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Solution phase synthesis of the 14-residue peptaibol antibiotic trichovirin I*

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Summary. The 14-residue peptaibol antibiotic trichovirin I 4A of the structure Ac-Aib-L-Asn-L-Leu-Aib-L-Pro-L-Ala-L-Val-Aib-L-Pro-Aib-L-Leu-Aib-L-Pro-L-Leuol (Aib = α-aminoisobutyric acid, Leuol = leucinol) was synthesized by stepwise conventional solution phase synthesis using the Z/O*t*Bu(OMe) strategy and HOBt/EDC as coupling reagents. Intermediates were fully characterized and the identity of the synthetic peptide with the component 4A of the natural, microheterogeneous peptide mixture was proven by electrospray mass spectrometry, HPLC, and bioassay.

Keywords: Peptaibols – Peptide antibiotics – Trichovirin – Peptide synthesis – *Trichoderma viride* – α-Aminoisobutyric acid (Aib)

Abbreviations: Amino acids are abbreviated according to three-letter-nomenclature; Aib, α-aminoisobutyric acid (2-methylalanine); Iva (isovaline, 2-ethylalanine); Leuol, L-leucinol [(*S*)-2-amino-4-methyl-1-pentanol]; AAA, amino acid analysis; EI-MS, electron impact mass spectrometry; ESI-MS, electrospray ionization mass spectrometry; HPLC, high performance liquid chromatography; Z, benzyloxycarbonyl; Fmoc, 9-fluorenylmethyoxycarbonyl; O*t*Bu, *tertiary* butoxy (*tert*-butylester); OMe, methoxy (methyl ester); OBzl, benzyloxy (benzyl ester); TDM, N,N,N′,N′-tetramethyl-4,4′-diamino-diphenylmethane (Arnold's base); for other abbreviations see Experimental.

Introduction

Antibiotics of the peptaibol family are defined as *N*-acylated linear polypeptides with a high proportion of non-protein Aib (that, occasionally, might be replaced by D- or L-isovaline, Iva) and a *C*-terminal bound 1,2-amino alcohol. These peptides are produced by filamentous fungi such as *Trichoderma*, *Gliocladium*, *Hypocrea* or *Stilbella*.

The 14-residue peptaibols trichovirins I and the 18-residue trichovirins II are concomitantly produced by the mold *Trichoderma viride* NRRL 5243 and can be isolated from the culture broth of the fungus cultured under submerge conditions. The sequences of trichovirins I (Brückner et al., 1991) and trichovirins II (Jaworski et al., 1999) could be determined using mainly mass spectrometric techniques.

Owing to their biosynthesis utilizing multienzyme complexes, trichovirins, in analogy to other peptaibols, are usually produced as very microheterogeneous mixtures differing by exchange of amino acids or amino alcohols. Such mixtures might also comprise groups of peptides differing by polarity or length of peptide chains.

Consequently, for the investigation of individual peptaibols highly effective liquid chromatographic methods have to be employed for their separation and isolation. Alternatively, peptides have to be synthesized chemically using classical segment condensation as well as syntheses utilizing solid-phase procedures. Chemical total synthesis, however, is severely hampered by the presence of sterically constrained Aib or Iva.

Among the peptaibols the 20-residue peptide alamethicin was the main target for various synthetic approaches since it was the first peptaibol that was structurally characterized and the sequence of which, as a result of conflicting reports on the primary structure, had to be confirmed by total synthesis. Further, for studies related in particular to experiments on voltage-gated pore formation, there was need for pure

* Dedicated to Prof. Dr. Günther Jung. Tübingen University, on the occasion of his 65th anniversary.

peptides. Finally, again as a result of the presence of Aib, the peptide is an excellent model for testing the effectivity and safeness of new coupling reagents with regard to yields as well as absence of racemization of chiral amino acids.

The problems and drawbacks encountered with foregoing attempts on the syntheses of alamethicin carried out by Balasubramanian et al. (1981), Nagaraj and Balaram (1981), and Gisin et al. (1981) have been discussed in the detailed protocols of the alamethicin synthesis performed by Schmitt and Jung (1985). These authors employed mainly the Boc/OMe strategy for the protection of segments and HOBt/DCCI coupling for the total synthesis of the main component.

The Z/OMe strategy and DCC/HOBt were also used for the stepwise solution synthesis of 11-residue peptaibols trichorozins I–IV (Iida et al., 1995), the 7-residue peptaibol trichodecenin II (Fujita et al., 1994), and the 20-residue peptaibol trichosporin B-VIa (Nagaoko et al., 1994). The 11-residue peptaibol trikoningin KB II and analogs were synthesized using also the Z/OMe approach and EDC/HOAt or the mixed anhydride method for peptide coupling (Piazza et al., 1999). For the synthesis of isotopically labelled fragments of zervamicin Ogrel et al. (1997) used the FMOC/O*t*Bu strategy and BOP/DMAP for activation or FMOC-Aib-Cl.

Taking into account the wealth on information on the solution synthesis of complete and partial syntheses of natural peptaibols and Aib-containing model peptides, Słomczynska et al. (1992a) performed the total synthesis of the 15-residue peptaibols emerimicins III and IV and their epimers containing Iva. Syntheses of these peptides was considered to comprise all of the synthetic challenges previously encounterd in other works on peptaibols, i.e. in particular presence of homosequences of Aib, presence of two hydroxyproline residues and, in the case of the (Iva^{12})-epimer, an α,α-dialkyl amino acid that is sterically even more hindered as Aib. Using established coupling procedures and reagents (HOBt/DCC, mixed anhydrides, oxazolones) and protecting groups (Z/O*t*Bu, Boc/OBzl), the syntheses of segments was optimized. However, since the final coupling step using HOBt/DCC proceeded, with a low yield of 12%, enzymic coupling with papain was found to be superior.

The chemo-enzymic approach was also applied to the solution synthesis of alamethicin (Słomczynska et al., 1992b) and antiamoebin I (Leplawy et al., 1996).

A special approach for the syntheses of segments of alamethicin (Wipf and Heimgartner, 1990), trichotoxin A-50G (Altherr and Heimgartner, 1991) and antiamoebin I (Altherr und Heimgartner, 1992), and the *C*-terminal nonapeptide of trichovirin I 1B (Luykx et al., 1996) was the so-called azirine/oxazolone method introduced by Heimgartner and coworkers (Koch and Heimgartner, 2000).

The development of highly reactive coupling reagents such as uronium (HBTU) and phosphonium salts (BOP, PyBroP) and of activated amino acids such as *N*-carboxyanhydrides (NCA's) and amino acid fluorides and test of their suitability for the synthesis of sterically constrained peptides provided evidence that they might complete or replace the traditional coupling reagents of peptide chemists (Spencer et al., 1992; Auvin-Guette et al., 1993; Ogrel et al., 1997). Their suitability for the entire solution synthesis of natural peptaibols, however, was not demonstrated up to now.

Whereas early attempts on the solid phase peptide synthesis (SPPS) of alamethicin using HOBt/DCC activation gave disappointing results, the situation changed favorably with the introduction of FMOC-amino acid fluorides (including FMOC-Aib-F). Use of this approach made possible the effective SPPS of alamethicins and a set of analogs distinguished by the position of Pro (Wenschuh et al., 1994, 1995), saturnisporins and a trichotoxin A50 component (Wenschuh et al., 1995). Notably, the reaction under SPPS conditions proceeded fast and lead to pure products also with regard to the chirality of the amino acids used. This is in contrast to the use of UNCA's or PyBroP activation used comparatively for the SPPS synthesis of alamethicin acid or elongation of a peptide by four consecutive Aib-residues (Wenschuh et al., 1994). This approach was extended on the stepwise solid phase assembly of various peptaibols (Carpino et al., 1996; Triolo et al., 1998).

In the following we describe in detail the total synthesis of the 14-residue peptaibol trichovirin I, component 4A, using entirely solution phase synthesis. An abstract of the synthesis described here as experiment A (see Fig. 1) has been reported (Brückner and Koza, 1992).

General considerations for the synthesis

For the solution phase synthesis of trichovirin I the component 4A of the natural microheterogenous

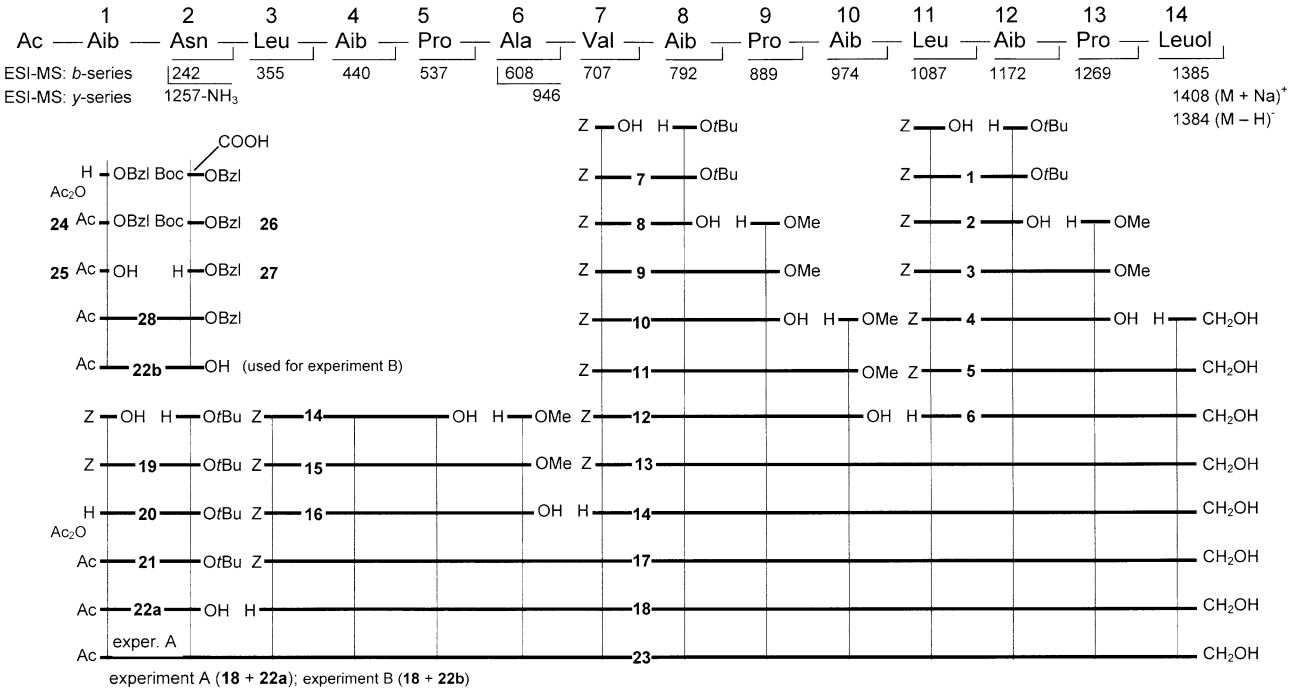

Fig. 1. Scheme of the solution synthesis of trichovirin I A4 indicating also abundant diagnostic nominal masses of peptide fragments determined by ESI-MS

peptide mixture was selected. This peptide is one of the major components with the advantage that it does not contain the sterically highly hindered D(=*S*)-Iva, in contrast to trichovirin peptides 2A, 3C, 5B, 6C, and 7A.

A benefit of solution syntheses is that segments can be characterized by chemical and spectroscopic methods as well as x-ray crystallography. Use of EDC instead of dicyclohexylcarbodiimide (DCCI) has the advantage that byproducts are easily removed from lipophilic Aib-peptides by partition between organic phase and water.

The additive HOBt has been used in order to prevent racemization of AA in the course of the carboxy activation of segments. This is of particular importance when Aib constitutes the *C*-terminal AA, and when prolonged reaction times and slightly increased temperatures are required for peptide coupling (Schmitt and Jung, 1985). It had also been shown that in particular *C*-terminal Aib in segments easily forms oxazolones which induce racemization of preceeding chiral AA *via* tautomerism (Brückner 1989; Brückner and Currle, 1989).

For the synthesis of segments the Z/O*t*Bu and Z/OMe strategy were used (with the exception of the dipeptide used in experiment B). It has been reported that saponification of peptides with *C*-terminal Aib methyl ester or hydrogenolysis of *C*-terminal Aib benzyl ester is incomplete and deprotection decreases with inceasing chain lengths of peptides. In contrast, acidic cleavage of the *t*-butyl group of *C*-terminal Aib *t*-butyl ester was expected to proceed fast even in large segments.

Further, treatment of natural or synthetic peptides containing Aib-Pro bonds with acids such as TFA bears the risk of, at least partial, cleavage of this particular acid sensitive bond (Brückner et al., 1985). In contrast, the Aib-Pro bond is completly stable towards alkaline saponification as used in peptide chemistry.

Taking the above considerations into account, as well as a set of preliminary rules compiling our experiences with the coupling of Aib-peptides using the EDC/HOBt approach (Brückner and Currle, 1989), the selection of segments as presented in Fig. 1 for the synthesis of trichovirin was chosen.

Results and discussion

Synthesis of segments Z-3-6-OH and Z-7-10-OH

Since the sequence Leu-Aib-Pro occurs twice in trichovirin (positions 3–5 and 11–13), Z-Leu-Aib-

O*t*Bu was synthesized and, after *C*-terminal deprotection with TFA, Z-Leu-Aib-Pro-OMe was obtained by coupling the dipeptide acid with H-Pro-OMe. The methyl ester was used in order to circumvent any acidic treatment of the tripeptide. Saponification of the methyl ester was carried out by addition of 2 equivalents of NaOH. Traces of unreacted ester, however, were still detectable by TLC towards the end of the reaction. Therefore, pH 10.5 of the solution was adjusted and the methyl ester extracted with ethyl acetate. The tripeptide acid remained as sodium salt in the aqueous phase. It was observed that the aqueous phase had to be maintained at pH 10.5 in order to avoid partial extraction of the particle into the organic phase. The aqueous solution was adjusted to pH 2, the tripeptide acid was extracted with AcOEt. Using this tripeptide as starting material, the segments Z-Leu-Aib-Pro-Ala-OMe (Z-3-6-OMe) and Z-Leu-Aib-Pro-Leuol (Z-11-13-ol) were synthesized. For the synthesis of Z-3-6-OH the methyl ester was saponified and provided the tetrapeptide acid.

Segments Z-11-14-ol and Z-7-10-OMe

For the synthesis of the *C*-terminal segment Z-11-14-ol, L-Leuol without protection of the hydroxyl group was coupled to Z-Leu-Aib-Pro-OH. Hydrogenolysis of Z-11-14-ol yielded H-11-14-ol that was coupled to Z-7-10-OH providing the segment Z-7-14-ol.

The synthesis of the protected tripeptide Z-Val-Aib-Pro-OMe (segment Z-7-9-OMe), followed by saponification, yielded Z-Val-Aib-Pro-OH (segment Z-7-9-OH) and proceeded, as expected, in analogy to the synthesis of Z-Leu-Aib-Pro-OH; however, Z-Val-Aib-Pro-OH was obtained as an oil. Coupling of this tripeptide acid to H-Aib-OMe yielded Z-Val-Aib-Pro-Aib-OMe (segment Z-7-10-OMe). Saponification of the tetrapeptide provided Z-Val-Aib-Pro-Aib-OH (segment Z-7-10-OH). This demonstrates that Aib methyl ester, when used as amino components, give good coupling yields with *C*-terminal Pro using the EDC/HOBt approach. This is in contrast to peptides with Aib as *C*-terminus giving low coupling yields on reaction with Pro-OMe. In order to accelerate the saponification of Z-7-10-OMe, the reaction was carried out at 30°C at the beginning.

Segments Z-7-14-OH and Z-11-14-ol

Coupling of the segment Z-7-10-OH to H-11-14-ol yielded Z-7-14-ol. A mixture of AcOEt and 1-BuOH was used for the extraction of the segment from the aqueous phase in order to avoid losses of the amphiphilic octapeptide bearing the *C*-terminal amino alcohol.

Segments Z-3-14-ol, Ac-1-2-OH, and synthesis of AC-1-14-ol (trichovirin I 4A)

Hydrogenolysis of the protected octapeptide yielded almost quantitatively the segment H-7-14-ol. Coupling of H-7-14-ol to Z-3-6-OH furnished Z-3-14-ol and hydrogenolysis of the dodecapeptide yielded the segment H-3-14-ol quantitatively.

The final step of the TV synthesis was coupling of H-3-14-ol to Ac-Aib-Asn-OH. For the synthesis of Ac-Aib-Asn-OH (Ac-1-2-OH), in the first step Z-Aib-OH was coupled to H-Asn-O*t*Bu. The protected dipeptide Z-Aib-Asn-O*t*Bu was hydrogenated, and the resulting H-Aib-Asn-O*t*Bu was acetylated quantitatively with Ac_2O. The resulting Ac-Aib-Asn-O*t*Bu was deprotected by treatment with TFA. The resulting Ac-Aib-Asn-OH **22a** was contaminated with a side product (probably the aspart imide) which could not be removed completely. This was assumed to be the rational that the final coupling of Ac-1-2-OH to H-3-14-ol provided Ac-1-14-ol (TV I A4) **23** that contained impurities which had to be removed by preparative HPLC (see Fig. 1, experiment A). This lowered the final yields of the 14-residue peptides resulting from two syntheses to 29% and 37%, respectively. In order to avoid acidic treatment of the protected dipeptide, Ac-Aib-Asn-OH **22b** was also synthesized from Ac-Aib-Asn-OBzl by hydrogenolysis. The benzyl ester was obtained by coupling Ac-Aib-OH to H-Asn-OBzl. However, use of this analytically pure dipeptide acid did not increase the final yield of **23** after purification by HPLC (see Fig. 1, experiment B). Success of the syntheses, identity of the peptides resulting from experiments A and B, and confirmation of the primary structure that had been determined for TV I A4 (Brückner et al., 1991), was proven as follows.

HPLC of the natural mixture of TVs, the mixture with synthetic TV resulting from experiment A, and the chromatograms of TV isolated from the natural mixture, the synthetic TV, and a mixture of the natural and synthetic peptide are shown in Fig. 2. The chromatograms demonstrate the purity of the synthetic peptaibol and coelution with the natural peptide. Chiral GC-MS showed low racemization of the final product.

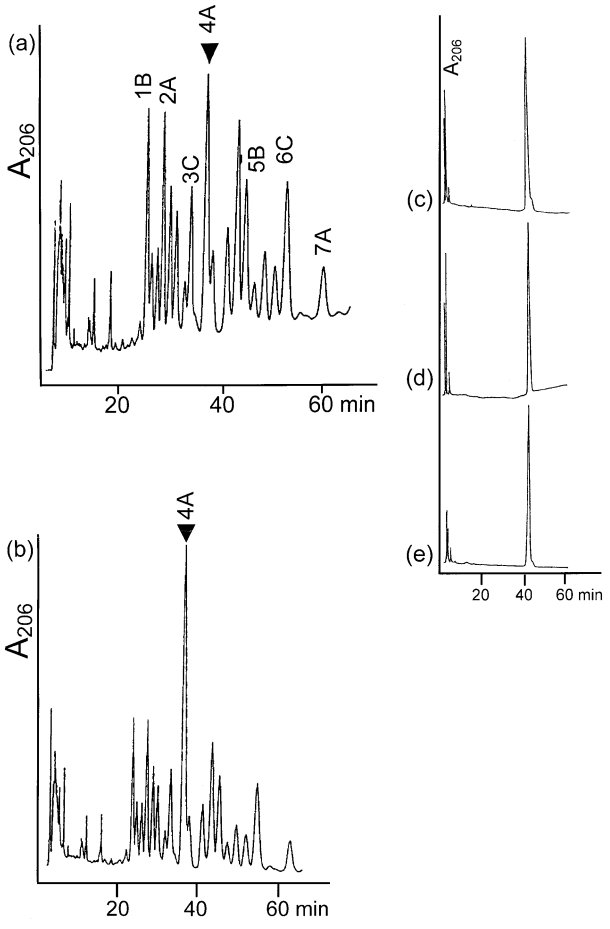

Fig. 2. Analytical HPLC of (**a**) the natural mixture of trichovirin (TV) I peptides and assignment of peptides with known sequences; component TV 4A is indicated by arrow, (**b**) natural mixture with synthetic TV 4A added, (**c**) component 4A isolated from the natural mixture, (**d**) synthetic TV 4A resulting from experiment A, and (**e**) mixture of (**c**) and (**d**). Chromatographic conditions, column 250 × 4 mm i.d.; stationary phase, LiChrospher 60 RP select B; particle size, 5 μm; eluent, MeCN/MeOH/water 40/30/30/(v/v); flow rate, 1 ml/min

ESI-MS from TV resulting from experiment A (see Fig. 1) provided the protonated molecular ion at *m/z* 1387.0 and sequence specific fragment ions of the *b*-series (see Experimental). Diagnostic ESI mass spectra characterizing the synthetic peptide from final coupling experiment B (see Fig. 1) are presented in Fig. 3. Full scan positive ion MS provided the sodium adduct of the molecular ion at *m/z* 1409.2 as well as the *b*-series of acylium ions at *m/z* 242.4 (b_2), 355.5 (b_3), 440.6 (b_4), 792.8 (b_8), 974.8 (b_{10}), and 1172.9 (b_{12}) (Fig. 3a). MS-MS of the precursor ion at *m/z* 1172.0 generated ions at *m/z* 355.3 (b_3), 440.4 (b_4), 608.3 (b_6), 707.5 (b_7), 792.4 (b_8), 975.3 (b_{10}), and 1088.3 (b_{11}) (Fig. 3b). MS-MS of precursor ion at *m/z* 792.6 furnished the acylium ion of the *N*-terminal dipeptide at *m/z* 242.0 (b_2) among other diagnostic ions of the *b*-series. MS-MS of the precursor ion at *m/z* 1385 provided abundant ions the *y*-series of at *m/z* 945.9 (y_{10}), 1144.0 (y_{12}), and 1240.7 ($y_{13} - NH_3$). Notably, in the negative ion mode the negatively charged $(M\text{-}H)^-$-ion at *m/z* 1385.1 was most intensive (MS not shown). For nominal molecular weights of fragments see also Fig. 1. For terminology and mechanistic aspects of fragmentations, in particular the prefered cleavage of the Aib-Pro bonds, we refer to our papers on peptaibol sequencing (Jaworski et al., 1999; Jaworski and Brückner, 1999, 2000, 2001). The optical purity of the synthetic peptide was determined by chiral GC after total hydrolysis and derivatization of residues released. Hydrolysis and chiral analysis of the natural mixture of TV performed in parallel revealed, besides acid catalyzed background racemization, a slightly increased racemization of certain amino acids of the synthetic peptide. The synthetic peptide showed inhibition of *Streptococcus faecalis* in plate diffusion antibiotic assay in complete agreement with the natural peptaibol mixture (see Experimental).

Experimental

Instruments and methods

Melting points: Model B 520 apparatus (Büchi, Flawil, CH), open capillaries, not corrected. – Elemental analyses: Model CHN elemental analyzer (Carlo-Erba, Milan, I); peptides were dried under high vacuum over P_4O_{10} (Siccapent, Merck). For AAA a dedicated LC 6000 analyzer (Biotronik, Maintal, Germany) and post-column derivatization with ninhydrin werde used. – TLC: precoated silica gel plates $60F_{254}$ (Merck), saturated glass chambers (Desaga, Heidelberg, Germany); solvent systems (A)–(D) (v/v): (A) chloroform/MeOH 80:20; (B) chloroform/MeOH/AcOH/water 65:25:3:4; (C) 1-BuOH/AcOH/water 30:10:10; (D) AcOEt/petroleum ether/MeOH 50:70:60; products were visualized on plates by successive spraying with water, ninhydrin (0.1% in EtOH) (amino groups) and TDM-reagent (peptide bonds); TDM-reagent: to TDM (7.5 g) in AcOH (30 ml), water (150 ml) was added, the solution was filtered and KJ (15 g) in water (300 ml) was added. Dry TLC-plates were treated with chlorine for 15 min in a dessicator, excess of Cl_2 was removed by fanning plates with cold air and *N*-chlorinated peptides were visualized by spraying with TDM-reagent. For the determination of retention factors (R_f), 2–3 μl-aliquots of 1% solutions of derivatives were spotted onto plates (20 × 20 cm, 0.25 mm); distance start to solvent front, 10 cm; Ac-Aib-OH (internal standard) R_f(A) 0.28; R_f(C) 0.84; R_f(D) 0.69. Optical rotation: Series 241 polarimeter (Perkin-Elmer, Überlingen, Germany), cuvette 1 dm; 20°C. – Racemization test of total hydrolysates (6 M HCl/24 h/110°C) of peptides by GC: (a) Model 4100 and (b) Model 5160 instruments (Carlo-Erba) equipped with (a) Chirasil-D-Val capillary column (25 mm × 0.25 m i.d.) (Chrompack, Middelburg, NL), and (b) Lipodex E column (0.25 m × 0.25 mm i.d.) (Macherey-Nagel, Düren, Germany); carrier gas,

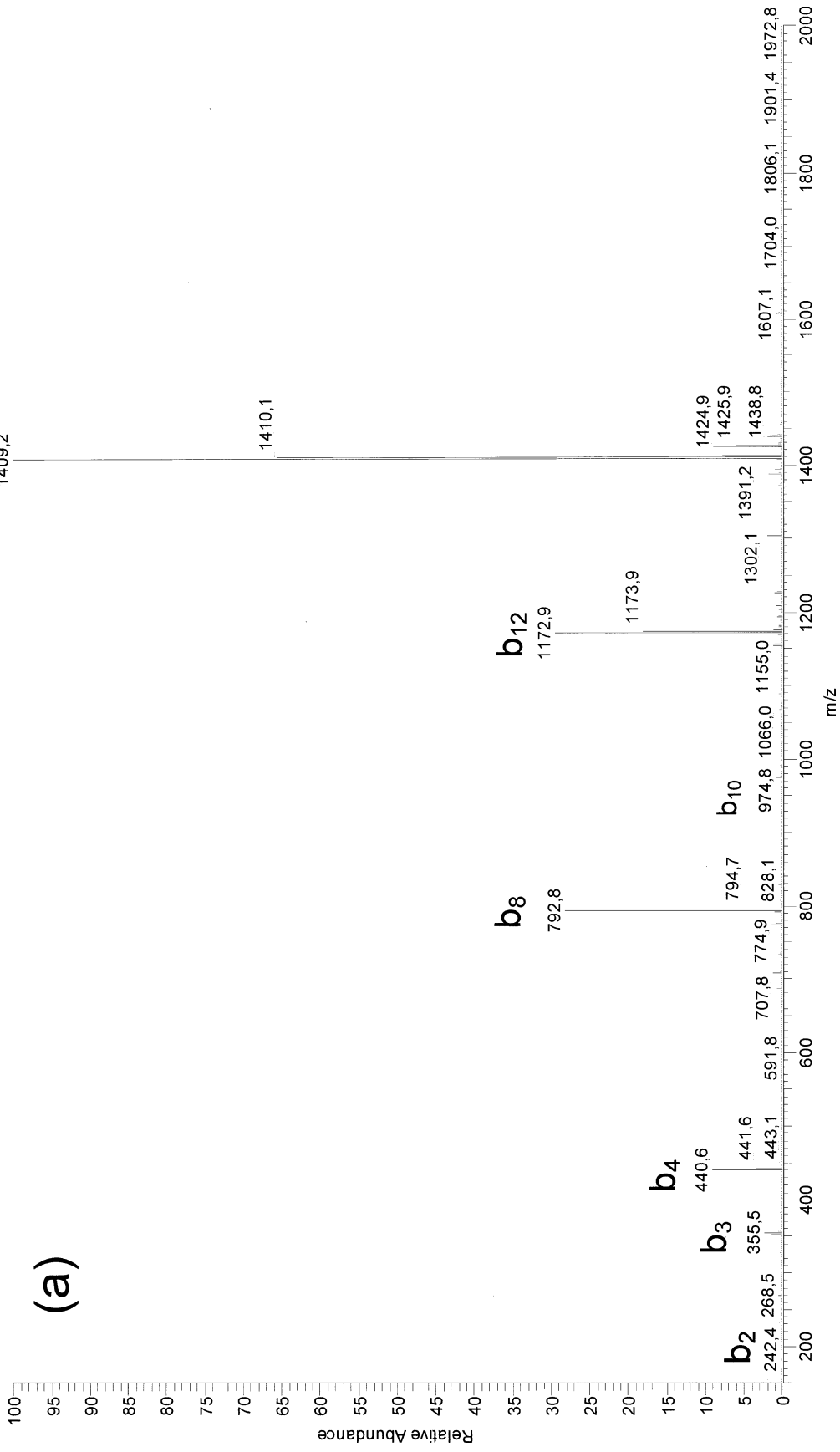

Fig. 3. Characterization of synthetic trichovirin I 4A from experiment B by (**a**) positive-ion ESI-MS, and (**b**) MS-MS of precursor ion at *m/z* 1,172 (b_{12}) Conditions: direct infusion (c = 0.1%, MeOH); capillary temp., 220°C; capillary voltage, 46.11 V; tube length offset, 55 V; maximal ion time, 400 ms

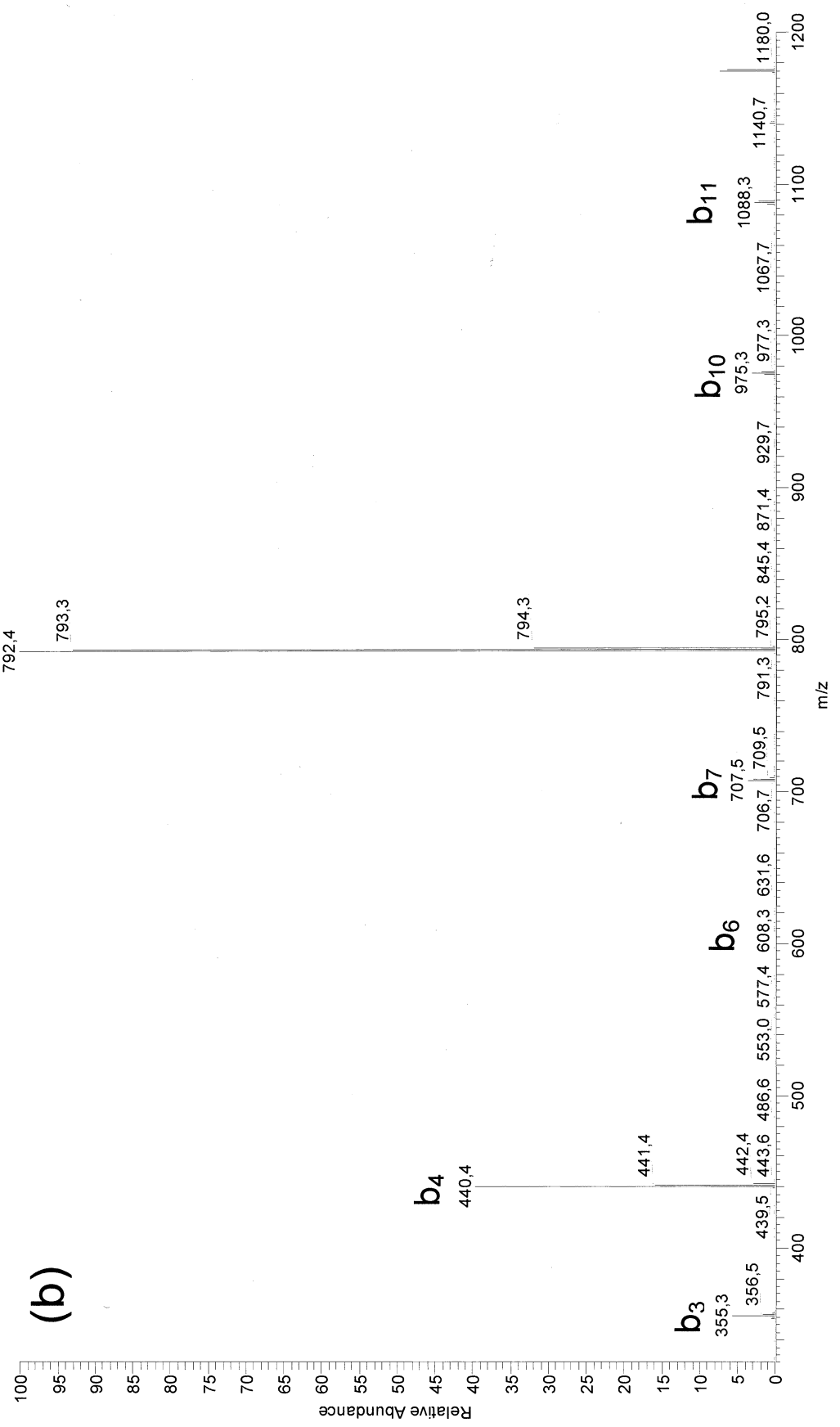

Fig. 3. *Continued*

hydrogen, 0.8 kg/cm; (a) TFA-2-propyl ester and (b) TFA-methyl ester; for chiral analysis peptides were hydrolysed (6 M HCl, 110°C, 24 h) and esterified with 2.5 M HCl in 2-PrOH or 2.5 M HCl in MeOH (100°C, 1 h), followed by trifluoroacetylation with TFAA (100°C, 20 min). Amounts of D-amino acids (%) were calculated from peak areas according to % D = 100D/(D + L); values are *not* corrected for acid catalyzed racemization. – ESI-MS: TSQ-700 instrument (Finnigan, Bremen, Germany); peptide segments were dissolved in MeOH that was acidified with aqueous AcOH. The final peptide resulting from experiment B was characterized using an LCQ instrument (Finnigan). For electron impact mass spectrometry (EI-MS) a Finnigan MS was used at an ionization energy of 70 eV.

For preparative HPLC a Series 3B instrument (Perkin Elmer) was used and a Hibar^R column RT 250-10 (250 mm × 16 mm i.d.) together with pre-column, packed with LiChrospher^R 60 RP Select-B (5 μm) (Merck). The eluent was MeCN/MeOH/water (40:30:20) at a flow rate at 7 ml/min; absorbance was measured at 215 nm. Crude trichovirins resulting from experiments A and B were subjected to HPLC in 10 mg aliquots in 100 μl MeOH. Fractions of 15 ml were collected manually and suitable fractions combined and evaporated to dryness. For analytical HPLC a Model 2200 instrument together with a Lambda 1000 spectral photometer, operated at 210 nm (Bischoff Analysentechnik, Leonberg, Germany) were used and 250 mm × 4 mm i.d. columns packed with LiChrospher^R 100 RP 18, particle size 5 μm (Merck). Flow rate was 1 ml/min for eluents A–F. Eluent A (v/v): MeOH/water/TFA (55:45:0.1); eluent B, MeOH/water (75:25); eluent C, MeOH/water (50:50); eluent E, MeOH/water/TFA (55:45:0.1); eluent F, MeOH/water (60:40); retention times t_R of peptides are given (not corrected for void volume).

For testing antibiotic activity using a plate diffusion assay, synthetic and natural TV (200 μg/10 μl MeOH) on cellulose discs (6 mm diameter) were placed on HNB complex agar that was incubated with *Staphylococcus aureus* (DSM 20372), *Streptococcus faecalis*, *Bacillus subtilis* (Freese/USA no. 60009) (conditioned for 24 h/4°C; 24 h/37°C) and Grove-Randall-Agar incubated with *Micrococcus luteus* (DSM 20030) (conditioned for 24 h/4°C, 24 h/30°C). Both peptides gave the same inhibition zones of 12 and 18 mm (2 experiments) for *S. faecalis*, and were weakly positive under these conditions on *S. aureus* and *M. luteus*, and negative on *B. subtilis*.

Chemicals and solvents

Chemicals were purchased from Fuka (Buchs, Switzerland) or Merck (Darmstadt, Germany), if not otherwise specified and are abbreviated as follows: 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and 1-hydroxybenzotriazole hydrate (HOBt · aq) (12–17% water); petroleum ether (b. p. 50–70°C), methanol (MeOH), acetonitrile (MeCN), dimethylformamide (DMF), ethyl acetate, *N*-methylmorpholine, 97% (NMM), *n*-hexane, diethyl ether (Et_2O), ethyl acetate (AcOEt), dichloromethane (DCM), 1-propanol (1-PrOH), 2-propanol (2-PrOH), 1-butanol (1-BuOH), benzyl alcohol (BzlOH), acetic acid (AcOH), acetic anhydride (Ac_2O), acetyl chloride (AcCl), trifluoroacetic acid (TFA), trifluoroacetic anhydride (TFAA), pentafluoropropionic acid anhydride (PFPAA), pentafluoropropionyl (PFP), benzyloxycarbonylchloride (Z-Cl).

Amino acids and derivatives

α-Aminoisobutyric acid (Aib; 2-methylalanine) was from Senn Chemicals, Dielsdorf, Switzerland; H-Ala-OMe × HCl, H-Asn-O*t*Bu, Boc-Asp-OBzl Z-Val-OH, Z-Leu-OH, H-Pro-OMe × HCl were from Bachem, Bubendorf, Switzerland and L-leucinol from Fluka.

Z-Aib-OH was synthesized by reaction of Aib with Z-Cl according to Leplawy et al. (1960). Z-Aib-O*t*Bu was obtained by acid catalyzed esterification of Z-Aib-OH with isobutylen; hydrogenolysis of Z-Aib-O*t*Bu in MeOH with 10% Pd on charcoal as catalyst afforded H-Aib-O*t*Bu that was obtained as colorless liquid b. p. 160–163°C by fractionated distillation. H-Aib-OMe × HCl was synthesized by thionyl chloride (Merck) catalyzed esterification of Aib in MeOH. H-Aib-OBzl × Tos was synthesized by toluolsulfonic acid (Tos) catalyzed esterification of Aib with BzlOH. The free ester was obtained as colorless liquid by partition between 10% aqueous Na_2CO_3 and Et_2O, followed by fractionated destillation of the organic phase. Ac-Aib-OH was synthesized by acetylation of Aib with Ac_2O in alkaline solution or by acetylation of H-Aib-OBzl with Ac_2O followed by hydrogenolytic cleavage of the benzyl ester.

Syntheses of peptides

As syntheses of segments was carried out several times typical protocols are reportet. Since HOBt hydrate contains water an excess based on weight was used. Stoichiometry of peptides is based on first amino acid given; calculated ratios are in paranthesis.

General procedures. Peptide coupling was carried out using HOBt · aq and EDC exclusively. The carboxy component was dissolved in a suitable solvent and HOBt · aq was added at ambient temperature with stirring. EDC was not added until HOBt · aq was completely dissolved. Since HOBt · aq contained variable amounts of water, an excess was used. Reactions were performed at ambient temperature if not otherwise specified. Saponification of Z-Val-Aib-Pro-OMe and Z-Leu-Aib-Pro-OMe did not proceed to completeness. Traces of unsaponified peptides were still detectable by TLC. The reaction solutions were therefore adjusted to pH 10.5 by addition of 1 M NaOH and the unreacted methyl esters were extracted by threefold treatment with AcOEt. It was necessary to adjust pH 10.5 after each extraction step.

Washing procedure A. Fully protected segments were dissolved in AcOEt (ca. 0.5% g/v) and transferred into a separatory funnel. The organic phase was washed with 5% aqueous $KHSO_4$ (3×), 5% $NaHCO_3$ (3×), and water until neutral. Then the organic phase was dried over anhydrous Na_2SO_4, filtered through a sintered glass frit and evaporated to dryness.

Washing procedure B. Protected peptides sparingly soluble in AcOEt were dissolved in 1-BuOH/AcOEt (2:1, v/v) and washing was carried out as described for procedure A. The organic phase was evaporated without drying over Na_2SO_4.

Washing procedure C. Peptides with free carboxyl group were dissolved in AcOEt and treated according to procedure A with the exception that washing with $NaHCO_3$ was omitted.

Hydrogenolysis. For the cleavage of Z- and Bzl-groups the peptides were dissolved in MeOH (ca. 1%, w/v), then 10% (w/w with respect to peptides) of catalyst (10% Pd on charcoal) was added under nitrogen. Hydrogen was gently bubbled through the mixture and completeness of deprotected was monitored by TLC. The catalyst was removed by filtration through a narrow pore glass frit and the filtrate was evaporated to dryness. Peptides with free amino groups were used for syntheses without further purification.

*Z-Leu-Aib-OtBu (**1**)*

To Z-Leu-OH (16.2 g, 61.1 mmol) in a mixture of MeCN (100 ml) and DMF (100 ml), HOBt · aq (16.5 g) and EDC (11.7 g, 61.1 mmol) were added with stirring. After 1 h H-Aib-O*t*Bu (9.7 g, 61.1 mmol) in MeCN (50 ml) was added. After 46 h the mixtures was evaporated

to dryness, AcOEt (0.8 L) was added and the organic phase treated according to procedure A. After drying with Na_2SO_4 and evaporation to dryness a colorless oil remained which was dissolved in *n*-hexane and precipitated by addition of petroleum ether; yield 21.0 g (85%); m.p. 81°C; $[\alpha]_D^{20} = -28$ ($c = 1$, MeOH); R_f(A) = 0.79, R_f(B) = 0.87, R_f(C) = 0.89; HPLC (D): t_R = 8.20 min; AAA: Leu (1); Aib 1.44 (1), EI-MS: m/z = 407 $[M + H]^+$; 305 $(M-101)^+$; GC: D-Leu 1.0%.

Calcd. for $C_{22}H_{34}O_5N_2$ (406.53) C 65.00, H 8.43, N 6.89; found C 65.02, H 8.51, N 6.87.

*Z-Leu-Aib-OH (**2**)*

To Z-Leu-Aib-O*t*Bu **1** (10.3 g, 25.3 mmol) in DCM (20 ml), TFA (100 ml) was added. After 2 h the mixture was evaporated to dryness, water (150 ml) was added and pH 10.5 was adjusted by addition of 2 M NaOH. The solution was extracted with AcOEt (3 × 30 ml) and the organic phase discarded. Then the aqueous phase was adjusted to pH 2.0 by addition of 2 M HCl and extracted with AcOEt (3 × 30 ml). These organic phases were combined and treated according to procedure C. The resulting residue was dissolved in AcOEt and the peptide precipitated by addition of petroleum ether.

Yield: 7.0 g (79%); m.p. 116°C; $[\alpha]_D^{20} = -23.1$ (c = 1, MeOH); R_f(A) 0.32, R_f(B) 0.69, R_f(C) 0.94; EI-MS: m/z = 350 $[M]^+$, 220 $(M-130)^+$; GC: D-Leu 0.6%. HPLC: t_R (eluent A) = 5.12 min; AAA: Leu (1); Aib 1.10 (1).

Calcd. for $C_{18}H_{26}O_5N_2$ (350.42): C 61.70, H 7.48, N 7.99; found C 61.41, H 7.52, N 7.81.

*Z-Leu-Aib-Pro-OMe (**3**)*

To Z-Leu-Aib-OH **2** (7.0 g, 19.98 mmol) was dissolved in DMF (140 ml) and HOBt · aq (5.3 g) and EDC (3.8 g, 19.82 mmol) were added. After stirring for 30 min, H-Pro-OMe × HCl (3.6 g, 21.74 mmol) and NMM (2.2 g, 22 mmol) were added and the mixture was stirred for 68 h at 30°C. After evaporation to dryness, AcOEt (0.7 L) was added and the organic phase was treated according to procedure A. Crystalline Z-Leu-Aib-Pro-OMe was obtained.

Yield: 8.0 g (87%); m.p. 132°C; $[\alpha]_D^{20} = -86.6$ (c = 1, MeOH); R_f(A) 0.82, R_f(B) 0.85, R_f(C) 0.86; ESI-MS: m/z = 462.3 $[M + H]^+$; 484.3 $[M + Na]^+$; AAA: Leu (1), Aib 1.12 (1), Pro 1.05 (1); GC: D-Leu 1.9%; D-Pro 2.5%. HPLC: t_R (eluent F) = 5.25 min.

Calcd. for $C_{24}H_{35}O_6N_3$ (461.56) C 62.46, H 7.64, N 9.10; found C 62.67, H 7.65, N 9.14.

*Z-Leu-Aib-Pro-OH (**4**)*

To Z-Leu-Aib-Pro-OMe **3** (8.0 g, 17.33 mmol) in MeOH (100 ml), 34.5 ml (34.7 mmol) 1 M NaOH were added with stirring. After 16 h at r.t. and 5 h at 37°C, 34.5 ml (34.5 mmol) 1 M HCl were added. A pH of 10.5 was adjusted by addition of 2 M NaOH and the solution was extracted with AcOEt (3 × 15 ml). The organic phases were discarded and the aqueous phase was adjusted to pH 2.0 by addition of 2 M HCl. The aqueous phase was extracted with AcOEt (3 × 15 ml) and the combined organic phases were treated according to procedure C. The resulting oily residue was dissolved in a mixture of MeOH and AcOEt and the tripeptide was precipitated by addition of petroleum ether.

Yield: 6.5 g (84%); m.p. 116°C; $[\alpha]_D^{20} = -81.3$ (c = 1, MeOH); R_f(B) 0.69, R_f(C) 0.79; ESI-MS: m/z = 448.2 $[M + H]^+$; 470.2 $[M + Na]^+$; AAA: Leu (1), Aib 0.94 (1), Pro 1.10 (1); GC: D-Leu 2.3%, D-Pro 3.0%. HPLC: t_R (eluent A) = 6.02 min.

Calcd. for $C_{23}H_{33}N_3O_6$ (447.54) C 61.73, H 7.43, N 9.39; found C 61.78, H 7.63, N 9.41.

*Z-Leu-Aib-Pro-Leuol (**5**)*

To Z-Leu-Aib-Pro-OH 4 (2.0 g, 4.47 mmol) in DMF (50 ml), HOBt · aq (1.2 g) and EDC (0.8 g, 4.2 mmol) were added, the mixture was stirred for 1 h and L-Leuol (0.5 g, 4.3 mmol) was added. After 60 h solvents were removed in vacuo, AcOEt (300 ml) was added and the organic phase was treated according to procedure A. The remaining oil was dissolved in DCM and the peptide precipitated by addition of petroleum ether.

Yield: 1.8 g (77%); m.p. 137°C; $[\alpha]_D^{20} = -31.8$ (c = 1, MeOH); R_f(A) = 0.65, R_f(B) = 0.71, R_f(C) = 0.78; EI-MS: m/z = 547 $[M]^+$; AAA: Leu (1), Aib (1) 0.69, Pro (1) 0.97, Leuol (approx. 1 according to GC); GC: D-Leu 1.7%, D-Pro 2.2%, D-Val 0.9%. HPLC: t_R (eluent D): 9.10 min.

Calcd. for $C_{29}H_{46}N_4O_6$ (546.71) C 63.71, H 8.48, N 10.25; found C 63.72, H 8.52, N 10.27. For crystal structure of **5** see Geßmann et al. (1994).

*H-Leu-Aib-Pro-Leuol (**6**)*

Z-Leu-Aib-Pro-Leuol **5** (0.7 g, 1.28 mmol) in MeOH (20 ml) was hydrogenated for 90 min according to procedure C. The remaining oil was immediately used for the synthesis of the protected octapeptide **12**.

Yield: 0.52 g (100%); ESI-MS: m/z = 413.2 $[M + H]^+$; R_f(A) 0.41, R_f(D) 0.49.

*Z-Val-Aib-O*t*Bu (**7**)*

To Z-Val-OH (3.0 g, 11.94 mmol) in DMF (50 ml), HOBt · aq (3.2 g) and EDC (2.3 g, 11.94 mmol) were added with stirring. After 1 h H-Aib-O*t*Bu (1.9 g, 11.94 mmol) was added and the solution was maintained at 39°C for 70 h. After evaporation to dryness, AcOEt (200 ml) was added and the organic phase was treated according to procedure A.

Yield: 3.8 g (81%); m.p. 100°C; $[\alpha]_D^{20} = -26.3$; EI-MS: m/z = 393 $[M]^+$; 291 $[M-101]^+$; R_f(A) = 0.79, R_f(B) = 0.89, R_f(C) = 0.81; GC: D-Val 0.6%; AAA: Val (1), Aib 1.03 (1). HPLC: t_R (eluent D) = 8.20 min.

Calcd. for $C_{21}H_{32}N_2O_5$ (392.50) C 64.26, H 8.22, N 7.14; found C 64.18, H 8.21, N 7.15.

*Z-Val-Aib-OH (**8**)*

To Z-Val-Aib-O*t*Bu **7** (3.7 g, 9.24 mmol) in DCM (10 ml), TFA (100 ml) was added with stirring. After 90 min the solution was evaporated to dryness, the residue was dissolved in Et_2O and the dipeptide was precipitated by addition of petroleum ether.

Yield 2.8 g (90%); m.p. 150°C; $[\alpha]_D^{20} = -24.4$ (c = 1, MeOH); EI-MS: m/z 336 $[M]^+$; 207 $[M-130 + H]^+$; R_f(A) 0.32, R_f(B) 0.69, R_f(C) 0.44; AAA: Val (1), Aib 1.00 (1); GC: D-Val 0.4%. HPLC: t_R (eluent E) = 5.24 min.

Calcd. for $C_{17}H_{24}N_2O_5$ (336.39) C 60.70, H 7.19, N 8.33; found C 60.60, H 7.20, N 8.13.

*Z-Val-Aib-Pro-OMe (**9**)*

To Z-Val-Aib-OH **8** (5.3 g, 15.75 mmol) in DMF (80 ml), HOBt · aq (4.2 g) and EDC (3.0 g, 15.75 mmol) were added with stirring. After 30 min H-Pro-OMe × HCl (2.8 g, 16.91 mmol) and NMM (1.7 g, 16.91 mmol) were added and the solution was maintained at 30°C for 68 h. After evaporation to dryness, AcOEt (500 ml) was added, the organic phase treated according to procedure A and evaporated almost to dryness. After addition of Et_2O the peptide began to precipitate after stirring for a few minutes. Precipitation was completed by addition of petroleum ether.

Yield: 5.4 g (77%); m.p. 76–80°C; $[\alpha]_D^{20} = -89.8$ (c = 1, MeOH); ESI-MS: *m/z* = 448.2 $[M + H]^+$, 470.2 $[M + Na]^+$; R_f(A) = 0.86, R_f(B) = 0.86, R_f(C) 0.82; AAA: Val (1), Aib 1.05 (1), Pro 1.19 (1). GC: D-Pro 1.2%, D-Val 0.5%. HPLC: t_R (eluent C): 9.48 min.

Calcd. for $C_{23}H_{33}N_3O_6$ (447.53) C 61.73, H 7.43, N 9.39; found C 61.53, H 7.46, N 9.29. For crystal structure of **9** see Gessmann et al. (1993).

*Z-Val-Aib-Pro-OH (**10**)*

To Z-Val-Aib-Pro-OMe **9** (4.1 g, 9.2 mmol) in MeOH (20 ml), 1 M NaOH (18.5 ml) was added and the solution was stirred for 15 h at r.t. and for 4 h at 40°C. Then 1 M HCl (18.5 ml) was added with stirring and MeOH was removed *in vacuo*. The aqueous phase was adjusted to pH 10.5 by addition of 2 M NaOH and extracted with AcOEt (3 × 10 ml); then pH 10.5 of the aqueous phase was adjusted by addition of 2 M HCl. The solution was extracted with AcOEt (3 × 10 ml), the combined organic phases were washed with water, dried with $NaSO_4$ and evaporated to dryness. The remaining residue was dissolved in AcOEt and the tripeptide was precipitated by addition of petroleum ether.

Yield: 3.8 g (95%); m.p. 102°C; $[\alpha]_D^{20} = -83.2$ EI-MS; *m/z* = 433 $[M]^+$; R_f(A) 0.15, R_f(B) 0.65, R_f(C) 0.82; AAA: Val (1), Aib 1.06 (1), Pro 1.09 (1); GC: D-Val 0.9%, D-Pro 2.6%. HPLC: t_R (eluent A): 6.02 min.

Calcd. for $C_{22}H_{31}N_3O_6$ (433.51) C 60.96, H 7.21, N 9.69; found C 60.78, H 7.35, N 9.41.

*Z-Val-Aib-Pro-Aib-OMe (**11**)*

To Z-Val-Aib-Pro-OH **10** (3.2 g, 7.38 mmol) in DMF (80 ml), HOBt · aq (1.9 g) and EDC (1.4 g, 7.38 mmol) were added with stirring. After 1 h, H-Aib-OMe × HCl (1.25 g, 8.11 mmol) and NMM (0.82 g, 8.11 mmol) were added and the solution was maintained for 60 h. The solution was evaporated to dryness, AcOEt (500 ml) was added and the organic phase was treated according to procedure A.

Yield: 3.1 g white solid (79%); m.p. 79°C; $[\alpha]_D^{20} = -52.0$ (c = 1, MeOH); ESI-MS: *m/z* = 533.1 $[M + H]^+$, 555.2 $[M + Na]^+$; GC: D-Val 0.8%, D-Pro 5.2%; AAA: Val (1), Aib 2.03 (1), Pro 1.13 (1); R_f(A) 0.67, R_f(C) 0.76; GC: D-Val 0.8%, D-Pro 5.2%. HPLC: t_R (eluent C): 9.48 min.

Calcd. for $C_{27}H_{40}N_4O_7$ (532.64) C 60.89, H 7.57, N 10.52; found C 60.78, H 7.71, N 10.24.

*Z-Val-Aib-Pro-Aib-OH (**12**)*

To Z-Val-Aib-Pro-Aib-OMe **11** (1.8 g, 3.37 mmol) in MeOH (40 ml), 1 M NaOH (6.7 ml, 6.7 mmol) were added with stirring. After 15 h at r.t. and 4 h at 30°C, 1 M HCL (6.7 ml, 6.7 mmol) was added and MeOH removed *in vacuo*. Then pH 10.5 was adjusted by addition of 2 M NaOH, the organic phase was extracted with AcOEt (3 × 20 ml). Then pH 10.5 of the aqueous phase was maintained by addition of NaOH during extraction. The organic phases were discarded, the aqueous phase was adjusted to pH 2 by addition of 2 M HCl, and the aqueous phase was extracted with AcOEt (3 × 20 ml). The organic phases were combined and treated according to procedure B. The remaining residue was dissolved in DCM and precipitated by addition of petroleum ether.

Yield: 1.4 g (80%); m.p. 188°C; $[\alpha]_D^{20} = -57.4$ (c = 1, MeOH); R_f(A) 0.66, R_f(C) 0.65; EI-MS: *m/z* = 518 $[M]^+$, 474 $[M\text{-}CO_2 + H]^+$; GC: D-Leu 0.9%, D-Pro 5.4%, D-Val 0.9%; AAA: Val (1), Aib (2) 1.92, Pro 1.11 (1). HPLC: t_R (eluent E): 9.43 min.

Calcd. for $C_{26}H_{38}N_4O_7$ (518.61): C 60.22, H 7.39, N 10.80; found C 60.09, H 7.48, N 10.54.

*Z-Val-Aib-Pro-Aib-Leu-Aib-Pro-Leuol (**13**)*

To Z-Val-Aib-Pro-Aib-OH **12** (100 mg, 0.19 mmol) in DMF (3.5 ml) HOBt · aq (48 mg) and EDC (36 mg, 0.19 mmol) were added with stirring. After 1 h, H-Leu-Aib-Pro-Leuol (78 mg, 0.19 mmol) in MeCN/DMF 1 : 1 (3 ml), was added. After 15 h the solution was evaporated to dryness and AcOEt (70 ml) and 1-BuOH (30 ml) were added. The organic phase was treated according to procedure A. The resulting residue was dissolved in a mixture of MeOH and AcOEt. The octapeptide was precitpitated by addition of petroleum ether and crystallized from MeOH by addition of Et_2O and petroleum ether.

Yield: 125 mg (72%); m.p. 217°C; $[\alpha]_D^{20} = -7.8$ (c = 1, MeOH); ESI-MS: *m/z* = 913.6 $[M + H]^+$, 935.6 $[M + Na]^+$; AAA: Val (1), Leu 1.06 (1), Aib 2.72 (3), Pro 2.09 (2), Leuol (approx. 1 accord. to GC); GC: D-Pro 2.7%, D-Leu 5.8%, D-Val 0.6%, D-Leuol 0.7%; R_f(A) 0.54, R_f(B) 0.75, R_f(C) 0.71. HPLC: t_R (eluent B): 6.58 min.

Calcd. for $C_{47}H_{76}N_8O_{10}$ (913.17) C 61.82, H 8.39, N 12.27; found C 61.59, H 8.44, N 12.15. For crystal structure of **13** see Gessmann et al. (1999).

*H-Val-Aib-Pro-Aib-Leu-Aib-Pro-Leuol (**14**)*

The octapeptide **13** (115 mg, 0.13 mmol) in MeOH (3.5 ml) was hydrogenated for 3 h according to procedure C. The remaining oil was uniform in TLC and immediately used for synthesis of the protected dodecapeptide **17**.

ESI-MS: *m/z* = 779.5 $[M + H]^+$; R_f(A) 0.52, R_f(B) 0.95, R_f(C) 0.36.

*Z-Leu-Aib-Pro-Ala-OMe (**15**)*

To Z-Leu-Aib-Pro-OH **4** (780 mg, 1.7 mmol) in DMF (15 ml), HOBt · aq (459 mg) and EDC (326 mg, 1.7 mmol) were added with stirring. After 1 h H-Ala-OMe × HCl (251 mg, 1.8 mmol) and NMM (280 mg, 1.8 mmol) were added and the solution was maintained for 60 h. The solution was evaporated to dryness, AcOEt (50 ml) was added and the organic phase treated according to procedure A.

Yield: 810 mg (89%, white foam); m.p. 62–66.5°C; $[\alpha]_D^{20} = -83.7$; EI-MS: *m/z* 532 $[M]^+$; 333 $[M\text{-}199]^+$; AAA: Ala (1); Leu 1.2 (1), Pro; 1.07 (1); Aib 1.0 (1); GC: D-Ala 1.1%, D-Leu 1.9%, D-Pro 3.4%; R_f(A) 0.74, R_f(B) 0.77, R_f(C) 0.82. HPLC: t_R (eluent F): 5.88 min.

Calcd. for $C_{27}H_{40}N_4O_7$ (532.64) C 60.89, H 7.57, N 10.52; found C 60.68, H 7.55, N 10.43.

*Z-Leu-Aib-Pro-Ala-OH (**16**)*

To Z-Leu-Aib-Pro-Ala-OMe **15** (0.78 g, 1.46 mmol) in MeOH (12 ml), 1 M NaOH (2.9 mmol) were added with stirring. After 3 h at 35°C water (30 ml) was added, MeOH was removed *in vacuo*, and pH 10.5 was adjusted by addition of 2 M NaOH. The solution was extracted with AcOEt (3 × 15 ml); pH 10.5 of the aqueous phase was maintained by addition of NaOH. The organic phases were discarded and pH 2 of the queous phase was adjusted by addition of 2 M HCl. The solution was extracted with AcOEt (3 × 15 ml), the combined organic phases were washed with water, dried with Na_2SO_4 and evaporated to dryness. The residue was dissolved in DCM and the peptide was precipitated by addition of petroleum ether.

Yield: 0.54 g (71%, white foam); m.p. 93°C; $[\alpha]_D^{20} = -78.2$; EI-MS: *m/z* 518 $[M^+]$, 474 $[M\text{-}CO_2]^+$; R_f(B) = 0.65, R_f(C) 0.63; AAA: Leu (1), Aib 0.63 (1), Pro 1.2 (1), Ala 0.92 (1); GC: D-Ala 1.5%, D-Leu 1.9%, D-Pro 4.3%.

Calcd. for $C_{26}H_{38}N_4O_7$ (518.61) C 60.22, H 7.39, N 10.80; found C 60.09, H 7.48, N 10.54.

*Z-Leu-Aib-Pro-Ala-Val-Aib-Pro-Aib-Leu-Aib-Pro-Leuol (**17**)*

To Z-Leu-Aib-Pro-Ala-OH **16** (108 mg, 0.21 mmol) in DMF (5 ml), HOBt · aq (56.7 mg) and EDC (40.2 mg, 0.21 mmol) were added with stirring. After 44 min, the octapeptide **14** (163 mg, 0.21 mmol) in DMF was added. After 48 h the solution was evaporated to dryness, AcOEt (20 ml) was added and the organic phase was treated according to procedure A. The remaining residue was dissolved in MeOH and crystallized by addition of Et_2O and *n*-hexane.

Yield: 223 mg (83%); m.p. 211°C; $[\alpha]_D^{20} = -6.5$ (c = 1, MeOH); ESI-MS: *m/z* = 1302.2 $[M + Na]^+$; R_f(A) = 0.53, R_f(B) = 0.67, R_f(C) = 0.63; AAA: Val (1), Ala 1.13 (1), Aib 3.75 (4), Leu 2.17 (2), Pro 3.12 (3), Leuol (GC: approx. 1); GC: D-Val 0.7%, D-Ala 3.5%, D-Pro 2.3%, D-Leu 4.0%, D-Leuol 1.7%.

Calcd. for $C_{65}H_{106}O_{14}N_{12}$ (1,279.64) C 61.01, H 8.35, N 13.14; found C 60.51, H 8.50, N 12.95. HPLC: t_R (eluent B): 10.29 min. For crystal structure of **17** see Gessmann et al. (1999).

*H-Leu-Aib-Pro-Ala-Val-Aib-Pro-Aib-Leu-Aib-Pro-Leuol (**18**)*

The dodecapeptide **17** (83.7 mg, 65.4 mmol) in MeOH (1.5 ml) was hydrogenated for 105 min and treated according to procedure C. The yield was assumed to be 100% and the peptide was immediately used for the synthesis of the tetradecapeptide **23**. ESI-MS: *m/z* = 1145.9 $[M + H]^+$, R_f(A) = 0.52, R_f(D) = 0.34.

*Z-Aib-Asn-O*t*Bu (**19**)*

To Z-Aib-OH (3.5 g, 14.7 mmol) in DMF (210 ml), HOBt · aq (4.0 g) and EDC (2.8 g, 14.6 mmol) were added with stirring and, after 15 min, H-Asn-O*t*Bu (2.8 g, 14.9 mmol) was added. After 32 h at 37°C the solution was evaporated to dryness, AcOEt (600 ml) was added and the organic phase was treated according to procedure A. The residue was dissolved in 1-BuOH and the dipeptide was crystallized by addition of petroleum ether.

Yield 5.0 g (83%); m.p. 115°C; EI-MS: *m/z* 408 $[MH]^+$; 334 $(M\text{-}O\textit{t}Bu)^+$; R_f(A) 0.71, R_f(B) 0.74, R_f(C) 0.82.

Calcd. for $C_{20}H_{29}N_3O_6$ (407.47) C 58.96, H 7.17, N 10.31; found C 59.04, H 7.15, N 10.35.

*H-Aib-Asn-O*t*Bu (**20**)*

Z-Aib-Asn-O*t*Bu **19** (1.8 g, 4.4 mmol) in MeOH (30 ml) was hydrogenated for 2 h according to procedure C.

Yield 1.2 g (100%, white solid); m.p. 141°C; EI-MS: *m/z* 273 $[M]^+$; R_f(A) 0.67, R_f(3) 0.46; GC: D-Asp 3.7%.

Calcd. for $C_{12}H_{23}N_3O_4$ (273.33) C 52.73, H 8.48, N 15.37; found C 52.80, H 8.55, N 15.27.

*Ac-Aib-Asn-O*t*Bu (**21**)*

To H-Aib-Asn-O*t*Bu **20** (1.16 g, 4.2 mmol) in DCM (120 ml), Ac_2O (0.47 g, 4.6 mmol) was added with stirring. After 60 min the solution was evaporated to dryness. The remaining oily residue solidified by addition of AcOEt with stirring. Petroleum ether was added and the acetylated dipeptide was removed by filtration.

Yield 1.2 g (91%); m.p. 165°C; EI-MS: *m/z* 315 $[M]^+$; R_f(A) 0.28; R_f(B) 0.60, R_f(C) 0.53; GC: D-Asp. 3.5%.

Calcd. for $C_{14}H_{25}N_3O_5$ (315.37) C 53.32, H 7.99, N 13.32; found C 52.92, H 8.02, N 13.07.

*Ac-Aib-OBzl (**24**)*

To H-Aib-OBzl (4.0 g, 20.7 mmol) in DCM (80 ml), Ac_2O (2.7 g, 26.4 mmol) was added dropwise. After 60 min solvents were removed *in vacuo*, the residue was dissolved in AcOEt (500 ml) and the organic phase treated according to procedure A. The solvent was removed, the residue was dissolved in MeOH and Ac-Aib-OBzl crystallized by addition of Et_2O and petroleum ether.

Yield: 4.6 g (94%); m.p. 87.5°C; EI-MS: *m/z* 235 $[M]^+$; R_f(A) 0.67, R_f(B) 0.76, R_f(C) 0.79.

Calcd. for $C_{13}H_{17}NO_3$ (235.28) C 66.36, H 7.28, N 5.95; found C 66.19, H 7.31, N 5.86.

*Ac-Aib-OH (**25**)*

Ac-Aib-OBzl **24** (0.8 g, 3.4 mmol) in MeOH (15 ml) was hydrogenated for 90 min (prodcedure C). The resulting residue was dissolved in Et_2O and precipitated by addition of petroleum ether.

Yield: 0.48 g (97%); m.p. 196°C; EI-MS: *m/z* 145.1 $[M]^+$; R_f(A) 0.43, R_f(C) 0.63.

*Boc-Asn-OBzl (**26**)*

To Boc-Asp-OBzl (4.5 g, 13.9 mmol) in DMF (200 ml), at 0°C HOBt · aq (3.7 g) and EDC (2.6 g, 13.6 mmol) were added. After 30 min at 0°C, a solution of 8% aqueous ammonia (0.4 g) was added and the mixture was stirred for 20 min at 0°C. Solvents were removed *in vacuo*, the residue was dissolved in AcOEt (500 ml), and treated according to procedure A. The peptide was dissolved in a small amount of MeOH and crystallized by addition of Et_2O and petroleum ether.

Yield: 4.1 g (93%); m.p. 121°C; $[\alpha]_D^{20} = -15.6$ (*c* = 1, MeOH); R_f(A) 0.61, R_f(B) 0.72, R_f(C) 0.79; GC: D-Asp 4.5%.

Calcd. for $C_{16}H_{22}N_2O_5$ (322.36) C 59.62, H 6.88, N 8.69; found C 59.61, H 6.96, N 8.67.

H-Asn-OBzl × TFA *(**27**)*

To Boc-Asn-OBzl **26** (1.3 g, 4.03 mmol), TFA (5 ml) was added and the solution stirred for 30 min. The solvent was removed *in vacuo*, and the oily residue treated twice with Et_2O. The organic phase was discarded and petroleum ether was added. The oily trifluoroacetate crystallized on standing overnight.

Yield: 1.35 g (100%); m.p. = 127°C; $[\alpha]_D^{20} = +5.0$ (c = 1, MeOH); R_f(A) 0.26, R_f(B) 0.28, R_f(C) 0.51; GC: D-Asp 4.9%.

Calcd. for $C_{13}H_{15}N_2O_5F_3$ (336.27) C 46.43, H 4.50, N 8.36; found C 46.21, H 4.54, N 8.33.

*Ac-Aib-Asn-OBzl (**28**)*

To Ac-Aib-OH **25** (0.78 g, 5.37 mmol) in DMF (60 ml), HOBt · aq (1.45 g) and EDC (1.02 g, 5.32 mmol) were added. After 45 min H-Asn-OBzl × TFA (1.98 g, 5.89 mmol) and NMM (0.59 g, 5.90 mmol) were added and the mixture was maintained for 48 h. The solution was subjected to silica gel chromatography (procedure B), and the elution of the dipeptide monitored by TLC (system A). Suitable fractions were combined and evaporated to dryness. The remaining oily residue was dissolved in AcOEt and the peptide precipitated by addition of *n*-hexane as a viscouses oil. The oil solidified on stirring for several hours.

Yield: 0.88 g (47%); m.p. 134°C; $[\alpha]_D^{20} = +12.1$ (c = 1, MeOH); EI-MS: *m/z* 349 $[M]^+$, 100 $[M\text{-}249]^+$; R_f(A) 0.39, R_f(B) 0.54, R_f(C) 0.63; HPLC (F): t_R 19.62 min; GC: D-Asp 5.4%.

Calcd. for $C_{17}H_{23}N_3O_5$ (349.39) C 58.44, H 6.64, N 12.03; found C 58.26, H 6.73, N 11.84.

*Ac-Aib-Asn-OH (**22a**) (method A)*

To a suspension of Ac-Aib-Asn-O*t*Bu 21 (0.85 g, 2.7 mmol) in DCM (5 ml), TFA (60 ml) was added with stirring. After 2 h 30 min the

solution was evaporated to dryness. Evaporation was repeated (3×) with addition of Et_2O in order to remove traces of TFA completely. Yield, 0.7 g (100%), EI-MS: *m/z* 128 (M-131), 100 (M-159). The oily peptide showed impurites on TLC (ca. 10%) which could not be removed by precipitation from AcOEt/petroleum ether and MeOH/Et_2O. This material was used for TV synthesis in experiment A.

*Ac-Aib-Asn-OH (**22b**) (method B)*

Ac-Aib-Asn-OBzl **28** (0.88 g, 2.52 mmol) in MeOH (15 ml) was hydrogenated according to the standard procedure for 1 h. The resulting peptide acid was dissolved in MeOH and precipitated by addition of ethyl acetate.

Yield: 0.55 g (85%) m.p. 184°C; EI-MS: *m/z* 259 $[M]^+$, 128 (M-131), 100 (M-159); R_f(C) = 0.32, GC: D-Asp 4.6% (Asn is hydrolysed to Asp).

Calcd. for $C_{10}H_{17}N_3O_5$ (259.26) C 46.33, H 6.61, N 16.21; found C 46.29, H 6.70, N 16.15.

*Ac-Aib-Asn-Leu-Aib-Pro-Ala-Val-Aib-Pro-Aib-Leu-Aib-Pro-Leuol; trichovirin I 4A (**23**)*

Experiment A: to Ac-Aib-Asn-OH **22a** (17 mg, 65.6 μmol) in DMF (0.5 ml), HOBt·aq (11 mg) and EDC (12.5 mg, 65.4 μmol) were added with stirring. After 30 min, the dodecapeptide **18** (74.9 mg, 65.4 μmol) in DMF (0.5 ml) was added. After 21 h the solution was evaporated to dryness, AcOEt (70 ml) was added, and the organic phase was treated according to procedure A; yield ca. 80 mg. Since the end product showed an impurity when inspected by HPLC, it was subjected to preparative HPLC in 10 mg portions and uniform fractions combined. Yield: 26 mg (29%). ESI-MS found (calcd.): *m/z* 1,387.0 (1,387.7) $[M + H]^+$; diagnostic sequence ions *m/z* 1,172.6 (1,173.5) (b_{12}), 792.4 (792.9) (b_8), 440.4 (440.5) (b_4).

AAA: Val (1), Aib 5.38 (5), Pro 3.00 (3), Leu 1.91 (2), Asp 0.90 (1), Ala 0.92 (1), Leuol ca. 1 (GC); GC-MS: D-Ala (1.4%), D-Val (0.3%), D-Pro (1.9%), D-Leu (2.9%), D-Asp (5.2%), D-Leuol (n.d.). HPLC: t_R (eluent: MeCN/MeOH/H_2O 40:30:30): 41.55 min; the synthetic peptide coeluted with the natural peptide, see Fig. 2.

This experiment was repeated using 100 mg (87.3 μmol) dodecapeptide **18** and 22.6 mg (87.3 μmol) dipeptide acid **22a**. Repetitive preparative HPLC of the resulting crude product yielded 45 mg (36.8%) uniform TV peptide **23**.

Experiment B: to 64.1 mg (56 μmol) H-3-14-ol **18** in DMF (2 ml), 43.5 mg (168 μmol) Ac-Aib-Asn-OH **22b**, 22.7 mg HOBt·aq and 32.2 mg (168 μmol) EDC were added and the solution was stirred for 15 h. Solvents were removed *in vacuo*, AcOEt (30 ml) was added and the organic phase was treated according to procedure B. After evaporation 48 mg (61%) amorphous peptide was obtained. Twofold preparative HPLC provided 29 mg (37%) uniform TV peptide **23**. m.p. 264–268°C; [α] = +6.3 (c = 1, MeOH); R_f(A) = 0.23, R_f(B) = 0.64, R_f(C) = 0.46. AAA: Val (1), Aib 4.92 (5.0), Pro 2.88 (3.0), Leu 1.76 (2.0), Asp 0.86 (1), Ala 0.84 (1). For GC the synthetic trichovirin and the natural mixture were hydrolysed and investigated under identical conditions (quantities of the natural component in parantheses): D-Ala 0.8% (0.5%), D-Val 0.3% (0.2%), D-Pro 2.0% (0.8%), D-Leu 3.4% (3.1%), D-Asp 7.9% (1.6%), D-Leuol (n.d.).

ESI-MS: found (calcd.) *m/z* 1409.2 (1409.7 $[M + Na]^+$, for mass spectra and diagnostic sequence ions see Fig. 3. The synthetic and natural peptide mixture exerted the same antibiotic activity (see Experimental).

Calcd. for $C_{67}H_{115}N_{15}O_{16} \times 2\ H_2O$ (1422.78) C 56.56, H 8.43, N 14.77; found C 56.54, H 8.34, N 14.43 (Karl Fischer titration of **23** revealed ca. 4.7% water).

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