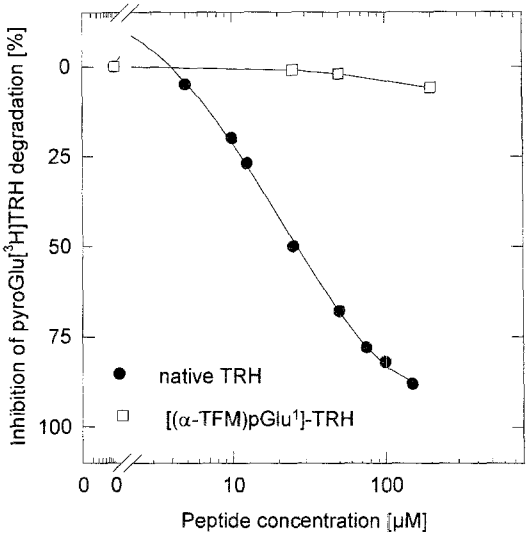

Fig. 6. Metabolic stability of [(α-TFM)pGlu[1]]-TRH to membrane-bound TRH degrading ectoenzyme testing the inhibition rate of pGlu[^{3}H]TRH degradation (Bauer et al., 1990)

logues to study structure-activity-relationships. TRH is the central stimulator of TSH (thyroid stimulating hormone) secretion by anterior pituitary cells and is regulated by peripheral and pituitary hormone levels (Ladram et al., 1994). TRH is used for treatment of various neurologic and neuropsychiatric disorders. The degradation of TRH in vivo is initiated by pyroglutamyl aminopeptidase II which selectively cleaves the pGlu-His bond (Bauer et al., 1981).

Substitution of pGlu by (α-TFM)pGlu at position 1 of TRH results in complete resistance to proteolysis by pyroglutamyl aminopeptidase II (Fig. 6).

However, the introduction of the strong electron withdrawing trifluoromethyl group influences the charge distribution and the three-dimensional conformation and, therefore, the interaction between substrate and receptor profoundly. As shown in Fig. 7 the receptor affinity of the trifluoromethyl substituted analogue is two to three orders of magnitude less than of the native compound.

The polarization effect of the α-TFM group obviously decreases the capacity of the pGlu residue to form a hydrogen bond between the pGlu

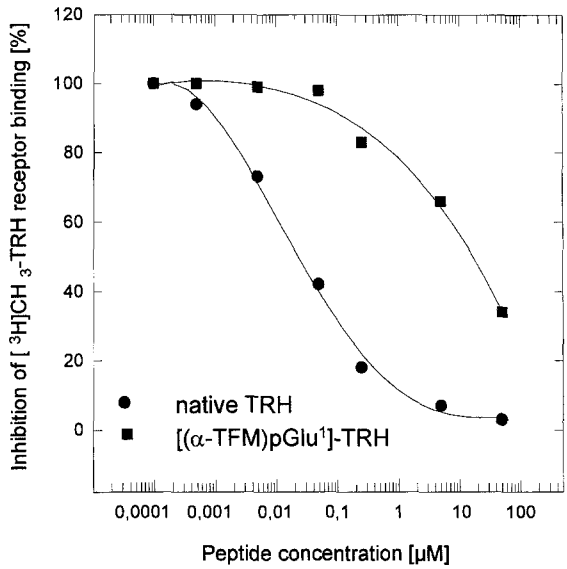

Fig. 7. Inhibition of receptor binding of [^{3}H]CH_3-TRH to Wistar rat anterior pituitary cells by native TRH and [(α-TFM)pGlu^1]-TRH (Bauer et al., 1990)

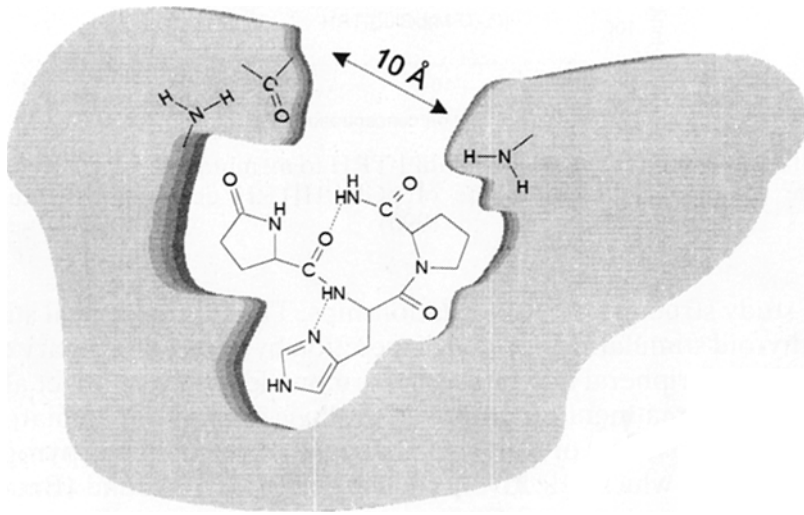

Fig. 8. Model of the interaction between the TRH-molecule and the receptor (Grant et al., 1972)

carboxy function and the amino function of the Pro moiety (Fig. 8). Comparison of the NMR shift values of the native sequence and the α-TFM substituted analogue implies a significant change in the chemical invironment in the pGlu-His region, especially for the NH groups of pGlu and His and for the C^α atoms. This fact together with steric constraints of the substituent might prevent a stable hairpin turn required for an optimal interaction with the receptor (Grant et al., 1972).

Conclusions

The trifluoromethyl group imposes considerable polarization effects and important conformational restrictions on the neighbouring residues when incorporated into peptides. These properties result in an increased proteolytic stability of peptides depending on the relative position of the α-TFM group to the predominant cleavage site of the protease used and on the absolute configuration of the α-TFM amino acid. Due to the high electron density, trifluoromethyl substituted peptides are capable of interacting with enzyme or receptor subsites in a manner which is impossible for the fluorine-free pendants.

Furthermore, peptides containing α-TFM amino acids tend to impart β-turn structures similar to peptides containing Aib.

The introduction of the trifluoromethyl group in position 1 of TRH protects this hormone against proteolysis by pyroglutamyl aminopeptidase II. The polarization effect of the TFM group, however, and the stereochemistry at C^{α} of (α-TFM)pGlu seem to influence the ability of the pGlu residue to participate in hydrogen bonding and, thereby, to form the conformation required for an optimal interaction with the receptor.

Acknowledgements

This work was funded by Deutsche Forschungsgemeinschaft. Support with chemicals came from HOECHST AG, ASTA Medica, and Degussa AG. The biological tests of the TRH analogues were performed by Prof. Dr. Karl Bauer, Max-Planck-Institute of Experimental Endocrinology, Hannover, Germany. The force field calculations were carried out by Prof. Dr. Hans-Jörg Hofmann, University of Leipzig.

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Authors' address: Dr. B. Koksch, Institut für Biochemie, Universität Leipzig, Talstraße 33, D-04103 Leipzig, Federal Republic of Germany.

Received January 30, 1996

Verleger: Springer-Verlag KG, Sachsenplatz 4–6, A-1201 Wien. – Herausgeber: Prof. Dr. Gert Lubec, Universitäts-Kinderklinik, Währinger Gürtel 18–20, A-1090 Wien. – Redaktion: Sachsenplatz 4–6, A-1201 Wien. – Hersteller: Satz und Umbruch: Best-Set Typesetter Ltd., Hong Kong; Offsetdruck: Novographic, Ing. W. Schmid, Maurer-Lange-Gasse 64, A-1238 Wien. – Verlagsort: Wien – Herstellungsort: Wien. – Printed in Austria.