Studies on the Selective Trifluoroacetolytic Scission of Native Peptaibols and Model Peptides Using HPLC and ESI-CID-MS

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Representative members of a group of linear, *N*-acylated polypeptide antibiotics (peptaibols) containing α-aminoisobutyric acid (Aib) and, in part, isovaline (Iva), as well as proteinogenic amino acids and a C-terminal-bonded 2-amino alcohol, were treated with anhydrous trifluoroacetic acid (TFA) at 37° for 0.5–26 h. The resulting fragments were separated by HPLC and characterized by electrospray ionization collision-induced dissociation mass spectrometry (ESI-CID-MS). The following 16–20-residue peptaibols were investigated: natural, microheterogeneous mixtures of antiamoebins and alamethicin F50, uniform paracelsin A, and synthetic trichotoxin A50/E. In the natural peptides, bonds formed between Aib (Iva) and Pro (Hyp) were rapidly and selectively cleaved within 0.5 h. Furthermore, TFA esters of the C-terminal amino alcohols were formed. Depending on time, release of C-terminal tri- and tetrapeptides as well as amino acids from the major fragments was observed. Synthetic homooligopeptides, namely Z- and Ac-$(Aib)_{10}$-O^{t}Bu and Z-$(Aib)_7$-O^{t}Bu, were analyzed for comparison. On treatment with TFA, a regular series of Z-$(Aib)_{10-5}$-OH from Z-$(Aib)_{10}$-O^{t}Bu were detected within 0.5 h, and, after 3 h, release of a regular series of Z-$(Aib)_{7-3}$-OH from Z-$(Aib)_7$-O^{t}Bu were observed. Moreover, concomitant release of the series of H-$(Aib)_{10-3}$-OH from the decapeptide occurred. From these data, a repetitive cleavage mechanism *via* intermediate formation of C-terminal oxazolones on trifluoroacetolysis is proposed. Furthermore, their formation and stability in native peptaibols are correlated with subtle structural differences in protein amino acids linked to Aib. From the conspicuous concordance of the formation and abundance of regular series of trifluoroacetolytic fragments and of positive ions of the *b*-series in CID-MS, the generation of intermediate oxazolonium ions in both gas and liquid phase is concluded.

1. Introduction. – The carboxamide groups in peptides and proteins are remarkably stable against treatment with organic acids, and use of protecting groups and linkers, which are selectively cleaved by acids of varying strengths under controlled conditions, is of major importance in both solution and solid-phase peptide synthesis. However, several unexpected and selective cleavage reactions of peptide bonds formed in particular by non-proteinogenic amino acids have been reported. Consequently, such reactions have to be taken into account not only when planning classical or advanced,

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fully automated syntheses, but also in the course of workup and purification of synthetic and natural peptides, as well as their sequence determination. Regarding the aim of the work presented here, we focus on more recent results of the selective acidolytic amide-bond cleavage of special amino acids. However, readers interested in the selective nonenzymatic cleavage of proteins and peptides are referred to the classical review [1].

Recently, *Rubini et al.* [2] reported that synthetic peptides containing consecutive Pip[2]) residues, *e.g.*, H-Pip[1]-Pip[2]-Pip[3]-Leu[4]-Pip[5]-Pip[6]-Lys(Boc)[7]-Pip[8]-Lys(Boc)[9]-Phe[10]-O-*Wang* resin, have been cleaved between Pip[5]-Pip[6] on treatment with 95–50% CF_3COOH (TFA) and scavenger.

Contrary to that, no cleavage of the Pip bonds was observed by treatment with 1% TFA in CH_2Cl_2 when using the extremely acid-sensitive 2-chlorotrityl chloride resin. Analogously, the peptide Ac-Pip[1]-Pip[2]-Pip[3]-Leu[4]-Pip[5]-Pip[6]-Lys(Ac)[7]-Pip[8]-Lys(Ac)[9]-Phe[10]-O-*Wang* showed cleavage of the Pip[1]-Pip[2] as well as the Pip[5]-Pip[6] bond on treatment with 95–50% TFA.

Jiang et al. [3] observed that the C-terminal amide of a series of *N*-acyl-*N*,α,α-trialkylglycine amides (acyl, benzoyl; amide, Pmb or cyclohexyl), such as *N*-acyl-(dibenzylglycine)cyclohexylamide, was cleaved on treatment with 5% TFA in MeCN. *Mannekens et al.* [4] reported that the amide bond of (*S*)-2-pivaloyl-Tic and structurally related derivatives, such as (1*R*,3*S*)-1-[3-(benzyloxy)benzyl]-2-pivaloyl-Tic-Phe-OBn, were easily cleaved by treatment with MeOH, saturated with HCl_g at 0°.

Creighton et al. [5] found that the cyclic hexapeptides c[(NMe)Aib-Phe-D-Trp-Lys-Thr-Phe] and c[(NMe)α Ac[5]c-Phe-D-Trp-Lys-Thr-Phe] as well as c[Phe-Ser(Bn)-Ser(Bn)-Phe-NMeAib] showed unexpected amide bond cleavage on the terminal side of (NMe)Aib or its analogue (NMe)Ac[5]c on treatment with a mixture of anhydrous TFA/CH_2Cl_2 1:1 at 0° for 1 h. This mixture was used for the simultaneous removal of the Boc and *t*-Bu-ether protecting groups in the last steps of the syntheses. To get insight into the cleavage mechanisms, a series of benzoyl-dipeptide derivatives of the general structure *p*-X-C_6H_4C(O)-(NMe)Aib-Phe-OMe were synthesized. The X-group was varied from NO_2 to Cl. Fast cleavage and release of Phe-OMe was observed in 1:1 mixtures of TFA/CH_2Cl_2 or TFA/MeCN. Extremely fast amide-bond cleavage was observed for *p*-Me-C_6H_4C(O)-(NMe)Aib-Phe-OMe using 2% TFA in MeCN.

Anteunis and *van der Auwera* [6] reported on the acidolytic cleavage of a large number of protected di- and tripeptides. They found that, in peptides containing C-terminal N-methylamino acid carboxamides, such as Z-Pro-(NMe)Ala-NMe_2, the Me_2NH is already cleaved by TFA within 30 min.

In the course of their intensive studies on the use of the so-called azirine/oxazolone method [7] for the synthesis of Aib-containing peptides and peptaibols [8], the group of *Heimgartner* recognized that the amide bond in the dipeptide Z-Ala-Aib-NMe_2 was selectively cleaved by treatment with 3M HCl in H_2O/THF 1:1 within 12 h at 35°, and Z-Ala-Aib-OH was released in 87% yield [9].

Selective, repetitive amide cleavage of N-acyl-$(Aib)_n$-NMe_2 using HCl_g/toluene and reaction of the resulting *N*-protected peptide acids with 3-(dimethylamino)-2,2-dimethyl-2*H*-azirine was used for the synthesis of oligo-Aib-peptides [10].

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[2]) For abbreviations, see *Exper. Part.*

Except for the above azirine/oxazolone approach, cleavages of amide bonds were considered as rather undesired side reactions. In contrast to that, the selective scission of peptide bonds formed by Aib and Pro attracted considerable attention, because the former is the characteristic and predominant constituent of a group of polypeptides comprehensively named **peptaibiotics**, *i.e.*, **pept**ides containing **Aib** and displaying antib**iotic** as well as a plentitude of other b**iotic** activities [11].

Notably, it was already recognized by *Closse* and *Huguenin* in 1974 [12] that the cyclotetrapeptaibiotic chlamydocin could be selectively cleaved on the carboxy side of Aib by treatment with TFA at 50° for 2 h, resulting in the formation of the linear peptide H-Phe-D-Pro-Aoe-Aib-OH, which has subsequently been sequenced by *Edman* degradation.

Selective trifluoroacidolysis of the Aib–Pro bond was also used by *Pandey et al.* for the mass spectrometric sequence determination of alamethicins and antiamoebins [13][14].

Subsequently, analytical and preparative trifluoroacetolysis of the peptaibols trichotoxin and suzukacillin was intensively used by *Jung* and co-workers [15][16]. It was shown that release of C-terminal peptides occurs, besides the fast scission of the Aib–Pro bond that is followed by cleavages of certain Aib–Aaa bonds (Aaa: Gly, Ala). This sequential work was accompanied by systematic investigations of the acid-sensitivity of synthetic peptides representing partial sequences of native **peptaibols** (*i.e.*, **pept**ides containing **Aib** and a C-terminal 2-amino alcoh**ol**). Recently, preferred acidolytic cleavage of the Aib(Iva)-Hyp bonds in the 16-residue peptaibiotics integramides A and B was observed. Treatment with 1N HCl for 1 h at 110° was used for chiral sequence determination of D- and L-Iva in fragments [17][18].

From the above reports, it was evident that peptide bonds formed by Aib and Pro are particularly sensitive to trifluoroacetolysis. Notably, bonds formed by other C(α),C(α)-dialkylated glycines, such as isovaline (Iva, 2-ethylalanine), and amino acids related to Pro, such as Hyp or Pip, show a similar cleavage behavior. However, the slow release of C-terminal Aib as well as tri- and tetrapeptides from *N*-acetylpeptide acids, resulting from preparative TFA scission of trichotoxin [15] and suzukacillin [16], had been recognized, indicating further cleavage preferences.

To evaluate the cleavage mechanisms proposed for small peptides and amino acid derivatives, a series of native and synthetic peptaibols were subjected to time-dependent trifluororacetolysis. Their cleavage behavior was compared to synthetic homooligo-Aib model peptides treated in parallel. Fast separation and reliable assignment of the acidolytic fragments was achieved by on-line-HPLC coupled to ESI-CID-MS.

2. Results. – 2.1. *Trifluoroacetolysis of Homooligo-Aib-Peptides.* Sequences of model peptides and peptaibiols and preferential cleavage sides are compiled in *Fig. 1.*

Z-(Aib)$_{10}$-O^{t}Bu (**1**). Peptide fragments resulting from the trifluoroacetolysis of **1**, separated by HPLC and analyzed by total ion current (TIC)-MS, are compiled in *Table 1*, together with their characteristic protonated molecular ions. The ESI-MS of **1**, establishing its identity and showing a regular series of acylium ions b_2–b_{10} with the characteristic difference of 85 Da for Aib residues, is presented in *Fig. 2*.

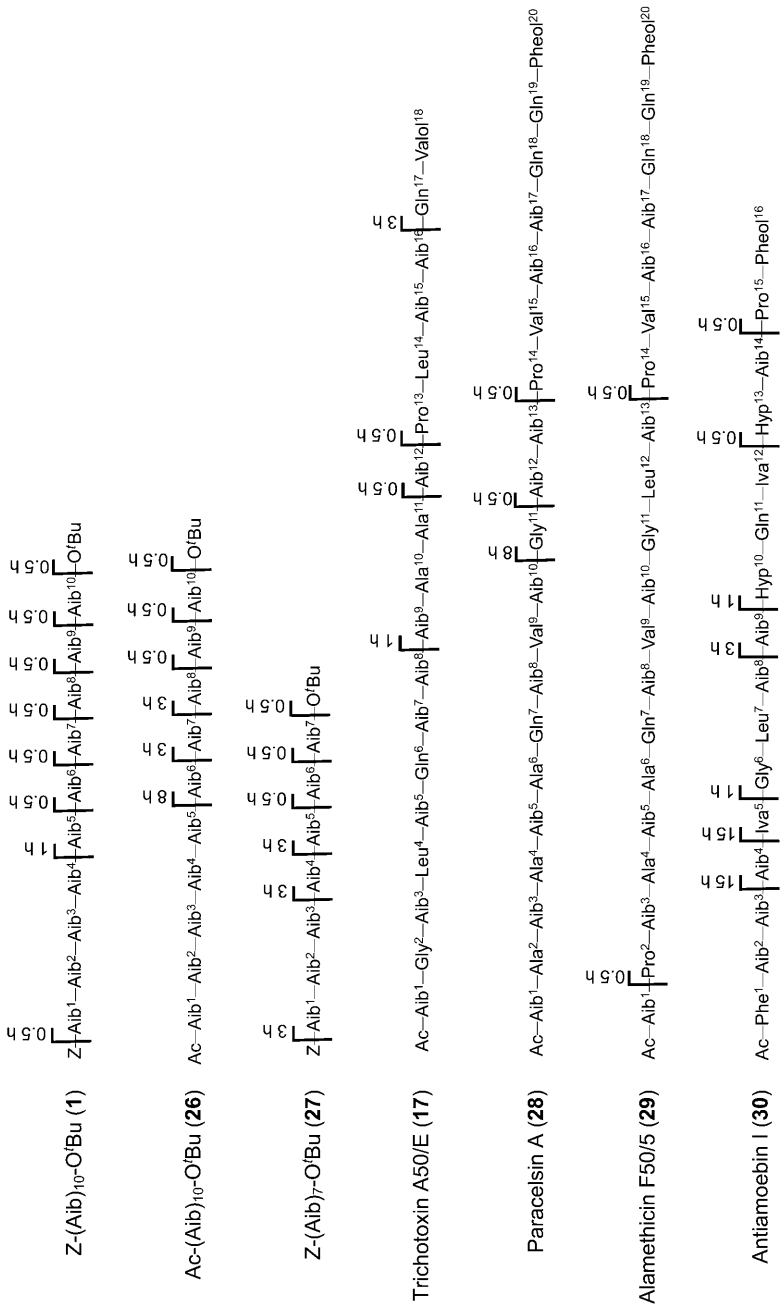


Fig. 1. *Structures of homooligo-Aib-peptides and peptaibols.* Preferred scission points at certain periods of time are indicated.

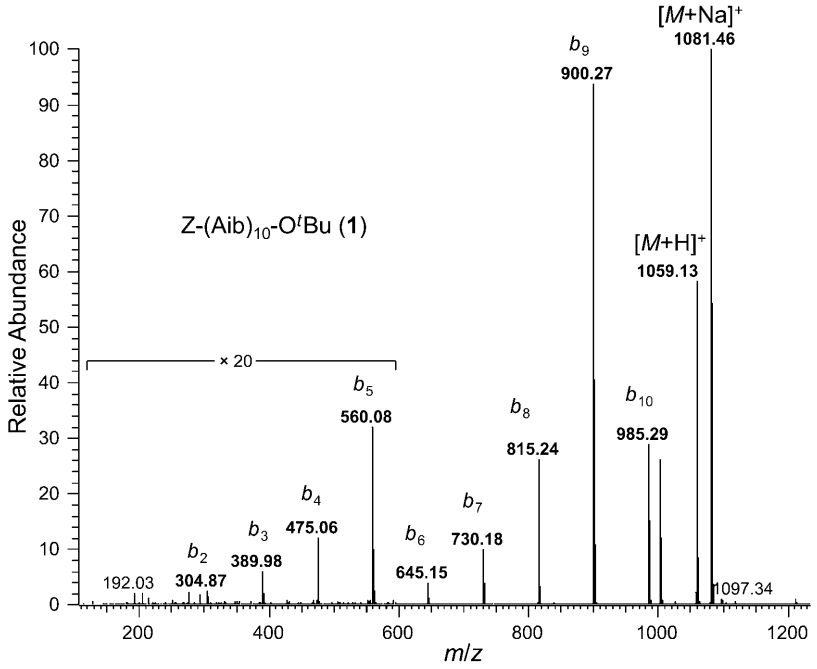


Fig. 2. *Mass spectrum of synthetic Z-(Aib)$_{10}$-O^{t}Bu* (**1**) s*howing the protonated molecular ion at* m/z *1059 and sodiated molecular ion at* m/z *1081.* Regular series of fragment ions b_2 to b_{10}, distinguished by the characteristic mass difference for Aib residues (85 Da), can be observed.

Table 1. *Peptides* **2**–**16** *Resulting from Trifluoroacetolysis of Z-(Aib)$_{10}$-O^{t}Bu* (**1**) *and Protonated Molecular Ions [*M + *H]$^+$ Used for the Characterization of Peptides*

	Detection after	Disappearance after	1	2	3	4	5	6	7	8	9	10		$[M+H]^+$ [m/z]
1		0.5 h	Z	Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib	O^{t}Bu	1059
2	0.5 h		Z	Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib		1003
3	0.5 h		Z	Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib			918
4	0.5 h		Z	Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib				833
5	0.5 h		Z	Aib	Aib	Aib	Aib	Aib	Aib	Aib					748
6	0.5 h		Z	Aib	Aib	Aib	Aib	Aib	Aib						663
7	0.5 h		Z	Aib	Aib	Aib	Aib	Aib							578
8	0.5 h	15 h		Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib		869
9	0.5 h	15 h		Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib			784
10	0.5 h			Aib	Aib	Aib	Aib	Aib	Aib	Aib	Aib				699
11	0.5 h			Aib	Aib	Aib	Aib	Aib	Aib	Aib					614
12	0.5 h			Aib	Aib	Aib	Aib	Aib	Aib						529
13	0.5 h			Aib	Aib	Aib	Aib	Aib							444
14	0.5 h			Aib	Aib	Aib	Aib								359
15	0.5 h			Aib	Aib	Aib									274
16	1 h		Z	Aib	Aib	Aib	Aib								493

After 0.5 h of trifluoroacetolysis, the starting material disappeared owing to the fast and complete cleavage of the ^{t}Bu group and the formation of the peptide acid Z-$(Aib)_{10}$-OH (**2**). Unexpectedly, concomitant generation of a mixture of individual peptides, *i.e.*, **3**–**16**, with decreasing ion intensities and retention times were observed. Peptides **2**–**10** were well resolved by HPLC, whereas peptides **11**–**15** co-elute. After 1 h, Z-$(Aib)_4$-OH (**16**), appeared, co-eluting with peptide **8**. Despite this, peptides **8** and **16**, as well as **11**–**15**, can be distinguished by their characteristic molecular ions (see *Table 1*). Z-Peptide acids **2**–**7** correspond to the series Z-$(Aib)_{10-5}$-OH, whereas peptides **8**–**10** correspond to the free peptides H-$(Aib)_{10-8}$-OH. The co-eluting free peptides **11**–**15** represent the mixture of H-$(Aib)_{7-3}$-OH. Inspection of *Fig. 3* shows that, with increasing reaction time, the intensity of the Z-decapeptide acid **2** decreases, and those of the series Z-$(Aib)_9$-OH (**3**) to Z-$(Aib)_5$-OH (**7**), increase. After 1 h, formation Z-$(Aib)_4$-OH (**16**; co-eluting with H-$(Aib)_{10}$-OH (**8**)) was also observed. This indicates successive cleavage of C-terminal Aib residues, one after the other. However, after 0.5 h reaction time, concomitant release of the series of free peptides **8**–**15** was observed, representing H-$(Aib)_{10-3}$-OH. This establishes that also the Z-group of the respective peptides is cleaved off. From the ion abundances, it appears, however,

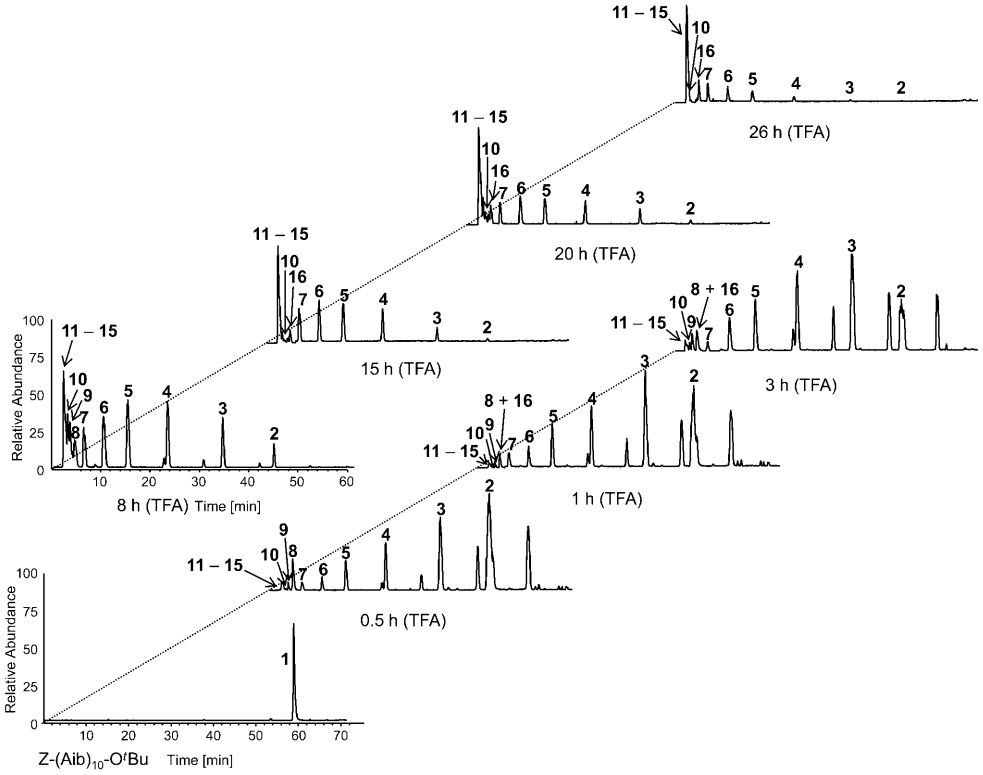


Fig. 3. *HPLC/TIC-MS of peptides* **2**–**16** *resulting from the trifluoroacetolysis of Z-(Aib)$_{10}$-O^{t}Bu* (**1**). Numbers refer to sequences presented in *Table 1*.

that cleavage of the Z-group proceeds slower, compared to the release of C-terminal Aib residues.

Ac-(Aib)$_{10}$-O^{t}Bu (**26**). Although this peptide contained some impurities resulting from the synthesis, the molecular ion at *m/z* 967 ($[M+H]^+$) could be used as the precursor, and the acidolytic cleavage kinetics could be analyzed. After 0.5 h, formation of the series Ac-(Aib)$_{10-8}$-OH was observed. After 3 h, Ac-(Aib)$_7$-OH and Ac-(Aib)$_6$-OH were recognized, and, after 8 h, formation of Ac-(Aib)$_5$-OH. After 15 h reaction time, Ac-(Aib)$_{10}$-OH and Ac-(Aib)$_9$-OH could not be detected anymore. In contrast to Z-(Aib)$_{10}$-OH, no formation of free H-(Aib)$_{10}$-OH or lower homologues from Ac-(Aib)$_{10}$-O^{t}Bu, even after longer reaction times (up to 26 h), could be observed. This indicates that the Ac-Aib terminus is more stable in comparison to the Z-Aib terminus. This is in agreement with the relative stability of acetylpeptides or Ac-Aib-OH released from peptaibols (see *Fig. 1*).

Z-(Aib)$_7$-OH (**27**). In analogy to the protected decapeptide, the *t*-Bu group was completely cleaved after 0.5 h reaction time, and formation of Z-(Aib)$_7$-OH was observed, as well as formation of the series Z-(Aib)$_6$-OH to Z-(Aib)$_5$-OH. After 3 h of acidolysis, formation of Z-(Aib)$_4$-OH and Z-(Aib)$_3$-OH were recognized as well as release of H-(Aib)$_7$-OH. This result clearly indicates that the Z-group was also cleaved in that case. Basically, the acidolytic cleavage behavior of the heptapeptide is in agreement with that of the decapeptide.

2.2. *Trifluoroacetolysis of Peptaibols. Trichotoxin* (**17**). The sequences of the peptaibols discussed in the following and fragments released are also presented in *Fig. 1*.

TICs of HPLC/MS resulting from time-dependent trifluoroacetolysis of the synthetic octadecapeptide **17** and the corresponding pseudomolecular ions of the resulting fragments recorded in CID off-mode are presented in *Fig. 4*.

Consecutive numbers of peptides resulting from trifluoroacetolysis and their corresponding sequences are presented in *Table 2*. Uniformity of trichotoxin (**17**) in HPLC and the peak at *m/z* 1690 ($[M+H]^+$) established the authenticity of the synthetic octadecapeptide. After 0.5 h of trifluoroacetolysis, the starting material **17** decreased, and the acetylpeptide acids **18** and **20**–**22**, as well as the TFA esters of **18** and the prolylhexapeptide **24**, appeared. After 1 h, peptide **23** and, after 3 h, peptide **19** was detected. The free prolylhexapeptide **25** was observed after 8 h.

Comparison with the sequence of trichotoxin (see *Fig. 1*) shows that, after 0.5 h, fast, but not complete, cleavage of the Aib–Pro bond takes place with release of the N-terminal dodecapeptide Ac-Aib^1-Aib^{12}-OH (**21**). Notably, concomitant formation of the trifluoroacetyl esters **18** of the C-terminal Valol from the intact trichotoxin (**17**), as well as the C-terminal prolylhexapeptide **24**, was recognized (see *m/z* in *Table 2* and *Fig. 4*). Furthermore, cleavage of the C-terminal Aib^{12} from the acetyldodecapeptide acid **21** and formation of Ac-Aib^1-Ala^{11}-OH (**22**) from that were observed. From **22**, the tripeptide Aib^9-Ala^{10}-Ala^{11} was subsequently released within 1 h, resulting in the formation of the acetyloctapeptide acid Ac-Aib^1-Aib^8-OH (**23**). After 3 h of acidolysis, cleavage of the C-terminal Gln^{17}-Valol^{18} from trichotoxin (**17**) was observed with release of the acetylhexadecapeptide acid Ac-Aib^1-Aib^{16}-OH (**19**). Notably, some trichotoxin (**17**) still remained intact even after 26 h of trifluoroacetolysis. These data are in excellent agreement with those of the preparative trifluoroacetolysis of native

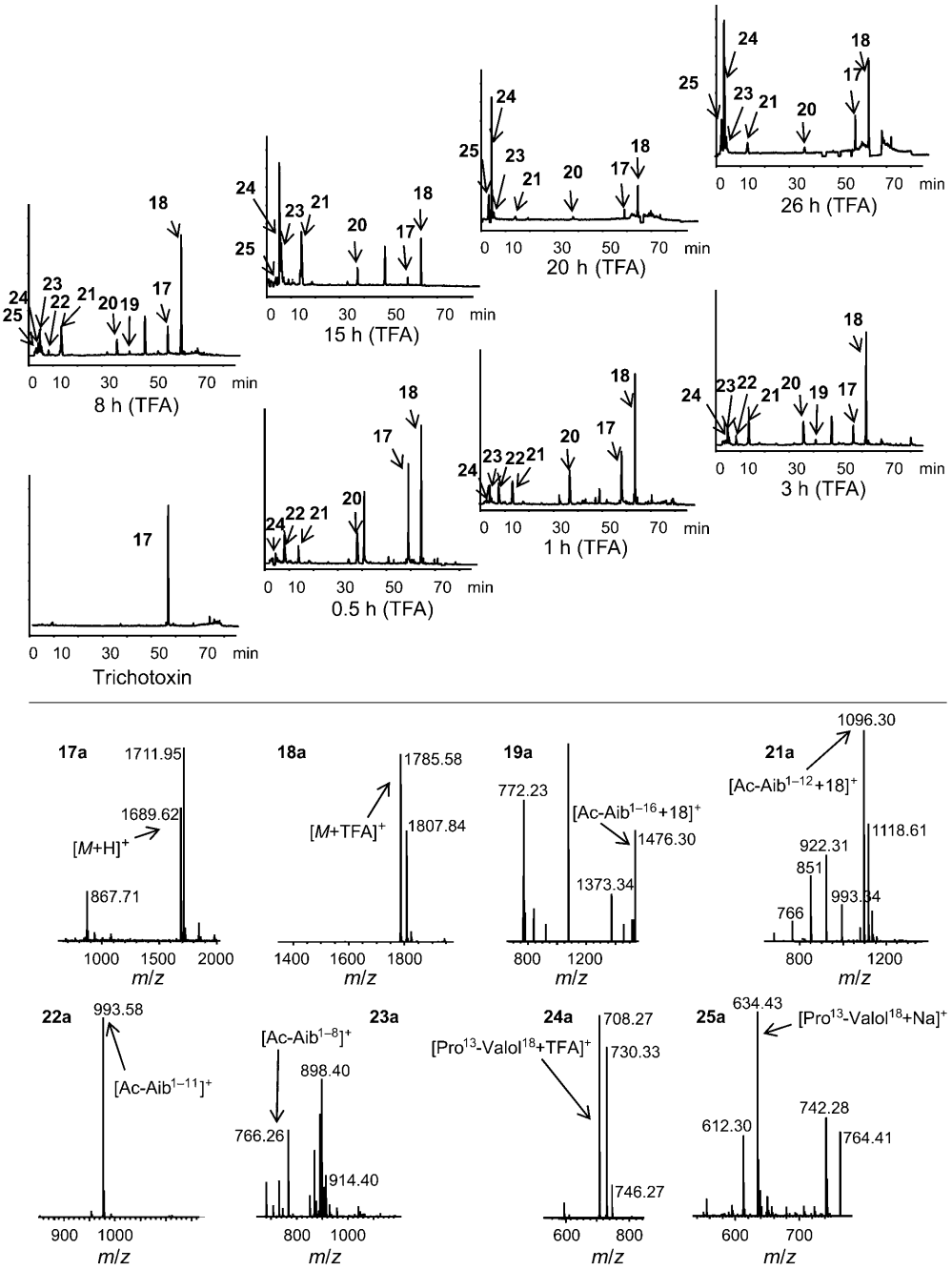


Fig. 4. *HPLC/TIC-MS of peptides* **17**–**25** *resulting from the trifluoroacetolysis of synthetic trichotoxin* (**17**; top), *and the corresponding diagnostic ions,* **17a**–**25a**, *used for their characterization* (bottom). Numbers refer to sequences presented in *Table 2*. Peptide **20** could not be assigned unambiguously.

Table 2. *Peptides* **20**, **21**, *and* **23**–**25**, *and TFA-esters* **18** *and* **22** *Resulting from the Trifluoroacetolysis of Trichotoxin* (**17**), *and Protonated Molecular Ions* $[M+H]^+$ *Used for the Characterization of Peptides*

	Detection after	Disappearance after	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18		$[M+H]^+$ [m/z]
17			Ac	Aib	Gly	Aib	Leu	Aib	Gln	Aib	Aib	Aib	Ala	Ala	Aib	Pro	Leu	Aib	Aib	Gln	Valol		1690
18	0.5 h		Ac	Aib	Gly	Aib	Leu	Aib	Gln	Aib	Aib	Aib	Ala	Ala	Aib	Pro	Leu	Aib	Aib	Gln	Valol	TFA	1786
20	0.5 h																					
21	0.5 h		Ac	Aib	Gly	Aib	Leu	Aib	Gln	Aib	Aib	Aib	Ala	Ala	Aib								1096
22	0.5 h	15 h	Ac	Aib	Gly	Aib	Leu	Aib	Gln	Aib	Aib	Aib	Ala	Ala									1011
24	0.5 h														Pro	Leu	Aib	Aib	Gln	Valol	TFA	709
23	1 h		Ac	Aib	Gly	Aib	Leu	Aib	Gln	Aib	Aib											784
19	3 h	15 h	Ac	Aib	Gly	Aib	Leu	Aib	Gln	Aib	Aib	Aib	Ala	Ala	Aib	Pro	Leu	Aib	Aib				1476
25	8 h															Pro	Leu	Aib	Aib	Gln	Valol		612

trichotoxins [15], aimed on the isolation of fragments suitable for sequence determination. Compound **20**, however, appearing after 0.5 h and still detectable after 26 h, could not be characterized unambiguously. The acidolytic scission of the Gln^{17}-Valol^{18} C-terminus of **17** *via* simultaneous or subsequent formation of a pyroglutamic acid derivative has been treated in previous reports and, therefore, is not considered here [19].

Paracelsin (**28**). Fragments resulting from trifluoroacetolysis of peptaibols **28**–**30** are presented in *Fig. 1*, but TICs of HPLC/MS are not shown. Therefore, peptide fragments treated in the following paragraph are not assigned by numbers as done in the cases of Z-$(Aib)_{10}$-O^{t}Bu (**1**) and trichotoxin (**17**). After 0.5 h of trifluoroacetolysis, cleavage between Aib^{13}-Pro^{14} was observed with release of the acetyltridecapeptide acid Ac-Aib^1-Aib^{13}-OH and the prolylheptapeptide H-Pro^{14}-Pheol^{20}. In addition, scission of the C-terminal dipeptide H-Aib^{12}-Aib^{13}-OH from Ac-Aib^1-Aib^{13}-OH, resulting in the formation of Ac-Aib^1-Gly^{11}-OH, was observed. After 8 h of acidolysis, cleavage of C-terminal Gly therefrom occurred, resulting in the formation of the acetyldecapeptide acid Ac-Aib^1-Aib^{10}-OH. After 15 h of acidolysis, no starting material remained, anymore.

Alamethicin (**29**). The major difference between paracelsin and alamethicin is that the latter contains an additional acid-sensitive Aib–Pro bond in the Ac-Aib^1-Pro^2 terminus (see *Fig. 1*).

After 0.5 h of acidolysis, partial formation of the TFA ester of **29** was observed as well as scission of the Aib–Pro bonds, resulting in the detection of H-Pro^2-Pheol^{20} and its TFA-ester, H-Pro^{14}-Pheol^{20} and its TFA-ester, as well as the free dodecapeptide H-Pro^2-Aib^{13}-OH. The N-terminal Ac-Aib-OH was not observed under the MS conditions applied, but its formation and use of this particular selective cleavage for sequencing was demonstrated previously [15][16]. After 3 h, the fragment H-Pro^2-Aib^{13}-OH increased in intensity, indicating the faster cleavage of the N-terminal Ac-Aib^1–Pro^2 bond in comparison to the Aib^{13}–Pro^{14} bond. After 8 h of acidolysis, starting material **29** was no longer detectable. The data are also in good agreement with those of isolated fragments resulting from the preparative trifluoroacetolysis of the alamethicin-related peptaibol suzukacillin [16].

Antiamoebin (**30**). Owing to its microheterogeneity and the presence of two Hyp as well as a C-terminal Pheol residue, separation of fragments and interpretation of mass spectra were more difficult in comparison to the above peptaibols. After 0.5 h of trifluoroacetolysis, formation of a mixture of mono-, di-, and tri-TFA esters, resulting from the esterification of the OH groups of the two Hyp residues and the C-terminal Pheol residue, were observed. Cleavage of Iva^{12}-Hyp^{13} and Aib^{14}-Pro^{15} was observed after 0.5 h. After 1 h, cleavage of Aib^9-Hyp^{10} with formation of Ac-Phe^1-Aib^9-OH was recognized. Also after 1 h, partial cleavage of Ac-Phe^1-Aib^9-OH between Iva^5(Aib^5)-Gly^6, resulting in the release of Ac-Phe^1-Iva^5(Aib^5)-OH, was observed (amino acids in parentheses indicate exchange positions in the natural peptaibol mixture used, resulting in the detection of the corresponding homologous fragments). After 3 h, further cleavage of C-terminal Aib^9 from remaining Ac-Phe^1-Aib^9-OH occurred, resulting in the release of Ac-Aib^1-Aib^8-OH. After 15 h, cleavage between Aib^4-Iva(Aib)^5 and Aib^3-Aib^4 was recognized, resulting in the formation of Ac-Phe^1-Aib^3-OH and Ac-Phe^1-Aib^4-OH.

3. Discussion. – The fast acidolytic cleavage of the Aib–Pro bond, the release of Aib and small C-terminal peptides from the C-termini of fragments, as well as the apparently successive scission of C-terminal Aib from the Z- or Ac-protected or free homooligo-Aib-peptides, require an explanation. The latter peptides, in particular, have the advantage of consisting of uniform amino acid constituents. Thus, subtle steric or electronic effects of the varying side chains as known from peptides that exclusively consist of proteinogenic amino acids must not be considered in this case.

Consequently, the multitude of information about the acidolytic scission of amino acid derivatives or simple model peptides reported in the literature or observed in our laboratory should be discussed. Thus, they can be taken into account for mechanistic considerations on homooligo-Aib-peptides and native peptaibols/peptaibiotics.

Rapid scission of the Aib–Pro bond by TFA or HCl/AcOH has also been reported for Ac-Aib-Pro-NH_2 [20], serving as a model for the N-termini of peptaibols such as alamethicin or hypelcin. Notably, the Aib–Pro bond is more stable if mixtures of TFA/CH_2Cl_2 are used at ambient or lower temperatures. This was established for Boc-Gly-Ala-Aib-Pro-Ala-Aib-Aib-Glu(OBzl)-Gln-OMe. Using a 9.3% solution in TFA/CH_2Cl_2 5:2 at 22°, it has been observed that the Boc group was completely cleaved within 0.5 h. However, no prolylpeptides could be detected by TLC after that time [20].

Trifluoroacetolytic cleavage of the Aib–Aaa bond has previously been investigated using the synthetic model peptides Ac-Aib-Ala-OH, Ac-Aib-Gly-OH, Ac-Ala-Aib-OMe, and Ac-Ala-Aib-Ala-OMe in the laboratory of *Günther Jung*, Tübingen University, Germany [19].

Whereas *ca.* 50% of the peptide bonds in Ac-Aib-Ala-OH and Ac-Aib-Gly-OH were cleaved with TFA within 3 h at 37°, cleavage of the peptide Ac-Ala-Aib-OMe started slowly after 6 h of acidolysis, but little cleavage was observed even after 19 h. Notably, only cleavage of the peptide bond was observed but not release of the Ac group. The tripeptide Ac-Ala-Aib-Ala-OMe remained almost unaffected on treatment with TFA for 23 h.

Based on the model peptide Ac-(NMe)Aib-Phe-OMe, *Creighton et al.* [5] postulated the formation of the oxazolone *via* an intermediate, tetrahedral oxazolinolium ion, from which the amino acid is ejected. The resulting Ac-(NMe)AibOx cation was postulated to be rapidly hydrolyzed by traces of H_2O with release of Ac-(NMe)Aib-OH.

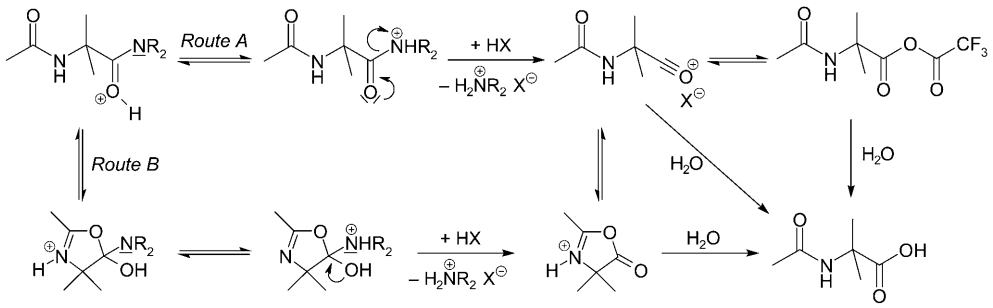
Notably, *Obrecht* and *Heimgartner* [10] had already demonstrated that $Me_2NH\cdot$HCl is released from peptides of the general structure Acyl-$(Aib)_n$-NMe_2 ($n = 1–3$), following treatment with HCl_g in toluene. Formation of intermediate oxazolones in this series was established by IR spectroscopy, as well as preparative isolation of the corresponding oxazolones. These authors also pointed out the experimental evidence for an equilibrium between the protonated oxazolone as well as the respective intermediate acylium ion or acid chloride.

This view is supported by the fact that the simple derivative Bz-Aib-NMe_2, considered as model for, *e.g.*, the Ac-Aib-Pro bond, is easily converted into the respective oxazolone by treatment with HCl_g in toluene at 80–100°. Such treatment resulted in the release of $Me_2NH\cdot HCl$ [9]. This oxazolone is easily hydrolyzed to Bz-Aib-OH by heating with mixtures of aqueous HCl/organic solvents. Analogously, the model peptides Z-Aaa-Aib-NMe_2 (Aaa: Ile, Ala) were directly converted into the

oxazolone by treatment with HCl_g/toluene (5–8 min, 100°). The corresponding dipeptide acids were obtained therefrom by treatment with 2N HCl for 0.5 h at room temperature. Intermediate formation of oxazolones in the latter cases was also deduced from the high degree of racemization of Ile and Ala as result of tautomerism. The problem of the racemization of the preceding chiral amino acids on prolonged activation of C-terminal Aib is well-known [21].

We found that treatment of Ac-Aib-NEt_2 with anhydrous TFA (10% solution, 15 min at 60°) resulted in the complete amide-bond cleavage with release of $Et_2NH\cdot$ TFA and Ac-Aib-OH within that time. This was evidenced by TLC and comparison with reference compounds. Notably, no formation of an oxazolone could be detected by TLC (*H. Brückner*, unpublished results). If one assumes protonation of the Ac O-atom in Ac-Aib-NEt_2 by TFA with formation of an oxonium ion, as has been proposed by *Klotz et al.* [22] for Ac-NHMe (*N*-methylacetamide), an intramolecular nucelophilic attack on the positive C-atom of the carboxamide group is not likely. In anhydrous TFA, representing a highly polar, ion-solvating solvent, however, concomitant release of the protonated amine and formation of an intimate ion-pair between the carbonium cation and trifluoroacetyl anion is favored that might result in an equilibrium with the respective covalently bonded mixed anhydride (*Scheme 1*, *Route A*). These intermediates are very reactive and will react quickly with traces of H_2O. This mechanism is related to that one formulated in [10], and, at least for Ac-Aib-NEt_2, does not require formation of an oxazolone, but excludes its formation neither directly (*Scheme 1*, *Route B*) nor *via* cyclization of an intermediate carbonium/acylium cation as shown in *A*. This mechanism might also be applied on the fast cleavage of N-terminal Ac-Aib-Pro bonds.

Scheme 1. *Possible Trifluoroacetolytic Cleavage of Ac-Aib-NEt_2* via *Intermediate Formation of an Ion Pair or Mixed Anhydride* (*Route A*) *or oxazolone* (*Route B*). R = Et, HX = TFA; $X^- = CF_3COO^-$.



However, most reports and studies presented in the introduction discuss or focus on the formation of an intermediate oxazolonium ion, indeed. This results from the intramolecular nucleophilic attack of the Aib C=O O-atom on the C-atom of the neighboring amide bond with release of the adjacent amino acid or substituted amine.

4. General Cleavage Mechanism. – Taking the aforementioned data into account, a general mechanism is proposed based on the facileness of the formation of the oxazolones and fragility of the protonated intermediates formed in Aib-peptides and their instability under the acidolytic cleavage conditions used.

The considerations on homooligo-Aib-peptides are extended to peptaibols and model peptides, and a mechanism is presented in *Scheme 2*. With regard to the following discussion, the encircled residue R represents a C-terminal Aib residue. The considerations can be extended on Pro (Hyp, Pip), or a disubstituted amide or, with limitations, to peptaibols containing Aib–Gly(Ala) bonds.

4.1. *Trifluoroacetolysis of Z- and Ac-(Aib)$_{10}$-O^{t}Bu and Z-(Aib)$_7$-O^{t}Bu*. Taking the large excess of TFA into account, the *t*-Bu groups of the peptides are cleaved immediately, and scission of the peptide bonds of the resulting peptide acids is discussed in the following.

Internal nucleophilic attack of the C=O O-atom of the carboxamide group of the second to last Aib on the carboxy group of the last Aib residue is probably favored by the *geminal* dimethyl or *Thorpe–Ingold* effect [7][23]. This leads to the fast formation of an unstable tetrahedral oxazolinolium intermediate [4], which is subsequently stabilized by proton transfer and release of the original C-terminal Aib. The resulting oxazolonium peptides are easily hydrolyzed by traces of H_2O which are always present, even in TFA labelled as anhydrous.

From the fast release of a complete series of Z-(Aib)$_{9–4}$ from Z-(Aib)$_{10}$-OH, a repetitive cleavage of the respective C-terminal Aib is proposed. The series ends with the formation of Z-(Aib)$_4$-OH. This might be explained by the acidolytic stability of tri- and tetrapeptides as a result of their particularly stable secondary structures.

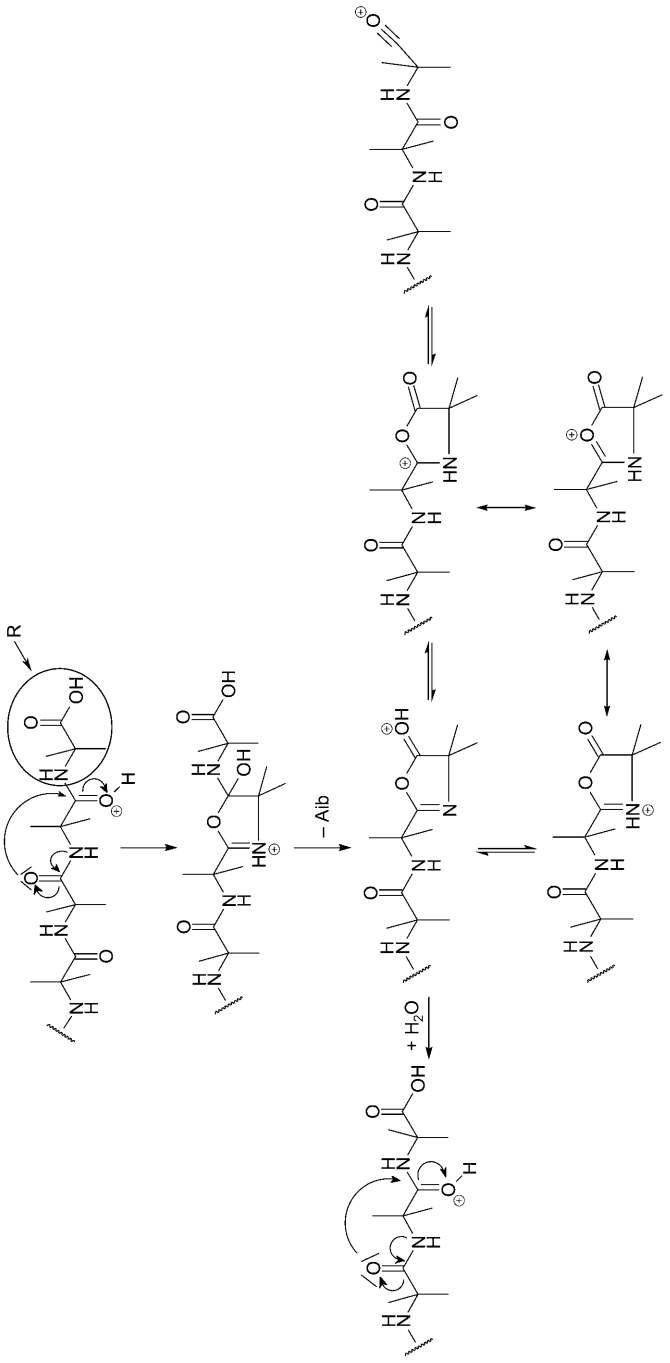
This stability against trifluoroacetolysis of the smaller acylpeptides is also supported by the release of Ac-Phe^1-Aib-Aib^3-OH from antiamoebins after 15 h. This tripeptide, however, remained resistant against further cleavage (see *Fig. 1*).

Remarkably, oxazolones are not formed from the C-terminal Aib but from the preceding ones. From a C-terminal oxazolone, the precursor peptide would be reconstituted on hydrolysis. Therefore, it is assumed that the C-terminal carboxy groups form H-bridges with TFA, and the resulting solvent clusters shield the C-atom of the carboxy group from nucleophilic attack of the O-atom by the adjacent carboxamide group. This effect might also explain the observed stability of Ac-Aib-OH in TFA.

However, concomitant cleavage of the Z-group leading to the series H-(Aib)$_{10–3}$-OH is also observed. This observation contradicts experiences from non-Aib-peptide chemistry: the Z-group, commonly used for orthogonal protection in peptide chemistry, is usually stable towards TFA under the conditions applied and requires, *e.g.*, boiling TFA for its removal. Unexpected cleavage of the Z-group from Z-Ala-Aib-NMe$_2$ on treatment with HCl_g in MeOH was also reported [9].

The release of Aib residues from the N-terminus of the free peptides is assumed not to take place because the protonated N-terminus decreases the nucleophilicity of the corresponding carboxamide group of Aib. This phenomenon is attributed to its electron-withdrawing properties.

From the data obtained from prolonged trifluoroacetolysis of peptaibols and release of C-terminal di- to tetrapeptides therefrom (see *Fig. 1*), formation of oxazolones at certain positions in the peptide backbone is also concluded according to the general mechanism proposed (in *Scheme 2*, encircled R in these cases represent C-terminal peptide sequences).



Scheme 2. *Proposed C-Terminal Cleavage Mechanism of Homooligo-(Aib)$_n$-Peptides* via *Repetitive Intermediate Formation of Oxazolinolium/Oxazolinium* (oxazolonium) *Ions after Treatment with TFA, Leading to Homooligo-(Aib)$_{n-1}$-Peptides* (left side) *and Related Formation of the* b-*Series of Positive Fragment Ions under Conditions of ES-MS* (right side). Encircled R represents an α-aminoisobutyryl or prolyl residue, or a disubstituted amine.

The considerations outlined for homooligo-Aib-peptides can be extended on peptide bonds formed between Aib and Pro or homologues. The scission of the amide bond proceeds fast and can take place at any position of the polypeptide chain.

4.2. *Trifluoroacetolysis of Peptaibols.* The above considerations can also be applied to *alamethicin* and *paracelsin*. In the case of *alamethicin*, fast cleavage of the Aib[1]–Pro[2] and Aib[13]–Pro[14] bonds with release of the respective fragments, according to the proposed mechanism, was observed. In paracelsin, fast cleavage of the Aib[13]–Pro[14] bond occurs. From the resulting Ac-Aib[1]-Aib[13]-OH, release of the dipeptide H-Aib[12]-Aib[13]-OH and of the tripeptide H-Gly[11]-Aib[12]-Aib[13]-OH on prolonged trifluoroacetolysis are also observed. Again, release of stable tripeptides and nucleophilic attack of constrained Aib on the sterically least constrained Gly were recognized. With the exception of this amino acid, sequence of -Aib-Aaa-(Aib-Aaa)$_n$-Aib- (Aaa, proteinogenic amino acid; exceptions C-terminal Aib-Gln-Xol bonds [15][19]) are stable against trifluoroacetolysis.

In *paracelsin*, release of C-terminal dipeptide Aib[12]-Aib[13] from Ac-Aib[1]-Aib[13]-OH (the latter resulting from scission of the C-terminal prolylheptapeptide) is assumed to proceed also *via* formation of an intermediate oxazolone. Cleavage of the Aib[10]–Gly[11] bond after 8 h indicates moderate acid-sensitivity of this bond.

In *trichotoxin*, the fragment Ac-Aib[1]-Ala[11]-OH formed on trifluoroacetolysis behaves conspicuously different because the C-terminal tripeptide Aib[9]-Ala[10]-Ala[11] was released therefrom and not the C-terminal Ala[11]. This is explained by the fact that the Ala residues, in contrast to Aib, contain C_α-hydrogens and oxazolone formation is not favored. Intramolecular attack of the carbonyl of Aib[8] on Aib[9], however, might proceed *via* formation of oxazolone and release of the tripeptide as outlined before. The stability of the tripeptide is in principal agreement with that of the model Ac-Ala-Aib-Ala-OMe mentioned above. The remaining Ac-Aib[1]-Aib[8]-OH remains stable in TFA for hours; possibly formed smaller fragments could not be detected.

Generally, cleavage of Aib-peptides is much slower if mixtures of TFA with organic solvents such as CH_2Cl_2 are used. This is explained by the decrease of the dissociation as well as concentration of TFA. Cleavage is also much lowered when acidic aqueous solvents are used or water is added to TFA. This is explained by a much lower capability to form oxazolones in aqueous solution. Heating at elevated temperature at prolonged time, however, enables acidolytic cleavage under these conditions (see examples in introduction). It is assumed that a change from the oxazolone mechanism to the common acid-catalyzed cleavage of amide bonds proceeds, with preference for disubstituted amides.

4.3. *Considerable Accordance of Trifluoroacetolytic Scission Pattern and Mass-Spectrometric Fragmentation.* Inspection of the trifluoroacetolytic fragments of Z-(Aib)$_{10}$-O^{t}Bu, resulting in the series Z-(Aib)$_{10-5}$-OH, and the Low-CID-MS of the decapeptide, shows that there is considerable agreement between the generation of homooligo-Aib-peptide fragments in the protic solvent and gas-phase dissociation, since corresponding regular series of *b* ions, corresponding to the Z-(Aib$_{10-2}$)$^+$ acylium (or oxonium/oxazolonium) cations (see *Fig. 2*), can be observed in the positive-ion mode (see *Scheme 2*).

Analogously, a regular series of b_2 to b_{10} cations is also generated from Ac-(Aib)$_{10}$-O^{t}Bu, and of b_2 to b_7 cations from Z-(Aib)$_7$-O^{t}Bu.

Notably, generation of acylium ions at low collision energy in peptides has been proposed to proceed *via* nucleophilic attack of the peptide bond C=O at the protonated carbonyl group of the preceding C-terminal amino acid, formation of an intermediate oxazolol, and proton transfer onto the N-atom of the amino acid that is released with formation of an oxazolonium/oxazonium cation [24–26]. Regarding peptaibols, prolyl residues are the best leaving groups and give rise to the respective intensive *y* series [27][28].

The abundances of the corresponding acylium ions are also in striking agreement: highest intensities are observed for ions resulting from Aib residues owing to the stabilization by the geminal dimethyl groups, lower intensities are observed for acylium ions from protein amino acids. For example, in the partial sequence -Aib^{9}-Ala^{10}-Aib^{11}-Aib^{12}-Pro^{13}- of trichotoxin A40/5, the resulting b_{12} acylium ion is most intensive, and the b_9 and b_{11} ions are comparatively more intensive than the b_{10} ion [27]. The notable moderate acid sensitivity of the -Aib–Gly- bond can also be observed in the positive-ion MS of suzukacillin A4. The MS/MS of the b_{13} fragment ion resulting from the partial sequence -Aib^{8}-Aaa^{9}-Aib^{10}-Gly^{11}-Aib^{12}- displays the highest ion abundance of b_{10}, and, lower abundance of b_9 and, in particular, of b_{11} in comparison to b_8 and b_{12} acylium ions [28].

Finally, it should be emphasized that the results presented here for oligo-Aib-peptides and peptaibols, and the mechanistic aspects deduced therefrom are in excellent agreement with the data and postulates resulting from the azirine/oxazolone approach promoted for many years by the group of *Heimgartner* [10].

5. Consequences and Outlook. – Beyond the mechanistic considerations outlined above, the potential acid-sensitivity of peptaibiotics has to be considered in the course of isolation and handling of natural products, as well as in planning of classical or solid-phase syntheses of peptaibiotics or peptides containing the special amino acids or analogues mentioned in the text.

From an applied point of view, the selective acidolytic scission of peptaibols and analysis of their fragments is of importance for their sequence determination in order to generate fragments suitable for the assignments of the position and configuration of amino acids, such as Iva enantiomers [15–18].

Use of proteases for the selective cleavage of peptaibiotics is of limited value, since bonds formed by repetitive Aib(Iva) residues or adjacent protein amino acids are completely resistant towards proteolytic degradation. For example, the 15-residue peptaibols of the native ampullosporin mixture displaying N-terminal Ac-Trp and C-terminal leucinol could neither be cleaved by the action of pepsin, chymotrypsin, or pronase, nor by treatment with liver homogenates [29].

This is in agreement with our and others' attempts to cleave trichotoxin or alamethicin with common proteases [30]. However, special peptaibiotics comprising bonds between consecutive proteinogenic amino acids, α-amino acids and β-amino acids, are cleaved at certain positions. This has been shown by the cleavage of the Gly^{13}–Leu^{14} and Leu^{6}–β-Ala^{7} bonds of neoefrapeptins with papain [31], and for synthetic ampullosporin analogues in which Aib residues had been replaced by a series of subsequent Ala residues [29].

However, since formation and degradation of peptaibiotics under fermentation conditions was observed for trichobrachins [32], fungal enzymes must exist being capable of cleaving Aib-peptide bonds. The search for such enzymes being capable of cleaving such sterically constrained bonds is thus encouraged. It might provide a novel class of enzymes being capable of degrading related polymers such as plastic fibres or detergents.

The extreme enzymatic resistance of the peptaibiotics, together with their microheterogeneity, might be the result of the defence strategy of their fungal producers [11] in order to overcome degradation of their arsenal of antibiotic and membrane-modifying peptides in their natural environments [33].

Experimental Part

Abbreviations. Aib, α-aminoisobutyric acid; Iva, isovaline (2-ethylalanine); Pheol, L-phenylalaninol; Valol, L-valinol; Xol, 2-amino alcohol; (NMe)Aib, 2-amino-*N*-methylisobutyric acid; (NMe)Ala, *N*-methylalanine; (NMe)αAc^{5}c, 1-amino-*N*-methylcyclopentanecarboxylic acid; Ser(Bn), serine-*O*-benzyl; Me_2N, dimethylamino; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; Pmb, 4-methoxybenzyl; Bn, benzyl; Aaa, proteinogenic amino acids; oxazolone refers to oxazole-5(4*H*)-one and pseudoaromatic oxazolone intermediates; AibOx in the synthesis part refers to the oxazolone of the C-terminal Aib; Pip, pipecolic acid; SPip, γ-thiapipecolic acid; Aoe, L-2-amino-9,10-epoxy-8-oxodecanoic acid.

Chemicals. MeCN and MeOH were obtained from *Riedel-de Haën* (*Sigma Aldrich*, D-Seelze); CF_3COOH (TFA) was from *Fluka* (CH-Buchs) and used without further treatment.

Homooligo-Aib-Peptides. The homooligo-Aib-peptides were synthesized according to the oxazolone method [34–36] but instead of stepwise elongation of the peptide segments condensation was used [37]. Briefly, coupling of Z-Aib-Aib-AibOx and H-$(Aib)_2$-O^{t}Bu in butyronitrile at 100° for 24 h yielded Z-$(Aib)_5$-O^{t}Bu. Hydrogenolysis in MeOH in the presence of Pd/C provided H-$(Aib)_5$-O^{t}Bu, and treatment of Z-$(Aib)_5$-O^{t}Bu with TFA/CH_2Cl_2 for 1 h at r.t. yielded Z-$(Aib)_5$-OH. The pentapeptide was converted into the respective oxazolone by treatment with Ac_2O for 1.5 h at 100°.

Reaction of Z-$(Aib)_4$-AibOx and H-$(Aib)_5$-O^{t}Bu in butyronitrile at 100° for 36 h provided Z-$(Aib)_{10}$-O^{t}Bu (**1**). Hydrogenolysis, followed by acetylation with Ac_2O/CH_2Cl_2 1:1 at ambient temp. for 4 h, provided Ac-$(Aib)_{10}$-O^{t}Bu (**26**) that, after precipitation from MeOH/CH_2Cl_2/petroleum ether, was obtained in *ca.* 65% purity as calculated from HPLC. Reaction of Z-$(Aib)_4$-AibOx and H-$(Aib)_2$-O^{t}Bu in butyl acetate/1,1,2,2-tetrachloroethane 10:1 at 100° for 18 h provided Z-$(Aib)_7$-O^{t}Bu (**27**). Protected peptides were characterized by MS, uniformity in TLC, and HPLC. For crystal structures of **1** and **27**, see [38][39]; the ESI-MS of **1** is depicted in *Fig. 2*.

Native Peptaibols. Native peptaibols were isolated in our laboratories as natural, microheterogeneous mixtures from the culture broths of the producing fungi. Alamethicin F50 containing the major componentent alamethicin F50/5 (**29**; see *Fig. 1*) was isolated from *Trichoderma viride* NRRL 3199 [40] (recently redescribed as *T. arundinaceum* [11]), crude paracelsins A–D were isolated from *Trichoderma reesei* QM 9414 [41], and the pure component paracelsin A (**28**) was isolated from the paracelsin mixture by HPLC; microheterogeneous antiamoebins were isolated from *Emericellopsis salmosynnemata* CBS 176.60 [42], and contained antiamoebin I as the major component **30** (see *Fig. 1*); trichotoxin A50/E (**17**) was synthesized by conventional soln.-phase synthesis [43] and also characterized by X-ray crystallography [44].

Trifluoroacetolysis of Peptides. TFA (100 µl) was added to 0.1 mg amounts of peptides in 1-ml screw-cap vials (size 25 mm × 10 mm ID). The tightly closed vials were immediately placed in a sand bath and incubated at 37 ± 1° for various periods of time.

After 0.5, 1, 3, 8, 15, 20, and 26 h treatment, TFA was immediately removed in a cold stream of N_2, CH_2Cl_2 was added to the dry residue, and vials were flushed again with N_2 in order to remove traces of TFA. The remaining residues were dissolved in MeOH (100 µl), and aliquots (10 µl) were subsequently analyzed by LC/ESI-CID-MS.

Instruments and Conditions for HPLC. For HPLC, a *HP 1100* series instrument comprising a model *G1322A* degasser, *G1312A* binary pump, *G1313A* autosampler, *G1316A* column thermostat, *G1314A* UV/VIS detector, and *HP ChemStation* software for LC (Rev. A.04.02) was used (all from *Agilent*, D-Waldbronn, or Palo Alto, CA, USA). The HPLC column used (150 mm × 4.6 mm ID) was filled with *Kromasil 100–3 C_8* material, particle size 3 μm. The column was kept at 35°. Elution profiles of peptides were recorded at a wavelength of 205 nm or as total ion currents (TIC) in on-line-ESI-MS. The following LC gradient was used: eluent *A:* MeOH/MeCN/H_2O 32 : 32 : 36; eluent *B:* MeOH/MeCN 50 : 50. TFA (0.1%) was added to both eluents.

Time [min]	Eluent [%]		Flow rate [ml/min]
	A	B	
0	100	0	0.8
5	100	0	0.8
45	50	50	0.8
65	0	100	0.8
75	0	100	1
76	100	0	1
85	100	0	1

Mass Spectrometry. For ESI-CID-MS, a calibrated *LCQ-MS*™ (*Thermo Quest*, Finnigan MAT, San Jose, CA, USA) was used. N_2 served as sheath and auxiliary gas. He (purity > 99.9990%, *Messer-Griesheim*, D-Krefeld) was used as collision gas. The temp. of the heated capillary was 250°; sheath gas and auxiliary gas were set at 40 and 10 relative units, resp. Ion source collision-induced dissociation (CID)MS was performed at 0 and 45% relative collision energy. Sequence analyses were carried out in the positive ionization mode. The *m*/*z* values were recorded in centroid mode and have an accuracy of ± 0.5 Da.

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