Structure Determination of Neofrapeptins A to N: Peptides with Insecticidal Activity Produced by the Fungus *Geotrichum candidum*

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Abstract The structures of neofrapeptins A to N, peptides with insecticidal activity, were elucidated. They showed a close similarity to efrageptin. However, all neofrapeptins contained the very rare amino acid 1-amino-cyclopropane-carboxylic acid and some of them also contained (2*S*,3*S*)-3-methylproline. The neofrapeptins are the first case, in which these amino acids are found as building blocks for linear peptides. They were identified by comparison of the silylated hydrolyzate to reference material by GC/MS (EI-mode). The sequence was elucidated using mass spectrometry (ESI+ mode). Full scan spectra showed two fragments in high yield, even under mild ionization conditions. MS/MS spectra of these two fragments yielded fragment rich spectra from which the sequence of the compounds was determined almost completely. The proteolytic cleavage with the proteinase papain yielded products that allowed to prove the rest of the sequence and the identity of the C-terminus to efrageptin. The proteolytic cleavage products allowed furthermore to determine the position of the isobaric amino acids, pipecolic acid and 3-methylproline in neofrapeptin F, as well as the location of *R*-isovaline and *S*-isovaline. Papain digestion was such established as a tool for structure elucidation of peptides rich in α,α -dialkylated amino acids. CD spectra suggested a 3_{10} helical structure for neofrapeptins A and F.

Keywords efrageptin, MS/MS, papain digestion, circular dichroism

Introduction

Natural products are a continuous and proven source of new lead compounds for the agrochemical industry. In a discovery program aimed at the identification of insecticides, several peptides were isolated from the strain SID 22780, isolated by CNPR (Center for Natural Products Research) in Singapore in 1997, and identified by DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) as *Geotrichum candidum* Link:F [1]. They were called neofrapeptins A through to N where the annotation of the compounds A to N reflects solely the process of the discovery. In the patent application [1] the fermentation, isolation and biological activity of the neofrapeptins was described. Having a positive charge they were isolated as acetate salts. In this paper the structures and physico-chemical properties of the newly discovered neofrapeptins, which show a close sequence similarity to the efrageptins [2–5] are presented.

Efrageptins are a mixture of peptide antibiotics produced by the fungus *Tolypocladium niveum*. They are rich in pipecolic acid (Pip) and α,α -dialkylated amino acids like 1-amino-isobutyric acid (Aib), which are quite rare as building blocks in peptides. They differ from peptaibols that are also rich in Aib by having a quite unique heterocyclic ring-system with a positive charge on the C-terminus. Efrageptins have a strong insecticidal activity against chewing pests and mites [6]. They are inhibitors of

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the mitochondrial ATPase [7, 8]. Efrapeptin E was isolated for comparison from a strain *Tolypocladium inflatum* W. Gams and was identical to the reported compound in all respects (amino acid composition, MS, MS/MS, biological activity).

Results and Discussion

Structure of Neofrapeptin A

In order to determine the sequence of neofrapeptin A (**1**) and to elucidate the structure amino acid analysis, MS and MS/MS experiments and selective digestion experiments were performed.

Amino acid analysis of the acidic hydrolyzate revealed the following components: *S*-Leu, Gly, Aib (1-amino-isobutyric acid), β -Ala (β -alanine), both *S*-Iva (isovaline) and *R*-Iva, *S*-Pip (pipecolic acid), and an unknown amino acid. Interestingly, neofrapeptin A contained one Iva in the *R*-configuration, while efrapeptin is known to contain only *S*-Iva. *R*-Iva is, however, quite common in fungal peptaibols [9].

MS and MS/MS experiments (see below) gave a molecular mass of 101 Da for the unknown amino acid. This was 2 Da less than Aib, suggesting either a cyclic or an unsaturated amino acid. The identification of

the unknown amino acid was performed by derivatizing the hydrolyzate of neofrapeptin A with *N*-Methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) and dansyl chloride, respectively. The product mixtures were subsequently analyzed by GC/MS (EI(+) mode) and LC/MS (ESI(+) mode). The chromatographic peak of the unknown amino acid in GC/MS and LC/MS runs could be readily identified based on the known mass. Commercially available reference compounds were then compared to the neofrapeptin A hydrolyzate (see Table 1) by GC-MS. Only 1-amino-cyclopropane-carboxylic acid (Acc) showed the same retention time and a very similar EI-MS. This finding was corroborated unambiguously by LC-NMR analysis of the chromatographic peak assigned to the unknown amino acid in the dansylated neofrapeptin A hydrolyzate.

Acc is a very rare amino acid among natural products. It is a building block of BZR-Cotoxin II [10], a depsipeptide metabolite of a plant pathogen, antibiotic CBS 154-94A [11], a depsipeptide protein farnesyl transferase inhibitor, and of cytotrienins [12], ansamycin type antibiotics.

As shown below neofrapeptin A carries an internal charge at the C-terminus. Consequently, the mass spectra (ESI(+) mode) showed the $(M+H)^{2+}$ rather than the $(M+2H)^{2+}$ molecular ion. Interestingly, two fragments at *m/z* 943.6 and 689.5 were observed - even at relatively low cone voltages (20 V; Fig. 1). Both, efrapeptins and

Table 1 Retention time and similarity index of the unknown amino acids as TMS derivatives with several reference compounds

EI-MS <i>m/z</i> (M^+)	Amino Acid	RT (GC/MS) (minute)	Similarity Index ^{a)} (EI-MS of TMS derivative)	
			245	273
	Unknown amino acid in hydrolyzate of 1	7.40		b)
	Azetidine-2-carboxylic acid	8.19		676
	2-Amino-3-butenic acid	6.40		662
	Acc	7.41		934
	Unknown amino acid in hydrolyzate of 7	9.63		b)
	Pip	10.99		835
	3-Piperidinecarboxylic acid	12.44		259
	4-Piperidinecarboxylic acid	13.29		281
	2-Methylproline	10.00		837
	(2 <i>S</i> ,3 <i>S</i>)-3-Methylproline	9.62		836
	rac-(2 <i>R</i> ,3 <i>S</i>)-3-Methylproline	10.60		838
	rac-4-Methylproline	10.10/10.31		844/845
	<i>N</i> -Methylproline	7.37		21

^{a)} Forward similarity index calculation as implemented in Mass Lynx 3.5 [30]. Identical spectra would give a match of 999. From our experience values above 800 usually can be considered as similar spectra. Besides the similarity index values that consider intensities at a given mass, spectra were also evaluated manually with the same result.

^{b)} Reference spectrum for similarity calculation.

neofrapeptins showed this quite unique feature of fragmenting easily into two parts. The larger fragment corresponded to the acetylated *N*-terminus (amino acids 1

Fig. 1 HR-MS of neofrapeptin A.

to 10) and the smaller to the *C*-terminus (residues 11 to 15), respectively. Examination of the sequence revealed no obvious indication why the breakage of the peptide bond between amino acid Aib¹⁰ and Pip¹¹ is preferred over all others. Neofrapeptin A also has a second Aib-Pip bond after residue 2, which showed no tendency to fragment. Adenopeptin, a tridecapeptide with the same *C*-terminus, also fragments at the only Aib-Pip bond of that molecule which is closer to the *C*-terminus (between amino acid 11 and 12) [13, 14]. Apart from bond stability, the position of the internal positive charge along the 3₁₀ helical structure (see CD studies, below) could be responsible for this preferred cleavage site.

The sequence of neofrapeptin A could be fully elucidated by interpretation of the MS/MS spectra of the two fragments (Scheme 1). The MS/MS spectrum of the *C*-terminus (selected fragmentation mass 689.5 Da; Fig. 2) was identical to efrapeptin E, establishing the same partial sequence. Both, *y*- and *b*-fragments were observed as indicated in Fig. 2. The mass of the *N*-terminal fragment was 943.7 Da and 2 Da lower than efrapeptin E. Interpretation of the MS/MS fragmentation pattern (Fig. 3) and comparison with the one from efrapeptin E (Fig. 4)

Scheme 1 Structures of neofrapeptins A to N

Residue #		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
1	Neofrapeptin A	Ac	Pip	Aib	Pip	Iva	Aib	Leu	β -Ala	Gly	Acc	Aib	Pip	Aib	Gly	Leu	Iva
2	Neofrapeptin B	Ac	Pip	Aib	Pip	Iva	Iva	Leu	β -Ala	Gly	Acc	Aib	Pip	Aib	Gly	Leu	Iva
3	Neofrapeptin C	Ac	Pip	Aib	Pip	Iva	Aib	Leu	β -Ala	Gly	Acc	Iva	Pip	Aib	Gly	Leu	Iva
4	Neofrapeptin E	Ac	Pip	Aib	Pip	Iva	Iva	Leu	β -Ala	Gly	Acc	Iva	Pip	Aib	Gly	Leu	Iva
5	Neofrapeptin D	Ac	Pip	Aib	Pip	Aib	Aib	Leu	β -Ala	Gly	Acc	Aib	Pip	Aib	Gly	Leu	Iva
6	Neofrapeptin N	Ac	Pip	Aib	Pip	Aib	Aib	Leu	β -Ala	Gly	Acc	Aib	Pip	Aib	Gly	Leu	Aib
7	Neofrapeptin F	Ac	Pip	Aib	Pip	Iva	Aib	Leu	β -Ala	Gly	Acc	Aib	3M-Pro	Aib	Gly	Leu	Iva
8	Neofrapeptin I	Ac	Pip	Aib	Pip	Iva	Iva	Leu	β -Ala	Gly	Acc	Aib	3M-Pro	Aib	Gly	Leu	Iva
9	Neofrapeptin M	Ac	Pip	Aib	Pip	Iva	Aib	Leu	β -Ala	Gly	Acc	Iva	3M-Pro	Aib	Gly	Leu	Iva
10	Neofrapeptin L	Ac	Pip	Aib	Pip	Iva	Iva	Leu	β -Ala	Gly	Acc	Iva	3M-Pro	Aib	Gly	Leu	Iva
11	Neofrapeptin G	Ac	Pip	Aib	Pip	Iva	Aib	Leu	β -Ala	Gly	Acc	Aib	Pip	Aib	Gly		
12	Neofrapeptin H	Ac	Pip	Aib	Pip	Iva	Iva	Leu	β -Ala	Gly	Acc	Aib	Pip	Aib	Gly		
	Efrapeptin E	Ac	Pip	Aib	Pip	S-Iva	Aib	Leu	β -Ala	Gly	Aib	Aib	Pip	Aib	Gly	Leu	Iva

The drawn structure represents neofrapeptin F. The bold line between amino acid 10 and 11 indicates the fragmentation observed in ESI-MS.

Acc: 1-amino-cyclopropane-carboxylic acid, Aib: 1-Amino-isobutyric acid, *β*-Ala: 3-amino-propionic acid, 3M-Pro: 3-methylproline, Iva: isovaline, Pip: pipecolic acid. X represents the *C*-terminus with the 2,3,4,6,7,8-hexahydro-1-pyrrole[1,2-*α*]pyrimidine.

Fig. 2 MS/MS spectrum of fragment 689.6 of neofrapeptin A (identical to efrapeptin E). The a-fragments (a₂, a₄) are formed by the loss of CO (-28) from the corresponding b-fragments.

Fig. 3 MS/MS spectrum of fragment 943.7 of neofrapeptin A (magnified by a factor of 2).

clearly revealed that Acc is at position 9 instead of Aib in efrapeptin E. The complete b-fragment series can be assigned to the proposed sequence, although the cleavage between β -alanine and glycine gave rise to a signal with low intensity. The intense fragments at masses 296.3, 409.3, 620.4, and 705.4 can be assigned to the partial y-series sequence Pip³-Iva-Aib-Leu- β -Ala-Gly-Acc-Aib¹⁰ (Fig. 3). This finding was supported by collision induced dissociation of ion 705.5 Da, which fragmented into the above mentioned masses. The HR-MS of the (M+H)²⁺ and of the two fragments 943.7 and 689.5 corroborated the

proposed sequence.

To complete the structure elucidation of **1** cleaved fragments of this peptide were needed. Several proteinases were screened by LC-MS. Papain, a non-specific thiol protease [15~17] was found to digest neofrapeptin A selectively. For an efficient reaction higher concentrations of EDTA and mercaptoethanol than that recommended by Allen were utilized [16]. Several fragments were observed by LC-MS analysis after prolonged exposure. The sequences of the products were established by HR-MS and MS/MS data (Scheme 2). Therefore, papain cleaves

Scheme 3 Proteolytic cleavage of neofrapeptins by papain **Scheme 4** Proposed ESI-MS/MS fragmentation mechanism of the dipeptide **14** obtained by cleavage of neofrapeptin A by papain

probably because the hydrolysis of the C-terminal amide bond was decelerated by the positive charge nearby.

To the best of our knowledge the use of papain or other proteases for cleavage of Aib-rich peptides or peptaibols has not been described previously. Interestingly, the inverse

reaction also involving a Gly-Leu bond has been described by Slomczynska *et al.* [18]: In their synthesis of the peptaibol alamecthin they coupled the 1~11 segment having a C-terminal Gly and the 12~20 segment, having a N-terminal Leu, using papain. Thus, depending on the

experimental conditions, papain is capable to cleave or couple the Gly-Leu bond.

Structures of Neofrapeptins B, C and E

The two compounds neofrapeptin B and C (**2**, **3**) were isolated as a homogenous peak having the same molecular mass. In a single experiment a very small amount of neofrapeptin C was obtained and characterized by MS and MS/MS experiments. Amino acid analysis revealed one Aib less and one Iva more than neofrapeptin A. The MS/MS spectrum of the C-terminus at 689.5 Da was identical to that of neofrapeptin A. The N-terminal fragment mass of 957.7 Da was 14 Da higher than neofrapeptin A. Its MS/MS fragmentation pattern showed that the compound was a mixture: Several fragments with a difference of 14 Da were observed in the range 534.4/548.3 Da to 858.6/872.5 Da. Thus, Aib and Iva must be interchanged in positions 5 and 10 in the two compounds. The major compound in this mixture was neofrapeptin B with Iva⁵ and Aib¹⁰. The ratio of neofrapeptin B to C was about 4 : 1 based on MS/MS.

Neofrapeptin E (**4**) had a molecular weight of 1660 or 28 Da higher than neofrapeptin A. As with most neofrapeptins, the MS/MS spectrum of the C-terminus was the same as in neofrapeptin A. The MS/MS spectrum of the N-terminal fragment revealed that Iva replaced two Aib residues at positions 5 and 10. So, compared to neofrapeptin A, neofrapeptin E combines the changes observed in neofrapeptins B and C. It contains 4 Iva-residues and only two Aib. As in the experiment described above, papain digestion products (Scheme 3) reveal that Iva⁴ and Iva⁵ are in the *R*-configuration, as peptides **15** and **18** contain only *R*-Iva. As papain digestion product **19** contains *S*-Iva, Iva¹⁰ in neofrapeptins C and E (**3**, **4**) is in the *S*-configuration.

Structure of Neofrapeptin D

HR-MS data of **5** suggested a mass difference of 14 Da or a CH₂-group less than neofrapeptin A. Again, the MS/MS spectrum of the C-terminus was the same as in neofrapeptin A. The N-terminal MS/MS spectrum showed masses of 14 Da less in all fragments above *m/z* 350. Therefore amino acid 4 (Iva) in neofrapeptin A was replaced by Aib in neofrapeptin D.

Structure of Neofrapeptin N

Neofrapeptin N (**6**) had a molecular weight of 1604 or 28 Da lower than neofrapeptin A. Neofrapeptin N was the only compound among the neofrapeptins with a different mass of the C-terminal MS fragment. The MS/MS spectrum revealed that the Iva at position 15 was replaced

by Aib: All masses above *m/z* 400 are shifted by 14 Da. MS and MS/MS spectra of the N-terminal fragment were the same as of neofrapeptin D. Neofrapeptin N has the highest Aib content among the neofrapeptins.

Structure of Neofrapeptin F

Neofrapeptin F eluted in the HPLC about 3 minutes later than neofrapeptin A. HR-MS and MS/MS spectra of neofrapeptin F were almost identical to neofrapeptin A. Both compounds showed the same elemental composition. The MS/MS spectrum of the fragment 943.6 showed no difference to neofrapeptin A, while in the MS/MS fragmentation pattern of the C-terminal fragment 689.6 of neofrapeptin F the fragment at *m/z* 578.4 was missing. The molecular weights of the amino acids 11 and 12 were determined in the MS/MS experiments of the proteolytic cleavage product **20**. It showed no difference to the MS/MS of **17** from neofrapeptin A and therefore established Aib in position 12. Amino acid composition revealed that one Pip was replaced by a different amino acid with the same molecular weight. The retention times of a number of candidate molecules were compared as TMS derivatives by GC-MS (Table 1). Only (2*S*,3*S*)-3-methylproline (or (2*R*,3*R*)-3-methylproline) eluted at the same RT as the unknown amino acid in the hydrolyzate of neofrapeptin F. The identification of 3-methylproline including the relative stereochemistry was corroborated unambiguously by LC-NMR analysis of the dansylated neofrapeptin F hydrolyzate. The chirality was determined as (2*S*,3*S*) by GC/MS on a chiral capillary column by comparison with commercial and in-house available material.

Neofrapeptin A has three Pip at positions 1, 3 and 11 and just one Pip is replaced by (2*S*,3*S*)-3-methylproline in neofrapeptin F. The proteolytic cleavage product **20** had no Pip left (Scheme 3). and its amino acid composition clearly indicated that (2*S*,3*S*)-3-methylproline was at position 11. Therefore, neofrapeptin F was identical to neofrapeptin A with the exception that *S*-Pip at position 11 was substituted by (2*S*,3*S*)-3-methylproline. As in neofrapeptin A, Iva⁴ was in the *R*-configuration and Iva¹⁵ in the *S*-configuration.

The amino acid 3-methylproline is extremely rare as a building block in natural products. It has been described as a building block of bottromycin A₂ from *Streptomyces bottropensis*, scytalidamide B, a cyclic heptapeptide from a marine fungus, roseotoxin B and roseocardin, two cyclodepsipeptides from the fungus *Trichothecium roseum* [19~23]. All these compounds contain 3-methylproline in the (2*S*,3*S*)-form. It is interesting to observe, that all compounds mentioned above were isolated together with their proline derivative, whereas the neofrapeptins

were isolated together with Pip-containing analogues. Furthermore, the neofrapeptins seem to be the first case, where 3-methylproline is described as a building block of linear peptides.

Structures of Neofrapeptins I, L and M

The neofrapeptins I, L and M all contained one (2*S*,3*S*)-3-methylproline and only two Pip. Apart from that difference they had the same structures as neofrapeptin B, C and E. Thus, apart from residue 11, neofrapeptin I corresponded to neofrapeptin B, neofrapeptin L to neofrapeptin E, and neofrapeptin M to neofrapeptin C. Chiral amino acid analysis suggested *R*-Iva⁴, *R*-Iva⁵, *S*-Iva¹⁰ and *S*-Iva¹⁵.

Structures of Neofrapeptins G and H

The two peptides neofrapeptins G and H were smaller than the others neofrapeptins. In the HR-MS ($M+H$)⁺ ions of m/z 1214.7151 and 1228.7301, were found. Additionally, fragments of m/z 943 or 957 were observed, suggesting that the *N*-terminus was identical to neofrapeptin A or B/C. A small fragment corresponding to the loss of glycine was seen in the mass spectrum of neofrapeptin H. In the MS/MS spectrum of the ($M+H$)⁺ ion loss of Aib-Gly-OH from the *C*-terminus was detected and most of the sequence information could be deduced. The rest of the sequence information was observed in the MS/MS spectra of the fragment 943 and 957, respectively. Neofrapeptin H differed from neofrapeptin G by an Iva in position 5 instead of Aib. Analysis of the hydrolyzates showed that the compounds **11** and **12** contained only 10% of (2*S*,3*S*)-3-methylproline and were therefore isolated in 90% purity. Iva was in the *R*-configuration. Neofrapeptin G is identical to **13**, formed by digestion of neofrapeptin A with papain.

Circular Dichroism Studies of Neofrapeptins A and F

Circular dichroism (CD) is a valuable tool for estimating the secondary structures of proteins and peptides. The CD spectra of neofrapeptins A and F were recorded in water containing 5% methanol (Fig. 5) and showed for both compounds a strong negative ellipticity at 204 nm and a minor one around 230 nm. The negative $n-\pi^*$ ellipticity around 230 nm of **1** and **7** was not observed in compound **13** (Scheme 2) and could therefore be due to a Cotton effect of the *C*-terminal chromophore. The spectra resembled the CD spectrum of a model compound for 3_{10} -helix in water published recently by Formaggio *et al.* which showed a negative Cotton effect in the 201~206 nm region accompanied by a shoulder at approximately 222 nm [24]. The peptides are therefore not in an α -helical conformation, which is very common among the Aib-rich

Fig. 5 CD spectra of neofrapeptin A (—), neofrapeptin F (---) and compound **13** (.....).

peptaibols [25]. The results are consistent with the structural analysis of efrapeptin C by Huber and Sewald utilizing ¹H NMR. They suggested two 3_{10} -helical regions at the *N* terminus and between Aib⁹ to Aib¹⁵ which are linked by a flexible region between Leu⁶ and Gly⁸ [26]. In the crystal structure of efrapeptin C, determined by Abrahams *et al.* in a complex with bovine F₁-ATPase, the *N*-terminal part showed the typical 4→1 hydrogen bonds of a 3_{10} -helix and was followed by a flexible region β -Ala⁷-Gly⁸-Aib⁹ [27]. The chirality of Iva was shown by Toniolo and Benedetti to induce no preference for right or left-handed helices and therefore the presence of the *R*-enantiomer of Iva in **1** and **7** did not alter the direction of helical rotation [28].

The molar ellipticity θ at 204 nm is a bit weaker for neofrapeptin F than for neofrapeptin A and suggested that the 3_{10} -helix is a bit less pronounced. The presence of 3-methylproline could induce a β -turn.

Experimental

Chemicals

Water for chromatography, TFA (BioChemica), acetonitrile (gradient grade), methanol (p.a.), acetyl chloride (puriss.), dansyl chloride (BioChemica), ethylenediaminetetraacetic acid disodium salt (EDTA; BioChemica) 2-mercaptoethanol (BioChemica), papain (#76218, BioChemica) and Tris-hydrochloride (Trizma, BioChemica) were from Fluka, Buchs, Switzerland. MSTFA was from Pierce, Rockford, Ill. USA. 1-Amino-cyclopropane-carboxylic acid (Acc) and *RS*-Pip from Aldrich, St. Louis MO, USA. *S*-Pip and Aib were from Sigma, St. Louis MO, USA. Trifluoroacetic anhydride, β -alanine, *RS*-Leu, *R*-Iva, *S*-Iva and (2*S*,3*S*)-3-methylproline were from Acros Organics, Geel, Belgium.

S-Leu was from Amresco, Solon OH, USA. The mixture of rac-(2*R*,3*S*)-3-methylproline and rac-4-methylproline were from Syngenta Crop Protection's reference compounds collection.

HPLC analysis was performed on a Waters Alliance 2690 (Waters Corp. Milford, MA, USA) equipped with a Waters 996 diode array detector with the following experimental conditions. Column: YMC-Pak ODS-AQ 120 Å, 5 µm, 125×2.0 mm plus precolumn 10 mm; mobile phase A: water - TFA, 100:0.1; mobile phase B: acetonitrile - TFA, 100:0.1; flow: 0.5 ml/minute; temp.: 40°C; gradient: 0 minute 45% B, 10 minutes 65% B, 12 minutes 100% B; injection: 5 µl of a solution in methanol; UV-detection: 210 nm. Retention times reported below refer to these conditions, unless stated.

Semipreparative HPLC separation was done on an Agilent 1100 with DAD detector. The flow was split 1:20 post column prior to MS analysis. A Quattro micro mass spectrometer (Waters) equipped with electrospray interface was used as a mass detector (cone 30 V). Fractions were collected using a Gilson FC 204 fraction collector.

HR-MS and **MS/MS** spectra were recorded on a Q-TOF I (Micromass, Manchester UK) equipped with an electrospray source (ESI) as follows: source temp: 150°C; desolvation temp.: 350°C; mass range: 100 to 1000 Da (100~1250 Da for **13** and **22**). Cone voltage and collision energy are given below. A mixture of polypropylene glycol (PPG; Aldrich, average M_n ca. 425 and 725; each 5 µg/ml) in 0.02 M ammonium acetate solution - ACN 1:1 was co-injected by a mixing T as a lock mass for accurate mass measurements to the MS at a flow rate of 0.2 ml/minute. A $[M + NH_4]^+$ ion of PPG close to the molecular ion was used for internal calibration. MS/MS experiments of neofraeptins A to C were done by direct infusion by pump syringe (5 µl/minute) of a solution ca. 0.05 mg/ml in MeOH - H₂O, 1:1. All HR-MS and MS/MS experiments of neofraeptins D to N were done by LC-MS with a Agilent 1100 (YMC-Pak ODS-AQ 120 Å, 5 µm, 125×2 mm; mobile phase A: H₂O - HCOOH, 99.5:0.5; mobile phase B: acetonitrile - HCOOH, 99.5:0.5; 0.2 ml/minute; gradient: 0 minute 5% B, 2 minutes 5% B, 12 minutes 95% B; injection: 5 µl of a solution in methanol).

For **MS³** and some **MS/MS** experiments, a LCQ deca XP plus (Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray interface was used. In those experiments the instrument is mentioned. A sheath gas setting of 30 units and a spray voltage of 5 kV was applied. The heated metal capillary was maintained at 250°C. The system was optimized for m/z 549 $[M + H]^+$ of antimycin A₁. Typical parameters are: Capillary voltage 20 V; tube lens offset -19 V; entrance lens -95 V; mass range 120 to 1500 Da.

MS/MS parameters: Isolation width 3.8 Da; no wideband excitation; normalized collision energy 50%; activation time 30 ms.

GS/MS was done on Trace GC Ultra 2160 (Thermo Finnigan; split 1:10) with an MS Trace DSQ (Thermo Finnigan with EI-ionization in positive mode at 70 eV; emission current +100 mA; ion source temp.: 250°C; scan range 50~650 amu) and an autosampler CombiPAL (CTC Analytics, Zwingen, Switzerland).

LC-NMR was done on an Inova 600 MHz (Varian) and tube NMR on a Unity 500 MHz (Varian).

CD measurements were made on a Jasco J710 (Japan Spectroscopic Co., Tokyo, Japan) using a quartz cell with a path length of 0.1 cm. Each measurement was the average of three repeated scans in steps of 0.1 nm at ambient temperature and while the instrument was flushed with nitrogen. The compound concentration was 0.2 mM in water with 5% MeOH. All spectra are background corrected. The optical rotation was determined with a Polarimeter 241 (Perkin-Elmer), FT-IR on a Spectrum One FT-IR (Perkin-Elmer) with universal ATR sampling accessory and UV on a Lambda 19 (Perkin-Elmer) with a cell length of 0.1 cm or on a UV 240 (Shimadzu) with a cell length of 1 cm.

Cleavage by the Protease Papain

To 0.2 ml of 0.05 M Tris buffer, pH 6.8, containing 20 mM 2-mercaptoethanol and 0.5 mM EDTA, 4 mg papain (9.8 units/mg) and 1.4 mg neofraeptin A in 10 µl DMSO - H₂O 1:1 were added. After 4 days at 37°C another portion of 2 mg papain in 100 µl buffer was added and the reaction was maintained for a total of 7 days. In the digest of neofraeptin A two new major peaks were observed by LC/MS at 6.3 minutes (**14**) and 9.7 minutes (**13**). Minor compounds were **15**, **16** and **17**.

HPLC conditions: Waters Alliance 2690 and Waters PDA 966; YMC-Pak ODS-AQ 120 Å, 5 µm, 125×2.0 mm plus precolumn 10 mm; solvent A: water - TFA 100:0.01; solvent B: acetonitrile - TFA 100:0.01; flow: 0.5 ml/minute; oven temperature: 40°C, gradient: 0% B to 100% B in 15 minutes.

By the same way papain digestion of neofraeptin B/C mixture was performed yielding compounds **14**, **15**, **17**, **18** and **19**, neofraeptin E yielded compounds **14**, **18** and **19**, neofraeptin F yielded **14**, **15**, **20**, **22** and **23**, neofraeptin I yielded **14**, **18** and **20**, neofraeptin M yielded **14**, **15** and **21** and neofraeptin L yielded compounds **14**, **18** and **21**.

The desired peptide fragments were isolated by semipreparative HPLC with the following experimental conditions. Column: YMC-Pak ODS-AQ 120 Å, 5 µm,

125×8.0 mm; mobile phase A: water - TFA, 100 : 0.1; mobile phase B: acetonitrile - TFA, 100 : 0.1; flow: 3 ml/minute; temp.: 30°C; gradient: 0 minute 4% B, 10 minutes 28% B, 26 minutes 35% B, 43 minutes 60% B, 44 minutes 80% B; injection: 100 μ l; 3 injections per compound; UV-detection at 220 nm; MS detection: ESI(+) cone 30 V, 200~1400 Da. The peaks with the correct m/z -values were collected with the fraction collector and the solvent was removed with a stream of nitrogen. Retention times (minutes): **17**: 6.7; **20**: 7.2; **19**: 7.5; **21**: 7.6; **14**: 12.3; **16**: 22.0; **23**: 27.5; **15**: 28.5; **18**: 30.3; **13**: 31.0; **22**: 31.9.

Amino Acids Analysis by GC/MS

The hydrolyzates of neofraeptins A to N (0.25 mg; 6 N HCl, 110°C, 24 hours) were evaporated on the speedvac, dried over P₂O₅ for 24 hours and silylated with MSTFA (50 μ l) for 20 minutes at 90°C. The TMS-derivatized amino acids were separated by capillary gas chromatography on a DB-35ms column (Restek, Bellefonte, PA, USA, 30 m×0.25 mm×0.25 μ m) with an initial column temperature of 70°C. The oven temperature was ramped at 5°C/minute to 170°C and then to 320°C at 20°C/minute (carrier gas: He; 1.2 ml/minute; injector temp. 240°C; ion source temperature 250°C). Compounds **15**, **17**, **18** and **19** contained Pip, while compounds **20**, **21**, **22** and **23** contained (2*S*,3*S*)-3-methylproline.

EI-MS of di-TMS derivative of Acc: 245.0 (M^+ , 7), 230.1 (12), 202.1 (36), 147.0 (77), 128.0 (39), 73.2 (100).

EI-MS of di-TMS derivative of (2*S*,3*S*)-3-methylproline: 273 (M^+ , 0.3), 258 (1.3), 230 (4.9), 158 (3.8), 157 (14), 156 (100), 147 (4.6), 75 (2.4), 73 (18).

Determination of the Chirality of the Amino Acids

The hydrolyzates (6 N HCl, 24 hours at 110°C) were esterified with 2-propanol (1 ml, 1 hour at 100°C; acidified with 17% acetyl chloride (v/v); then dried under a stream of nitrogen) and then acetylated with trifluoroacetic anhydride (0.2 ml and 1 ml CH₂Cl₂; 10 minutes at 100°C; then dried under a stream of nitrogen and redissolved in 0.25 ml CH₂Cl₂). The derivatized amino acids were resolved by capillary gas chromatography on a modified cyclodextrin stationary phase (Lipodex E, 50 m×0.25 mm, Macherey-Nagel, Düren, Germany) [29] with an initial column temperature of 70°C. The oven temperature was ramped at 5°C/minute to 150°C, maintained 150°C for 5 minutes, then to 200°C at 10°C/minute (carrier gas: He; 1 ml/minute; injector temp. 180°C; ion source temperature 200°C). The usual amino acids were identified by comparison with commercial standards. The sample of rac-(2*R*,3*S*)-3-methylproline contained all four stereoisomers as minor by-products from synthesis and allowed therefore to

prove that the column separates all four isomers: RT of (2*S*,3*S*) 16.39 minutes; RT of (2*R*,3*R*) 16.75 minutes; RT of (2*R*,3*S*) and (2*S*,3*R*): 17.64 minutes and 18.15 minutes. The derivatized amino acids of neofraeptin F hydrolyzate showed a peak coeluting with commercially available (2*S*,3*S*)-3-methylproline.

Hydrolysis and GC-MS analysis of the TMS-derivatives on a chiral column revealed the chirality of Iva as follows: Compounds **15** and **18** contained only *R*-Iva (RT 10.28 minutes), while compounds **14**, **19** and **21** contained only *S*-Iva (RT 10.13 minutes).

Dansyl Derivatives of the Hydrolyzate of Neofraeptins A or F

To the dried hydrolyzate of neofraeptin A or F (0.25 mg; 6 N HCl, 110°C, 24 hours) in 100 μ l H₂O a solution of 1 N Na₂CO₃ (10 μ l) and dansyl chloride (100 μ l, 13.5 mg in ACN) was added and kept in the dark for 1 hour at room temperature. Before HPLC analysis the reaction was acidified with TFA (10% v/v), the solvent removed by a stream of nitrogen and redissolved in acetonitrile.

¹H NMR of dansyl derivative of (2*S*,3*S*)-3-methylproline (600 MHz; ACN, D₂O, TFA): δ 8.91 (1H, d), 8.43 (1H, d), 8.40 (1H, d), 8.04 (1H, d), 7.91 (1H, t), 7.88 (1H, t), 3.97 (1H, d, J =4.9 Hz, H-1), 3.47 (1H, m), 3.43 (1H, m), 3.41 (6H, s), 2.41 (1H, m), ~2.13 (1H, m), 1.46 (1H, m), 0.90 (3H, d, J =6.6 Hz, 3-Me).

¹H NMR of dansyl derivative of rac-(2*R*,3*S*)-3-methylproline (600 MHz; ACN, D₂O, TFA): δ 8.85 (1H, d), 8.42 (1H, d), 8.32 (1H, d), 8.04 (1H, d), 7.90 (1H, t), 7.87 (1H, t), 4.32 (1H, d, J =8.7 Hz, H-1), 3.63 (1H, t, J =8.5 Hz), 3.40 (6H, s), 3.35 (1H, q, J =8.5 Hz), 2.49 (1H, m, H-3), ~2.14 (1H, m), 1.74 (1H, m), 0.96 (3H, d, J =6.6 Hz, 3-Me).

Physico-chemical Data

Neofraeptin A

HPLC Rt: 6.7 minutes HR-MS (cone Voltage: 20 V) Found: 689.5029 Calcd for C₃₆H₆₅N₈O₅: 689.5078; Found: 816.5308 ($M+H$)²⁺ Calcd for C₈₂H₁₄₀N₁₈O₁₆: 816.5347; Found: 943.5562 Calcd for C₄₆H₇₅N₁₀O₁₁: 943.5617. MS/MS of 943.6 (cone Voltage: 40 V; collision energy: 40~50 V) 943.6; 858.5; 705.5; 647.4; 620.5; 534.3; 449.4; 409.4; 350.3; 296.3; 239.1; 154.1. MS/MS of 689.6 (cone Voltage: 100 V; collision energy: 25~35 V): 689.6; 671.6; 578.5; 565.5; 464.4; 436.4; 367.3; 339.3; 323.4; 254.2; 197.2; 169.2; 125.1. MS/MS of 705.1 (Quattro II, Micromass, cone Voltage: 80 V; collision energy: 35 V, resolution of MS2: 11): 705, 620, 409, 296, 211, 183, 84. UV $\lambda_{\max}^{\text{H}_2\text{O/MeOH}}$ 95:5 nm (ϵ) 204 (43,000), 230 (sh, 9,600). $[\alpha]_D^{25} +0.1$ (c 0.1, MeOH). FT-IR $\nu_{\max}$ (film) cm⁻¹ 3290

(br), 2940, 2870, 1655 (C=O), 1540, 1440, 1420, 1390, 1260, 1200, 1170, 1140. CD (in H₂O/MeOH 95:5): see Fig. 5.

Neofrapeptin B and Neofrapeptin C Mixture

HPLC Rt: 7.6 minutes (compounds are coeluting). HR-MS (cone Voltage: 20 V) Found: 689.5005 Calcd for C₃₆H₆₅N₈O₅: 689.5078; Found: 823.5363 (M+H)²⁺ Calcd for C₈₃H₁₄₂N₁₈O₁₆: 823.5426; Found: 957.5687 Calcd for C₄₇H₇₇N₁₀O₁₁: 957.5773. MS/MS of 957.6 (cone Voltage: 40 V; collision energy: 40~50 V): 957.6; 872.5; 858.6; 719.6; 661.6; 647.6; 634.5; 548.3; 534.4; 449.3; 350.3; 310.3; 239.1; 154.1. MS/MS of 689.6 (cone Voltage: 100 V; collision energy: 25~35 V): 689.6; 671.6; 578.5; 565.5; 464.4; 436.4; 367.3; 339.3; 323.4; 254.2; 197.2; 169.2; 125.1.

Neofrapeptin C

HR-MS (cone Voltage: 20 V) Found: 689.5053 Calcd for C₃₆H₆₅N₈O₅: 689.5078; Found: 823.5427 (M+H)²⁺ Calcd for C₈₃H₁₄₂N₁₈O₁₆: 823.5426; Found: 957.5729 Calcd for C₄₇H₇₇N₁₀O₁₁: 957.5773. MS/MS of 957.6 (cone Voltage: 20 V; collision energy: 30 V): 957.6; 858.5; 775.5; 719.5; 647.4; 620.4; 534.3; 449.3; 350.2; 296.2; 239.1; 154.1. MS/MS of 689.6 (cone Voltage: 20 V; collision energy: 50 V): 689.5; 671.5; 578.4; 464.3; 454.3; 436.4; 367.2; 339.2; 254.2; 197.1; 169.1; 125.1.

Neofrapeptin D

HPLC Rt: 6.2 minutes HR-MS (cone Voltage: 20 V) Found: 689.5035 Calcd for C₃₆H₆₅N₈O₅: 689.5078; Found: 809.5253 (M+H)²⁺ Calcd for C₈₁H₁₃₈N₁₈O₁₆: 809.5269; Found: 929.5430 Calcd for C₄₅H₇₃N₁₀O₁₁: 929.5460. MS/MS of 929.6 (cone Voltage: 40 V; collision energy: 30~45 V): 929.6; 844.5; 761.5; 704.5; 691.5; 633.4; 606.4; 520.3; 435.3; 395.3; 350.3; 282.2; 239.2; 154.1. MS/MS of 689.5 (cone Voltage: 40 V; collision energy: 45~60 V): 689.5; 671.5; 578.4; 565.4; 464.4; 454.4; 436.4; 367.2; 339.2; 254.1; 197.1; 169.1; 125.1. UV $\lambda_{\max}^{\text{H}_2\text{O}/\text{MeOH } 95:5}$ nm (ϵ) <200 (50,400). $[\alpha]_D^{25} +0.3$ (c 0.1, MeOH).

Neofrapeptin E

HPLC Rt: 8.2 minutes HR-MS (cone Voltage: 20 V) Found: 689.5018 Calcd for C₃₆H₆₅N₈O₅: 689.5078; Found: 830.5455 (M+H)²⁺ Calcd for C₈₄H₁₄₄N₁₈O₁₆: 830.5504; Found: 971.5896 Calcd for C₄₈H₇₉N₁₀O₁₁: 971.5930. MS/MS of 971.6 (cone Voltage: 40 V; collision energy: 30~45 V): 971.6; 872.5; 789.5; 733.5; 661.5; 634.4; 548.3; 449.3; 423.3; 350.2; 310.2; 239.1; 154.1. MS/MS of 689.5 (cone Voltage: 40 V; collision energy: 45~50 V): 689.5; 671.5; 578.5; 565.4; 464.3; 454.3; 436.4; 367.2; 339.2;

323.3; 254.2; 197.1; 169.1; 125.1. UV $\lambda_{\max}^{\text{H}_2\text{O}/\text{MeOH } 95:5}$ nm (ϵ) <200 (48,300). $[\alpha]_D^{25} +0.3$ (c 0.1, MeOH). FT-IR $\nu_{\max}$ (film) cm⁻¹ 3290 (br), 2940, 2870, 1655 (C=O), 1540, 1460, 1440, 1390, 1260, 1170, 1140.

Neofrapeptin F

HPLC Rt: 8.9 minutes HR-MS (cone Voltage: 20 V) Found: 689.5058 Calcd for C₃₆H₆₅N₈O₅: 689.5078; Found: 816.5270 (M+H)²⁺ Calcd for C₈₂H₁₄₀N₁₈O₁₆: 816.5347; Found: 943.5602 Calcd for C₄₆H₇₅N₁₀O₁₁: 943.5617. MS/MS of 943.6 (cone Voltage: 40 V; collision energy: 30~45 V) 943.6; 858.5; 775.5; 718.5; 705.4; 647.4; 620.4; 534.3; 449.3; 409.3; 350.2; 296.2; 239.1; 154.1. MS/MS of 689.5 (cone Voltage: 40 V; collision energy: 45~50 V): 689.5; 671.5; 565.4; 464.3; 436.4; 367.2; 339.3; 254.2; 197.1; 169.1; 125.1. UV $\lambda_{\max}^{\text{H}_2\text{O}/\text{MeOH } 95:5}$ nm (ϵ) 204 (42,400), 230 (sh, 10,100). $[\alpha]_D^{25} +0.2$ (c 0.1, MeOH). FT-IR $\nu_{\max}$ (film) cm⁻¹ 3290 (br), 2930, 2870, 1645 (C=O), 1530, 1440, 1410, 1390, 1260, 1170, 1140. CD (in H₂O/MeOH 95:5): see Fig. 5.

Neofrapeptin G

HPLC Rt: 9.7 minutes (gradient as in cleavage with papain). HR-MS (cone Voltage: 20 V) Found: 1214.7151 (M+H)⁺ Calcd for C₅₈H₉₆N₁₃O₁₅: 1214.7149; Found: 943.5634 Calcd for C₄₆H₇₅N₁₀O₁₁: 943.5617. MS/MS of 1214.7 (cone Voltage: 20 V; collision energy: 40 V): 1054.7, 943.6; 858.5; 647.4; 534.3; 449.3; 350.2; 154.1. MS/MS of 943.6 (cone Voltage: 20 V; collision energy: 30 V): 943.6; 858.5; 775.5; 718.4; 705.4; 647.4; 620.4; 534.3; 449.3; 410.3; 350.2; 296.2; 239.1; 154.1. $[\alpha]_D^{25} -0.1$ (c 0.1, MeOH). Additional physico-chemical data see compound **13**.

Neofrapeptin H

HPLC Rt: 9.9 minutes (gradient as in cleavage with papain). HR-MS (cone Voltage: 20 V) Found: 1228.7301 (M+H)⁺ Calcd for C₅₉H₉₈N₁₃O₁₅: 1228.7305; Found: 1153.6980 Calcd for C₅₇H₉₃N₁₂O₁₃: 1153.6985; Found: 957.5752 Calcd for C₄₇H₇₇N₁₀O₁₁: 957.5773. MS/MS of 1228.7 (cone Voltage: 20 V; collision energy: 30 V): 1068.7, 957.6; 872.5; 719.5, 661.4; 548.3; 449.3; 350.2; 154.1. MS/MS of 957.6 (cone Voltage: 20 V; collision energy: 40 V): 957.6; 872.5; 789.5; 719.5; 661.4; 634.4; 548.3; 449.3; 423.3; 350.2; 310.2; 239.1; 154.1.

Neofrapeptin I

HPLC Rt: 9.7 minutes HR-MS (cone Voltage: 20 V) Found: 689.4979 Calcd for C₃₆H₆₅N₈O₅: 689.5078; Found: 823.5396 (M+H)²⁺ Calcd for C₈₃H₁₄₂N₁₈O₁₆: 823.5426; Found: 957.5628 Calcd for C₄₇H₇₇N₁₀O₁₁: 957.5773.

MS/MS of 957.6 (cone Voltage: 40 V; collision energy: 30 V): 957.6; 872.5; 719.4; 661.4; 634.4; 548.3; 449.3; 350.2; 310.2; 239.1; 154.1. MS/MS of 689.6 (cone Voltage: 40 V; collision energy: 50 V): 689.5; 671.5; 565.4; 503.4; 464.3; 436.4; 367.2; 339.2; 254.1; 197.1; 169.1; 125.1. UV $\lambda_{\text{max}}^{\text{H}_2\text{O}/\text{MeOH } 95:5}$ nm (ϵ) <200 (50,900). $[\alpha]_{\text{D}}^{25} +0.3$ (c 0.1, MeOH).

Neofrapeptin L

HPLC Rt: 10.3 minutes HR-MS (cone Voltage: 20 V) Found: 689.5064 Calcd for $\text{C}_{36}\text{H}_{65}\text{N}_8\text{O}_5$: 689.5078 Found: 830.5529 (M+H) $^{2+}$ Calcd for $\text{C}_{84}\text{H}_{144}\text{N}_{18}\text{O}_{16}$: 830.5504; Found: 971.5920 Calcd for $\text{C}_{48}\text{H}_{79}\text{N}_{10}\text{O}_{11}$: 971.5930. MS/MS of 971.6 (cone Voltage: 20 V; collision energy: 30 V): 971.6; 872.5; 733.5; 661.4; 634.4; 548.4; 449.3; 424.3; 350.2; 310.2; 239.1; 154.1. MS/MS of 689.6 (cone Voltage: 20 V; collision energy: 50 V): 689.6; 671.5; 436.4; 339.2; 254.2; 197.1; 169.1; 125.1. UV $\lambda_{\text{max}}^{\text{H}_2\text{O}/\text{MeOH } 95:5}$ nm (ϵ) <200 (45,800). $[\alpha]_{\text{D}}^{25} +0.2$ (c 0.1, MeOH).

Neofrapeptin M

HPLC Rt: 9.4 minutes HR-MS (cone Voltage: 20 V) Found: 689.5099 Calcd for $\text{C}_{36}\text{H}_{65}\text{N}_8\text{O}_5$: 689.5078 Found: 823.5443 (M+H) $^{2+}$ Calcd for $\text{C}_{83}\text{H}_{142}\text{N}_{18}\text{O}_{16}$: 823.5426; Found: 957.5807 Calcd for $\text{C}_{47}\text{H}_{77}\text{N}_{10}\text{O}_{11}$: 957.5773. MS/MS of 957.6 (cone Voltage: 20 V; collision energy: 30 V): 957.6; 858.5; 775.5; 719.5; 705.4; 647.4; 620.4; 534.3; 449.3; 424.3; 350.2; 296.2; 239.1; 154.1. MS/MS of 689.6 (cone Voltage: 20 V; collision energy: 45 V): 689.5; 671.5; 565.4; 464.3; 369.3; 367.2; 339.2; 254.1; 197.1; 169.1; 125.1. UV $\lambda_{\text{max}}^{\text{H}_2\text{O}/\text{MeOH } 95:5}$ nm (ϵ) <200 (52,000). $[\alpha]_{\text{D}}^{25} +0.3$ (c 0.1, MeOH).

Neofrapeptin N

HPLC Rt: 5.2 minutes HR-MS (cone Voltage: 20 V) Found: 675.4930 Calcd for $\text{C}_{35}\text{H}_{63}\text{N}_8\text{O}_5$: 675.4921 Found: 802.5237 (M+H) $^{2+}$ Calcd for $\text{C}_{80}\text{H}_{136}\text{N}_{18}\text{O}_{16}$: 802.5191; Found: 929.5457 Calcd for $\text{C}_{45}\text{H}_{73}\text{N}_{10}\text{O}_{11}$: 929.5460. MS/MS of 929.5 (cone Voltage: 20 V; collision energy: 30~45 V) 929.5; 844.5; 761.5; 704.4; 691.4; 633.4; 606.4; 520.3; 435.3; 410.3; 395.3; 350.2; 336.2; 282.2; 239.1; 154.1. MS/MS of 675.5 (cone Voltage: 20 V; collision energy: 45~60 V): 675.5; 657.5; 564.4; 505.4; 489.4; 450.3; 440.3; 422.4; 367.2; 355.3; 339.2; 254.2; 197.1; 169.1; 125.1.

13

HPLC Rt: 9.7 minutes HR-MS (cone Voltage: 15 V) Found: 1214.7264 (M+H) $^{+}$ Calcd for $\text{C}_{58}\text{H}_{96}\text{N}_{13}\text{O}_{15}$: 1214.7149. MS/MS of 1214.7 (cone Voltage: 20 V; collision energy: 20~35 V): 1054.7, 943.6, 858.5, 718.4, 705.4, 647.4,

534.4, 449.3, 350.2, 239, 154. CD $\lambda_{\text{max}}^{\text{H}_2\text{O}/\text{MeOH } 95:5}$ nm (Θ) 225 sh (−24,900), 207 (−69,400). UV $\lambda_{\text{max}}^{\text{H}_2\text{O}/\text{MeOH } 95:5}$ nm (ϵ) <200 (45,000).

14 (C-terminal fragment of neofrapeptin A)

HPLC Rt: 6.3 minutes HR-MS (cone Voltage: 15 V) Found: 436.3593 (M $^{+}$) Calcd for $\text{C}_{24}\text{H}_{46}\text{N}_5\text{O}_2$: 436.3652. MS/MS of 436.3 (cone Voltage: 20 V; collision energy: 30 V): 436.3, Found: 312.2708 Calcd for $\text{C}_{17}\text{H}_{34}\text{N}_3\text{O}_2$: 312.2651; Found: 241.1941 Calcd for $\text{C}_{13}\text{H}_{25}\text{N}_2\text{O}_2$: 241.1916; Found: 199.1817 Calcd for $\text{C}_{11}\text{H}_{23}\text{N}_2\text{O}$: 199.1810; Found: 185.1677 Calcd for $\text{C}_{10}\text{H}_{21}\text{N}_2\text{O}$: 185.1654; Found: 125.1122 Calcd for $\text{C}_7\text{H}_{13}\text{N}_2$: 125.1079; Found: 72.0850 Calcd for $\text{C}_4\text{H}_{10}\text{N}$: 72.0813. MS 3 of fragment 312 (LCQ deca XP Plus; normalized collision energy 40% of m/z 436 then 30% of 312; isolation width 3.8): 241, 199, 185; MS 3 of fragment 241: 141, 113. ^{13}C NMR (CD_3OD) δ ; numbering see [4]; 175.4 (1C=O), 54.4 (1 α), 44.8 (1 β), 25.7 (1 γ), 22.2 (1 δ 1*), 23.4 (1 δ 2*), 61.1 (2 α), 30.0 (2 β), 8.3 (2 γ), 22.7 (2 β 1), 176.9 (2C=O), 46.4 (1), 57.9 (2), 41.4 (3), 25.9 (4), 21.7 (5*), 23.8 (6*), 45.7 (2*), 19.9 (3'), 43.5 (4'*), 55.6 (6'), 19.1 (7'), 31.9 (8'), 166.2 (8'a) *: assignments may be interchanged. UV $\lambda_{\text{max}}^{\text{H}_2\text{O}/\text{MeOH } 95:5}$ nm (ϵ) 220 (7,290). CD $\lambda_{\text{max}}^{\text{H}_2\text{O}/\text{MeOH } 95:5}$ nm (Θ) 220 (−13,800), 189 (26,600).

15 (Ac-Pip 1 -Aib-Aib-Iva-Aib-Leu 6)

HPLC Rt: 9.6 minutes HR-MS (cone Voltage: 15 V) Found: 665.4189 (M+H) $^{+}$ Calcd for $\text{C}_{33}\text{H}_{57}\text{N}_6\text{O}_8$: 665.4238. MS/MS of 665.4 (cone Voltage: 20 V; collision energy: 15 V): 534.3, 512.4, 449.3, 427.3, 350.2, 296.2, 239.2, 154.1.

16 (β -Ala-Gly-Acc-Aib-Pip-Aib-Gly-Leu-Iva-C-Terminus)

HPLC Rt: 8.0 minutes HR-MS (cone Voltage: 15 V) Found: 985.6564 (M $^{+}$) Calcd for $\text{C}_{49}\text{H}_{85}\text{N}_{12}\text{O}_9$: 985.6562. MS/MS (LCQ deca XP plus) of 985.7: 968.6, 857.6, 774.6, 689.6, 671.6, 588.6, 578.5, 565.5, 408.2, 367.2.

17 (β -Ala 7 -Gly-Acc-Aib-Pip-Aib-Gly 13)

HPLC Rt: 4.8 minutes HR-MS (cone Voltage: 15 V) Found: 568.3150 (M+H) $^{+}$ Calcd for $\text{C}_{25}\text{H}_{42}\text{N}_7\text{O}_8$: 568.3095. MS/MS of 568.3 (cone Voltage: 20 V; collision energy: 25 V): 493.3, 408.2, 380.2, 323.2, 297.2, 272.2, 269.2, 212.1.

18

HPLC Rt: 9.9 minutes MS: 679.4 (M+H) $^{+}$. MS/MS of 679.4 (cone Voltage: 20 V; collision energy: 20 V): 548.3, 526.4, 449.3, 441.3, 350.2, 310.2, 239.2, 154.1.

19

HPLC Rt: 5.1 minutes MS: 582.3 (M+H) $^{+}$. MS/MS of

582.3 (cone Voltage: 20 V; collision energy: 25 V): 507.3, 422.2, 408.3, 394.4, 323.2, 311.2, 283.2, 272.2, 212.1.

20 (β -Ala⁷-Gly-Acc-Aib-3-Me-Pro-Aib-Gly¹³)

HPLC Rt: 5.0 minutes HR-MS (cone Voltage: 19 V) Found: 568.3107 (M+H)⁺ Calcd for C₂₅H₄₂N₇O₈: 568.3095. MS/MS of 568.2 (LCQ deca XP plus): 493.2, 465.3, 408.2, 297.1, 272.1, 212.1.

21

HPLC Rt: 5.3 minutes MS: 582.3 (M+H)⁺. MS/MS of 582.3 (cone Voltage: 20 V; collision energy: 25 V): 507.3, 422.2, 408.3, 323.2, 311.2, 283.2, 272.2, 212.1.

22 (Ac-Pip-Aib-Pip-Iva-Aib-Leu- β -Ala-Gly-Acc-Aib-MePro-Aib-Gly-OH)

HPLC Rt: 9.8 minutes HR-MS (cone Voltage: 19 V) Found: 607.8569 (M+2H)²⁺ Calcd for C₅₈H₉₇N₁₃O₁₅: 607.8614. MS/MS (LCQ deca XP plus) of 1236.8 (M+Na)⁺: 1161.7, 1133.8, 1076.6, 998.7, 965.7, 937.7, 897.7, 880.7, 852.6.

23 (β -Ala-Gly-Acc-Aib-MePro-Aib-Gly-Leu-Iva-C-Terminus)

HPLC Rt: 8.5 minutes HR-MS (cone Voltage: 19 V) Found: 985.6555 (M⁺) Calcd for C₄₉H₈₅N₁₂O₉: 985.6562. MS/MS (LCQ deca XP plus) of 985.7: 967.8, 857.7, 774.6, 689.6, 671.6, 578.4, 565.4, 493.3, 465.5, 367.2, 339.2.

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