On the Biodegradation of β-Peptides**

Jürg. V. Schreiber,[a] Jens Frackenpohl,[a] Frank Moser,[a] Thomas Fleischmann,[b] Hans-Peter E. Kohler,*[b] and Dieter Seebach*[a]

A consortium of microorganisms was established that was able to grow with the β-tripeptide H-β-HVal-β-HAla-β-HLeu-OH, with the β-dipeptide H-β-HAla-β-HLeu-OH, and with the β-amino acids H-β-HAla-OH, H-β-HVal-OH, and H-β-HLeu-OH as the sole carbon and energy sources. This growth was achieved after several incubation-transfer cycles with the β-tripeptide as the substrate. During degradation of the β-tripeptide H-β-HVal-β-HAla-β-HLeu-OH, the temporary formation of a metabolite was observed. The metabolite was identified as the β-dipeptide H-β-HAla-β-HLeu-OH by nuclear magnetic resonance spectroscopy and mass spectrometry. This result indicates that in the course of the degradation of the β-tripeptide, the N-terminal β-HVal residue was cleaved off by a not yet known mechanism. During the subsequent degradation of the β-dipeptide, formation of additional metabolites could not be detected. The growth – yield coefficients $Y_{x/s}$ for growth on the β-di- and β-tripeptide both had a value of 0.45. When a 1:1 mixture of the β-tripeptide and the corresponding α-tripeptide H-Val-Ala-Leu-OH was added to the enrichment culture, the α-peptide was completely utilized in six days and thereafter growth of the culture stopped. This result indicates that even in β-peptide enrichment cultures, α-peptides are the preferred substrates. Our experiments clearly show for the first time that β-peptides and β-amino acids are amenable to biodegradation and that a microbial consortium was able to utilize these compounds as sole carbon and energy sources. Furthermore, the preparation of β-amino acids, of derivatives thereof, and of β-di- and β-tripeptides is described.

KEYWORDS:

biodegradation · biotransformations · environmental chemistry · peptides · metabolism

Introduction

A major characteristic of β-peptides is their ability to form stable secondary structures with as few as four β-amino acid residues. This is in sharp contrast to α-peptides, for which stable secondary structures are observed only with longer sequences of α-amino acids.[1] The high stability of β-peptidic helices makes β-peptides an attractive class of compounds for drug design. It has been demonstrated that certain β-tetrapeptides have high affinity for a human somatostatin protein receptor.[2] In addition to structural stability, β-peptides exhibit a remarkable stability with regard to degradation by proteolytic enzymes.[3–5] Extracellular enzymes from bacteria and fungi, such as pronase and pronase E from *Streptomyces griseus*, proteinase K from *Tritirachium album*, and proteinase from *Bacillus subtilis* var. biotecus A, degrade neither β-peptides nor γ-peptides. The potential for use of β-peptides in medicinal chemistry and materials science prompted us to investigate their biodegradability. The importance of this investigation is underlined by the fact that a lot of commercially important compounds, such as pesticides, pharmaceuticals, and consumer chemicals, eventually enter the environment.[6] Thus, questions about their clearance from the environment are of great concern. Interestingly, the oligo-[(*R*)-3-hydroxyalkanoates] are fully biodegradable.[7]. These compounds are structurally related to β-peptides and were the starting point of our investigations of these peptides. As a result of their biodegradability, industrial interests have focused on the use of polyhydroxybutyrate as well as copolymers of 3-hydroxybutyrate and 3-hydroxyvalerate as biodegradable plastics.[8]

In a preliminary communication we reported growth experiments with defined strains of *Pseudomonas aeruginosa* (PAO1S[9]) and *P. putida* (S313, DSM 6884[10]) that tested their ability to metabolize β-amino acids and to cleave β-peptides.[4] The free β-amino acids **1** – **4**, the *N*-acetyl-β-amino acids **5** – **8**, β-dipeptides **9** and **10**, dipeptides **11** and **12** (which consist of a β- and an α-amino acid), and for comparison, α-dipeptide **13** (see Scheme 1) were offered to the bacteria as sole carbon and nitrogen sources. With the exception of certain 3-aminobutanoic-acid (β-HAla) derivatives, the bacteria did not grow on the free β-amino acids, on the *N*-acetyl-β-amino acids, or on the dipeptides. As expected *P. aeruginosa* and *P. putida* grew on the α-dipeptide **13**.

The objectives of the present study were, (1) to investigate whether β-peptides would be biodegradable, (2) to gain information about the pathway of a possible degradation, and (3) to substantiate the results of preliminary growth experiments.[4]

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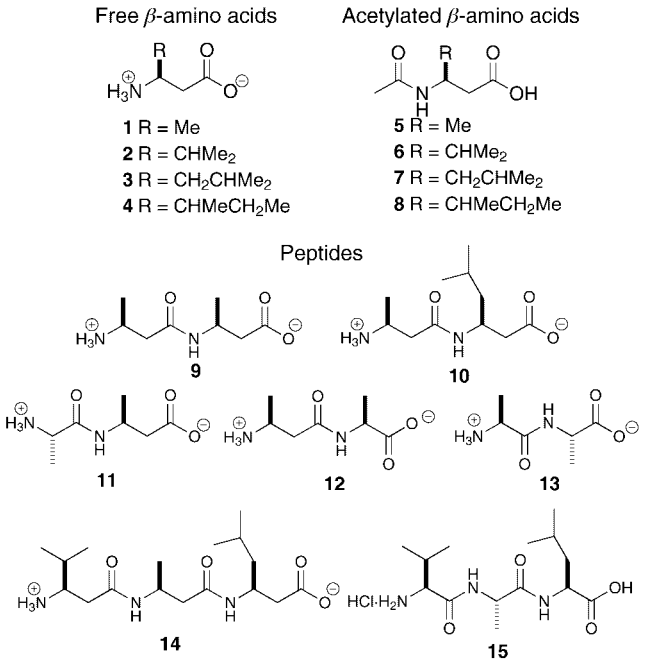

Scheme 1. *β-Amino acids, derivatives thereof, and peptides used for the degradation studies.*

Results and Discussion

Preparation of β-amino acids, derivatives thereof, and peptides

We chose β-substituted homologues of alanine, valine, leucine, and isoleucine as test substrates and as building blocks for the synthesis of the di- and tripeptides (Scheme 1). The required β-amino acids and derivatives were all prepared by the previously described method (Arndt – Eistert homologation of α-amino acid precursors).[11, 12] The free β-amino acids **1** – **4** were derived by trifluoroacetic acid (TFA) mediated *tert*-butoxycarbonyl (Boc) deprotection of the corresponding *N*-protected β-amino acids and purification by ion-exchange chromatography. Acetylation of compounds **1** – **4** with Ac_2O afforded the *N*-acetyl β-amino acid derivatives **5** – **8**. The peptides **9** – **12** and **14** were prepared in solution by the standard procedure (3-(3-dimethylaminopropyl)-1-ethylcarbodiimide (EDC)/HOBt),[11, 13] with the orthogonal protecting groups Boc and methylester and subsequent purification by ion-exchange chromatography. α-Dipeptide **13** is commercially available and saponification of Boc-Val-Ala-Leu-OMe with NaOH followed by Boc deprotection with HCl in dioxane gave compound **15**.

Biodegradation experiments

In order to obtain enrichment cultures that would degrade β-peptides, soil samples from two different locations and a sample from the aeration tank of a sewage treatment plant were collected. Solid materials from the soil samples were suspended in fresh water for initial experiments. Aliquots were taken from the supernatant liquid and from the solution from the water purification facility and were added to buffered solutions that contained β-tripeptide **14** as the sole carbon and energy source (for details, see the Experimental Section). To our surprise, a significant increase of optical density at 546 nm (OD^{546}) indicated the growth of microorganisms in all three cases. In order to verify that the β-tripeptide **14** was actually metabolized, the experiments were repeated, mixing experiments were carried out, and the culture media with the strongest growth of microorganisms were analyzed by HPLC (Figure 1). Thus, freshly prepared media containing the β-tripeptide **14** as substrate were inoculated with samples of turbid cultures and incubated for 62 days. At certain intervals, samples were withdrawn and analyzed by reversed-phase HPLC. The peak originating from the β-tripeptide **14** had disappeared after 62 days in all three incubations, although it diminished at different rates. We focused on sample C (Figure 1) for all further experiments as this culture showed an HPLC-degradation profile with just one single new peak.

More detailed HPLC investigations of the β-tripeptide degradation were performed after several transfers of the enriched cultures to new media. A medium that contained β-tripeptide **14** as the sole carbon and energy source was inoculated with microorganisms from the enrichment mixture. Samples of this incubation mixture were withdrawn in 24 h intervals and analyzed by reversed-phase HPLC, UV/Vis spectroscopy, and dissolved organic carbon (DOC) measurements. As shown in Figure 2a, the concentration of β-tripeptide **14** decreased continuously, whereas the OD^{546} value increased. Interestingly, microbial degradation of β-tripeptide **14** led to formation of a metabolite with a concentration maximum at 200 h, at which point 50% of **14** had been degraded. Afterwards, the concentration of the metabolite also decreased, with complete consumption of both the tripeptide and the metabolite after an incubation time of 450 h. The OD^{546} values reached their maximum after 400 h and then slightly decreased, most likely due to the fact that the culture entered the death phase.

The metabolite formed during the course of the degradation was isolated and purified by preparative reversed-phase HPLC. Analyses by mass spectrometry, ^{1}H NMR spectroscopy, and analytical HPLC, as well as comparison with an authentic sample, showed that this metabolite was β-dipeptide H-β-HAla-β-HLeu-OH (**10**). The DOC levels measured, along with the concentration of β-tripeptide **14** and the optical density, correspond to a steady conversion of the starting material into biomass (and presumably CO_2; Figure 2b). The DOC measurements correlate well with the concentrations of di- and tripeptides **10** and **14** as DOC levels decreased more slowly than the tripeptide concentration owing to intermediate formation of dipeptide **10**. Based on the formation of dipeptide **10**, it is evident that the first degradation step takes place by cleavage of the β-HVal residue (by a hitherto unknown mechanism).

Next, degradation of β-dipeptide **10** was investigated. The growth of microorganisms proceeded much faster than with tripeptide **14** and led to complete consumption of the starting material within 240 h (Figure 3; compare with 450 h in the case of tripeptide **14**). In accordance with the results obtained in the course of the degradation of tripeptide **14** (Figure 2a), dipeptide **10** was degraded in such a way that we could not detect any

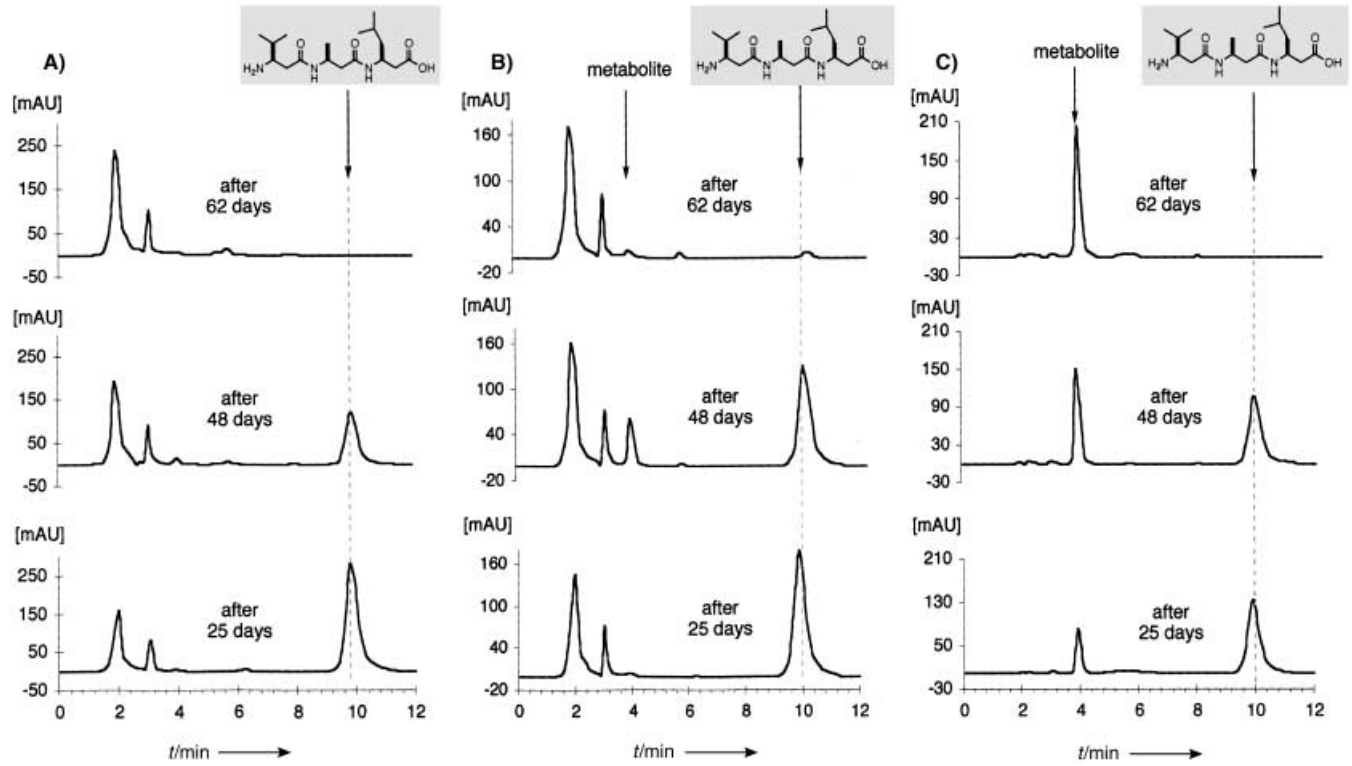

Figure 1. *Degradation of a β-tripeptide under environmental conditions: HPLC profiles of culture media with the strongest growth of microorganisms (A, B, and C; see the Experimental Section for details). The β-peptide* ***14*** *was offered as the sole energy and carbon source.*

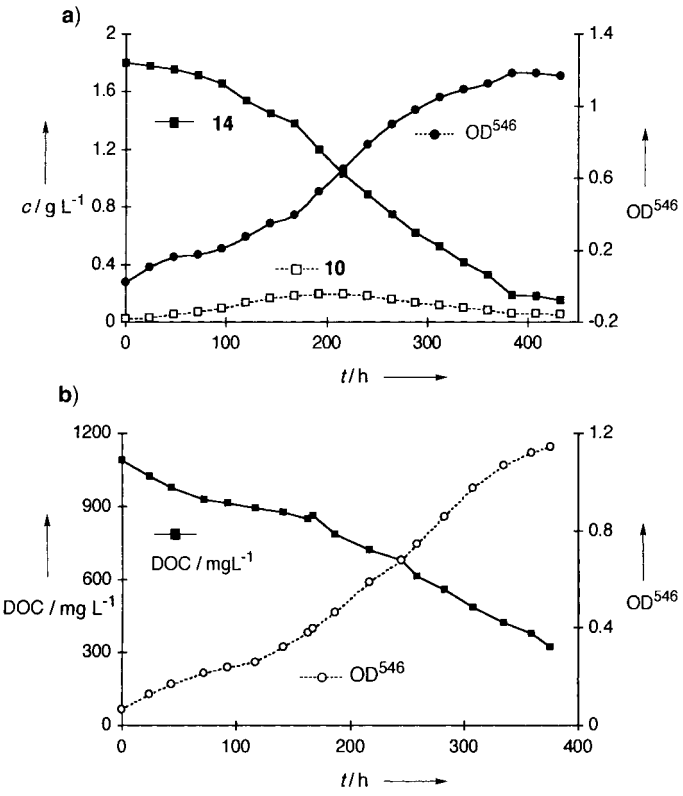

Figure 2. Degradation of β-*tripeptide* ***14*** *and formation of* β-*dipeptide* ***10****, monitored by concentration (HPLC integration; (a)), OD (a, b), and DOC (b) measurements.*

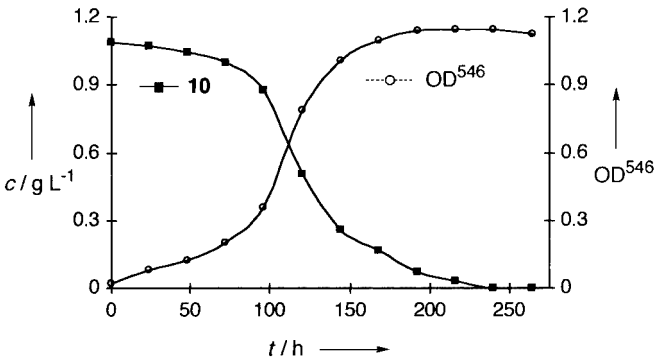

Figure 3. Degradation of β-*dipeptide* ***10*** *monitored by concentration (HPLC integration) and OD measurements.*

metabolites giving rise to biomass (and CO_2). Neither of the two β-amino acids H-β-HAla-OH (**1**) and H-β-HVal-OH (**2**) were detected in any of the degradation experiments.

Additional growth experiments with the free β-amino acids **1** – **3** showed that the enriched microorganism consortium was able to degrade the single amino acids quite easily (Figure 4). Amino acids **2** and **3** were consumed completely within 150 h, whereas degradation of **1** was slightly slower (170 h).

More quantitative information about the microbial degradation of the β-di- and β-tripeptides **10** and **14** was gained by determination of the growth – yield coefficients $Y_{x/s}$ (for definition, see the Experimental Section) of both compounds since

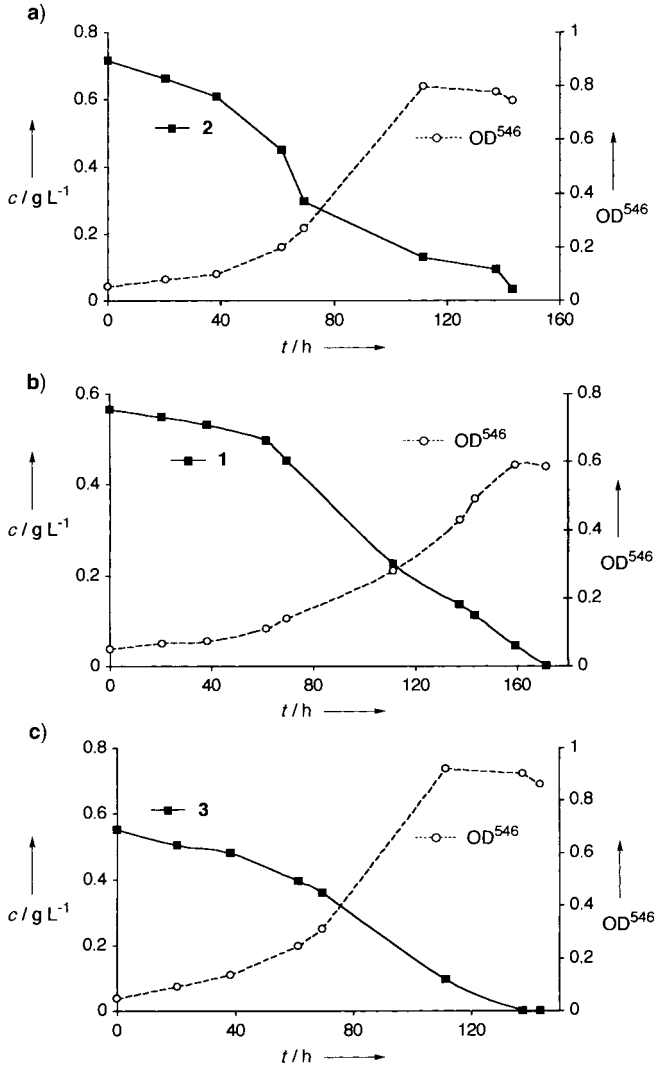

Figure 4. *Degradation of the β-amino acids **2** (a), **1** (b), and **3** (c), monitored by concentration (HPLC integration) and OD measurements.*

these values represent the energy converted by microorganisms with **10** and **14** as carbon sources (Table 1). Samples of growing and grown (stationary phase) cultures were withdrawn, the concentration of the remaining peptides was determined, and the dry weight of biomass was measured by filtration of a defined volume of the samples. The average growth–yield coefficients $Y_{x/s}$ for the degradation of **10** and **14** both had a value of 0.45. This value is in the range of that for the $Y_{x/s}$ of glucose (0.50).[14] Hence, the β-dipeptide **10** and the β-tripeptide **14** are equally well suited to the role of carbon and energy source for the consortium and can be utilized as efficiently as glucose.

Table 1. *Growth–yield coefficients ($Y_{x/s}$) of β-peptides **10** and **14**.*

β-Peptide	**10**			**14**		
t [h]	120	168	216	312	360	408
c(**14**) [g L^{-1}][a]	-	-	-	0.50	0.31	0.17
c(**10**) [g L^{-1}][a]	0.54	0.18	0.04	0.12	0.09	0.06
amount of peptide consumed [g L^{-1}]	0.61	0.97	1.11	1.09	1.31	1.48
OD (546 nm)	0.79	1.09	1.14	1.05	1.12	1.18
dry weight of biomass [mg mL^{-1}]	0.30	0.43	0.46	0.55	0.57	0.60
OD/dry weight	2.64	2.55	2.49	1.91	1.97	1.97
$Y_{x/s}$	0.49	0.44	0.42	0.50	0.44	0.41

[a] Peptide concentrations in the culture solutions were taken from the experiments presented in Figure 2 and 3.

Interestingly, the degradation process improved over time during the enrichment experiments so that complete degradation of the β-tripeptide **14** was achieved within 20–25 days after six enrichment cycles. However, attempts to isolate the microorganisms responsible for degradation have not yet been successful. Colonies that were formed upon incubation on agar plates with **14** as the substrate were isolated and tested separately in degradation experiments that used tripeptide **14** in liquid cultures. No degradation was observed. Furthermore, combination of isolated colonies did not lead to degradation either.

To test the specificity of the β-peptide-degrading consortium, α-peptide **15** and a 1:1 mixture of the β-tripeptide **14** and the α-peptide **15** were incubated under the same conditions (Figure 5). Growth of microorganisms on α-tripeptide **15** proceeded faster than in the corresponding experiment with β-tripeptide **14**. Maximal OD^{546} values were obtained after 150 h with **15**, as compared to approximately 400 h with **14** (see Figure 2a). Likewise, the concentration curve of α-tripeptide **15** shows that the α-peptide is degraded entirely within 150 h (Figure 5a). Furthermore, the maximal optical density was twice as high as with β-peptide **14**, that is, more biomass was produced. Interestingly, OD^{546} curves of both degradation experiments

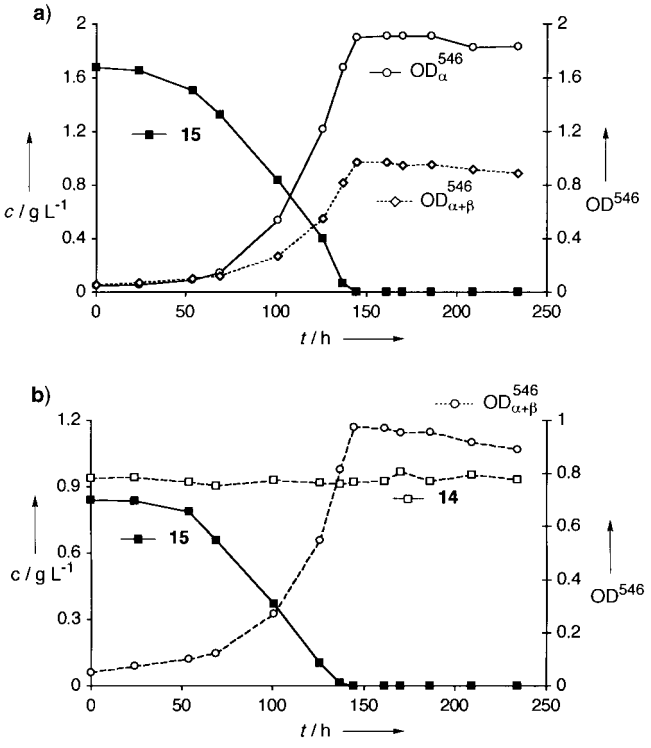

Figure 5. *Degradation of α-tripeptide **15** (a) and of a 1:1 mixture of β-peptide **14** and α-peptide **15** (b), monitored by concentration (HPLC integration) and OD measurements.*

with α-tripeptide **15** (pure **15** and a 1:1 mixture of **14** and **15**) show similar shapes, but the curve obtained with the mixture reaches only 50% of the OD^{546} that was observed in the degradation experiment with α-tripeptide **15** alone. In agreement with these results, concentration curves of both components of the 1:1 mixture indicate that α-peptide **15** is entirely degraded in 150 h, whereas β-peptide **14** is not degraded at all within the time range of the experiment. Thus, the β-peptide-degrading consortium seems to prefer the α-peptide as a growth substrate.

Conclusion

The experiments presented in this study demonstrate for the first time the complete microbial degradation of β-tripeptides, β-dipeptides, and β-amino acids. A microbial consortium enriched with the β-tripeptide **14** was able to utilize these compounds as sole carbon and energy sources. In view of the extraordinary stability of such peptides with regard to cleavage by isolated proteolytic enzymes,[5] this is quite a surprising finding and probably means that mechanisms and biocatalysts different from those seen when α-peptides are available apply. However, this result is in agreement with our ongoing studies on the antimicrobial activities of β-peptides,[15] in which we have observed that growth of various eukaryotic fungi was induced and stimulated by certain β-peptides. The failure to isolate pure cultures of microorganisms with the ability to use β-peptides as their sole carbon and energy source leads us to suggest that in natural environments the complete degradation of β-peptides may only occur through the synergistic activities of a consortium of microorganisms.

Experimental Section

General: Tetrahydrofuran (THF) was freshly distilled over Na/benzophenone under Ar before use. $CHCl_3$ for optical rotation measurements was filtered over basic Al_2O_3 (alumina, Woelm N, actvity I) to remove EtOH. Solvents for chromatography and workup procedures were distilled from Sikkon ($CaSO_4 \cdot H_2O$) (hexane, pentane, AcOEt, and MeOH) and P_2O_5 (CH_2Cl_2 and $CHCl_3$).

Reagents and methods: Et_3N was distilled from CaH_2 and stored over KOH. Amino acid derivatives were purchased from Bachem, Senn, Degussa, or Fluka. Ar was purchased from PanGas. The following compounds were prepared according to literature procedures: Boc-(*S*)-β^3-HAla-OH, Boc-(*R*)-β^3-HVal-OH, Boc-(*S*)-β^3-HLeu-OH, Boc-(*S*)-β^3-Ile-OH, Boc-(*S*)-β^3-HAla-OMe, and Boc-(*S*)-β^3-HLeu-Ome;[11] Boc-(*S*)-β^3-HAla-(*S*)-β^3-HAla-OMe;[17] Boc-(*R*)-β^3-HVal-(*S*)-β^3-HAla-(*S*)-β^3-HAla-OMe;[17] Boc-Val-Ala-Leu-Ome.[18]. Thin layer chromatography (TLC) analysis was performed on Merck silica gel 60 F_{254} plates and results were detected with UV light. The plates were dipped into a solution of ninhydrin (300 mg), HOAc (3 mL), and 1-butanol (100 mL) then heated with a heat gun. Flash chromatography (FC) was carried out with Fluka silica gel 60 (40–63 mm) at RT at a pressure of about 0.3 bar. Eluents are indicated in the text below. Mp values were measured in open-end glass capillary tubes on a Büchi 510 apparatus. The values reported are uncorrected. IR Spectra were measured from 1–2% solutions in $CHCl_3$ or KBr pellets on a Perkin–Elmer 782 spectrophotometer. ^1H NMR spectra were recorded on a Bruker AMX 500 (500 MHz), AMX 400 (400 MHz), or ARX 300 (300 MHz) spectrometer. ^{13}C NMR spectra were recorded on a Bruker AMX 500 (125 MHz), AMX 400 (100 MHz), or ARX 300 (75 MHz) spectrometer. Tetramethylsilane was used as the internal standard. Mass spectrometry was performed on a ZAB2 SEQ (fast atom bombardment (FAB), in a 3-nitrobenzylalcohol matrix), Finnigan MAT TSQ 700 (electrospray ionisation (ESI)), or IonSpec Ultima 4.7 T FT (ion cyclotron resonance (ICR), high-resolution, matrix-assisted laser desorption ionization (HR-MALDI) in a 2,5-dihydroxybenzoic acid matrix) mass spectrometer and used to measure *m/z* (% of basis peak). Optical rotations ($[\alpha]_D^{RT}$) were measured on a Perkin–Elmer 241 polarimeter (10 cm, 1-mL cell) at RT. The solvent and the concentration (g per 100 mL) are indicated in the text below. Elemental analyses were performed by the Microanalytical Laboratory of the Laboratorium für Organische Chemie, ETH-Zürich. UV/Vis spectroscopy measurements were made on a UVIKON 860 spectrometer (Kontron). HPLC analysis was performed on a Gynkotek HPLC system with a M480G pump, a Gina 50T autosampler, a UVD340S photodiode array detector (Gynkotek GmbH, Germering, Germany), and a Macherey–Nagel C_{18} column (Nucleosil 100-5 C_{18} (250×4 mm)). Detection was achieved by measurement of the UV absorption at 220 nm. Samples were centrifuged (10 min, $13\,000 \times g$) and injected with the autosampler. A mixture of two solutions, A (99% triethylammonium acetate (TEAA; 2 mM) in H_2O and 1% CH_3CN) and B (5% TEAA (2 mM) in H_2O and 95% CH_3CN), was used as eluent. The gradients used were 0% B for 4 min, increased to 30% B within 10 min, 30% B for 4 min, and back to 0% B within another 2 min. The flow rate for all separations was 1 mL min^{-1}. Preparatory HPLC was performed on a Merck system (LaChrom, pump type L-7150, UV detector L-7400, interface D-7000, HPLC manager D-7000) with UV-grade TFA (>99% GC). To determine DOC levels, samples were centrifuged ($12\,000 \times g$ for 15 min), acidified with HCl (pH 2), freed from dissolved CO_2 by purging with N_2 for 10 min, and finally analyzed in a Tocor 2 carbon analyzer (Maihak, Hamburg, Germany). Growth yields (*Y*) were determined as follows: Dry weight was measured by filtration of defined volumes of the culture broth through tared polycarbonate filters (pore size = 0.20 μm). The filters were dried at 100 °C before and after the filtration. Growth yields (grams of biomass produced (*x*) per gram of substrate (*s*) utilized) were calculated after determination of the amount of residual substrate and DOC.

Origin of microorganisms: Inocula for the growth and degradation experiments were obtained from soil samples collected at two different locations (first sample:a flower bed near our laboratory at ETH, Zürich, Switzerland; second sample:a private garden in Konstanz, Germany) and from a sample taken from the aeration tank of a waste water treatment plant (Leutschenbach, Zürich, Switzerland). M9-Buffer solution: Na_2HPO_4 (60.2 g L^{-1}), KH_2PO_4 (30.1 g L^{-1}), and NaCl (5.01 g L^{-1}). Trace element solution: $FeSO_4 \cdot 7H_2O$ (2.50 g L^{-1}), $MnSO_4 \cdot H_2O$ (0.75 g L^{-1}), $ZnSO_4 \cdot 7H_2O$ (1.30 g L^{-1}), $CuSO_4 \cdot 5H_2O$ (0.25 g L^{-1}), $Co(NO_3)_2 \cdot 6H_2O$ (2.50 g L^{-1}), $Na_2MoO_4 \cdot 2H_2O$ (0.15 g L^{-1}), $NiSO_4 \cdot 7H_2O$ (0.01 g L^{-1}), H_3BO_3 (0.10 g L^{-1}), and H_2SO_4 (5.0 mL L^{-1}).[19] Vitamin mixture: pyridoxin–HCl (0.1 g L^{-1}), biotin (20 mg L^{-1}), folic acid (20 mg L^{-1}), thiamine–HCl (50 mg L^{-1}), riboflavin (50 mg L^{-1}), nicotinic acid (50 mg L^{-1}), Ca pantothenate (50 mg L^{-1}), 4-aminobenzoic acid (50 mg L^{-1}), nicotinamide (50 mg L^{-1}), and vitamin B_{12} (50 mg L^{-1}).[20] Mineral medium: microorganisms were grown on a mineral salts medium with β-amino acids and β-di- and β-tripeptides as the sole carbon sources. The mineral salts medium of a standard growth experiment with 0.69 mmol of peptide consisted of H_2O (100 mL), M9-buffer solution (25 mL), $MgSO_4$ solution (1 M, 250 μL), $CaCl_2$ solution (0.1 M, 250 μL), $(NH_4)_2SO_4$ solution (0.12 g L^{-1}, 125 μL), trace element solution (125 μL), and vitamin mixture (250 μL).

Synthesis of amino acids and peptides:

Boc-deprotection (general procedure (GP) 1): A method similar to a reported procedure[16] was used. The Boc-protected amino acid was dissolved in CH_2Cl_2 or $CHCl_3$ (0.5 M) and cooled to 0 °C in an ice bath. An equal volume of TFA was added and the mixture was allowed to warm to r.t. and then stirred for a further 1.5 – 2.5 h. Concentration under reduced pressure and drying of the residue under high vacuum (h.v.; 0.01 – 0.1 Torr) yielded the crude TFA salt, which was used without further purification if not stated otherwise.

Peptide coupling with EDC (GP 2): A stirred solution of the TFA salt (1 equiv) in THF (0.1 M) was treated with the Boc-protected fragment (1.4 equiv, added as solid), *N*-methylmorpholine (NMM; 4.0 – 4.4 equiv), and 1-hydroxy-1*H*-benzotriazole hydrate (HOBt; 1.7 equiv). After cooling to 0 °C (ice bath), EDC (1.4 equiv) was added and the mixture was allowed to warm to RT. Stirring was continued for 23 h at RT. The mixture was evaporated under reduced pressure and the residue was dissolved in CH_2Cl_2 then washed with 1 N HCl, saturated aq $NaHCO_3$, and NaCl solution. The organic phase was dried ($MgSO_4$) and evaporated. FC yielded the pure peptide.

Acetylation of free β-amino acids (GP 3): A solution of the free β^3-amino acid (1 equiv) in CH_2Cl_2 (0.4 M) was treated at RT. with Et_3N (0.2 equiv). Ac_2O (1.2 equiv) was added dropwise and after stirring for 0.5 h at RT., the mixture was heated under reflux for 16 – 21 h. Saturated aq $NaHCO_3$ was added, the organic layer was separated, and the aqueous phase was adjusted to pH 1 – 2 with 6 N HCl and extracted with AcOEt (twice). The combined AcOEt phases were dried ($MgSO_4$) and evaporated under reduced pressure. The resulting oil was coevaporated with toluene (twice), CCl_4 (once), $CHCl_3$ (twice), and CH_2Cl_2 (twice), and dried under h.v. at 55 – 60° (oven temperature).

Methyl *N*-[(*tert*-butoxy)carbonyl]-(*S*)-β^3-homoalanyl-(*S*)-β^3-homoalanate (Boc-(*S*)-β^3-HAla-(*S*)-β^3-HAla-OMe): Boc-(*S*)-β^3-HAla-OMe (6.29 g, 29.0 mmol) was deprotected in CH_2Cl_2 (58 mL) according to GP 1 with stirring for 1.75 h to afford TFA·H-(*S*)-β^3-HAla-OMe (9.61 g). According to GP 2, TFA·H-(*S*)-β^3-HAla-OMe (1.38 g, 4.16 mmol) was dissolved in THF (37 mL) and treated with Boc-(*S*)-β^3-HAla-OH (1.22 g, 6.00 mmol), NMM (1.85 mL, 16.8 mmol), HOBt (1.09 g, 7.20 mmol), and EDC (1.15 g, 6.00 mmol) to yield Boc-(*S*)-β^3-HAla-(*S*)-β^3-HAla-OMe (1.03 g, 82%). For analytical purposes, Boc-(*S*)-β^3-HAla-(*S*)-β^3-HAla-OMe was purified by FC (AcOEt/petroleum ether (PE) 2:1). The product was a white amorphous solid. Mp: 124.5 – 125.5 °C; R_f (AcOEt/PE 2:1): 0.25; $[\alpha]_D^{RT} = -31.4$ ($c = 1.0$, $CHCl_3$); IR ($CHCl_3$): $\tilde{\nu} =$ 3435, 3007, 2980, 1701, 1498, 1455, 1439, 1368 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): $\delta = 1.21$ (d, $J = 6.7$ Hz; Me), 1.22 (d, $J = 6.8$ Hz; Me), 1.43 (s; *t*Bu), 2.33 (dd, $J = 14.6$, 5.9 Hz, 1H; CH_2), 2.38 (dd, $J = 14.6$, 5.1 Hz, 1H; CH_2), 2.48 – 2.57 (m; CH_2), 3.69 (s; MeO), 3.91 – 4.01 (m; CHN), 4.31 – 4.41 (m; CHN), 5.26 (br s; NH), 6.32 (d, $J = 7.1$ Hz; NH); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta =$ 20.0, 20.6, 28.4, 40.0, 42.0, 42.9, 44.1, 51.7, 79.3, 155.4, 170.1, 172.0; MS (FAB) (%): 605 (13) $[(2M+H)]^+$, 325 (3) $[(M+Na)]^+$, 304 (22), 303 (100) $[(M+H)]^+$, 247 (45), 204 (12), 203 (89); elemental analysis: calcd for $C_{14}H_{26}N_2O_5$ (302.4): C 55.61, H 8.67, N 9.26; found: C 55.61, H 8.58, N 9.12.

Methyl *N*-[(*tert*-butoxy)carbonyl]-alanyl-(*S*)-β^3-homoalanate (Boc-Ala-(*S*)-β^3-HAla-OMe): According to GP 2, TFA·H-(*S*)-β^3-HAla-OMe (1.04 g, 3.13 mmol, see preparation of Boc-(*S*)-β^3-HAla-(*S*)-β^3-HAla-OMe) was dissolved in THF (28 mL) and treated with Boc-Ala-OH (0.85 g, 4.50 mmol), NMM (1.50 mL, 13.6 mmol), HOBt (0.81 g, 5.35 mmol), and EDC (0.86 g, 4.50 mmol) to yield Boc-Ala-(*S*)-β^3-HAla-OMe (0.96 g, 74%). For analytical purposes, Boc-Ala-(*S*)-β^3-HAla-OMe was purified by FC (AcOEt/PE 2:1) to yield a colorless crystalline solid. Mp: 78.0 – 79.5 °C; R_f (AcOEt/PE 2:1): 0.38; $[\alpha]_D^{RT} = -53.6$ ($c = 1.0$, $CHCl_3$); IR ($CHCl_3$): $\tilde{\nu} =$ 3431, 3008, 2980, 1724, 1675, 1495, 1454, 1368 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): $\delta = 1.22$ (d, $J = 6.8$ Hz; Me), 1.34 (d, $J =$ 7.1 Hz; Me), 1.45 (s; *t*Bu), 2.53 (d, $J = 5.5$ Hz; CH_2), 3.69 (s; MeO), 4.05 – 4.15 (m; CHN), 4.29 – 4.39 (m; CHN), 5.06 (br s; NH), 6.65 (d, $J = 8.0$ Hz; NH); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta =$ 18.4, 20.0, 28.3, 39.8, 42.0, 50.1, 51.7, 80.0, 155.4, 171.8, 171.9; MS (ESI, positive) (%): 327 (17) $[(M+K)]^+$, 311 (100) $[(M+Na)]^+$; elemental analysis: calcd. for $C_{13}H_{24}N_2O_5$ (288.3): C 54.15, H 8.39, N 9.72; found: C 54.37, H 8.26, N 9.54.

Benzyl *N*-[(*tert*-butoxy)carbonyl]-(*S*)-β^3-homoalanyl-alanate (Boc-(*S*)-β^3HAla-Ala-OBn): A stirred solution of TosOH·H-Ala-OBn (0.45 g, 1.28 mmol; TosOH = *p*-toluenesulfonic acid, BN = benzyl) in $CHCl_3$ (2.6 mL) at 0 °C (ice bath) was treated with Et_3N (0.54 mL, 3.87 mmol), HOBt (0.23 g, 1.53 mmol), a solution of Boc-*(S)*-β^3-HAla-OH (0.26 g, 1.28 mmol) in $CHCl_3$ (2.6 mL), and EDC (0.30 g, 1.54 mmol). The mixture was allowed to warm to RT and then stirred for 12 h. Subsequent dilution with $CHCl_3$ was followed by thorough washing with 1 N HCl, saturated aq $NaHCO_3$, and NaCl solution. The organic phase was dried ($MgSO_4$) and then concentrated in vacuo. Recrystallization (AcOEt/pentane) yielded Boc-(*S*)-β^3-HAla-Ala-OBn (0.27 g, 59%), a white amorphous solid. Mp: 133.0 – 134.5 °C; $[\alpha]_D^{RT} = -18.4$ ($c = 1.0$, $CHCl_3$); IR ($CHCl_3$): $\tilde{\nu} =$ 3434, 3007, 2979, 1737, 1699, 1498, 1454, 1391, 1368m, 1346, 1307 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): $\delta =$ 1.20 (d, $J = 6.7$ Hz; Me), 1.41 (d, $J = 7.2$ Hz; Me), 1.43 (s; *t*Bu), 2.37 – 2.46 (m; CH_2CO), 3.93 – 4.03 (m; CHN), 4.61 (quint, $J = 7.2$ Hz; CHN), 5.18 (br s; NH); 5.15, 5.19 (AB, $J_{AB} = 12.3$ Hz; CH_2O), 6.42 (br s; NH); 7.31 – 7.40 (m; 5 arom. H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta =$ 18.2, 20.6, 28.4, 42.6, 44.1, 48.1, 67.2, 79.3, 129.2, 128.5, 128.6, 135.4, 155.4, 170.5, 172.8; MS (FAB) (%): 729 (10) $[(2M+H)]^+$, 387 (8) $[(M+Na)]^+$, 366 (20), 365 (92) $[(M+H)]^+$, 309 (51), 266 (16), 265 (100); elemental analysis: calcd for $C_{19}H_{28}N_2O_5$ (364.4): C 62.62, H 7.74, N 7.69; found: C 62.39, H 7.82, N 7.70.

(*S*)-3-Amino-butanoic acid (H-(*S*)-β^3-HAla-OH (**1**)): Boc-(*S*)-β^3-HAla-OH (10.6 g, 52.0 mmol) was treated in CH_2Cl_2 (105 mL) according to GP 1 with stirring for 2.5 h to afford TFA·**1** (11.0 g). A portion of the resulting TFA salt (6.28 g) was purified by ion-exchange chromatography (Dowex-H^+ 50 × 8) and recrystallization from MeOH/hexane to yield colorless crystals of **1** (2.04 g, 66% from TFA·**1**). Mp: 227 – 227.5 °C; $[\alpha]_D^{RT} = +38.6$ ($c = 1.1$, $CHCl_3$); IR (KBr): $\tilde{\nu} = 2947$, 1641, 1580, 1546, 1440, 1413, 1354 cm^{-1}; ^1H NMR (400 MHz, CD_3OD): $\delta = 1.29$ (d, $J = 6.7$ Hz; Me), 2.33 (dd, $J = 16.6$, 8.6 Hz, 1H; CH_2), 2.43 (dd, $J = 16.6$, 4.6 Hz, 1H; CH_2), 3.43 – 3.51 (m; CHN); ^{13}C NMR (100 MHz, CD_3OD): $\delta =$ 18.9, 41.4, 46.8, 177.8; MS (ESI, positive) (%): 126 (36) $[(M+Na)]^+$, 104 (100) $[(M+H)]^+$; MS (ESI, negative) (%): 102 (100) $[(M-H)]^-$: elemental analysis: calcd for $C_4H_9NO_2$ (103.1): C 46.59, H 8.80, N 13.58; found: C 46.68, H 8.78, N 13.45.

(*R*)-3-Amino-4-methyl-pentanoic acid (H-(*R*)-β^3-HVal-OH (**2**)): Boc-(*R*)-β^3-HVal-OH (25.0 g, 0.10 mol) was treated in CH_2Cl_2 (200 mL) according to GP 1 with stirring for 1.5 h to afford TFA·**2** (28.9 g). A portion of the resulting TFA salt (11.1 g) was purified by ion-exchange chromatography (Dowex-H^+ 50 × 8) and recrystallization from MeOH/hexane to yield **2** (3.37 g, 67% from TFA·**2**) as colorless crystals. Mp: 210.0 – 211.0 °C; $[\alpha]_D^{RT} + 55.6$ ($c = 1.1$, H_2O); IR (KBr): $\tilde{\nu} =$ 2966, 2131, 1620, 1538, 1465, 1415, 1386, 1353, 1326 cm^{-1}; ^1H NMR (400 MHz, D_2O): $\delta = 1.00$ (d, $J = 6.8$ Hz; Me), 1.03 (d, $J = 6.9$ Hz; Me), 1.84 – 1.95 (m; CH), 2.28 (dd, $J = 16.8$, 10.3 Hz, 1H; CH_2), 2.47 (dd, $J =$ 16.7, 3.5 Hz, 1H; CH_2); 3.13 – 3.29 (m; CHN); ^{13}C NMR (75 MHz, D_2O): $\delta =$ 18.5, 18.9, 31.9, 36.6, 56.4, 178.2; MS (ESI, positive) (%): 154 (80) $[(M+Na)]^+$, 132 (100), $[(M+H)]^+$; MS (ESI, negative) (%): 130 (100) $[(M-H)]^-$; elemental analysis: calcd for $C_6H_{13}NO_2$ (131.2): C 54.94, H 9.99, N 10.68; found: C 54.91, H 9.96, N 10.60.

(*R*)-3-Amino-5-methyl-hexanoic acid (H-(*S*)-β^3-HLeu-OH (**3**)): Boc-(*S*)-β^3-HLeu-OH (13.0 g, 52.9 mmol) was deprotected in CH_2Cl_2 (105 mL)

according to GP 1 with stirring for 1.5 h. The resulting TFA salt was purified by ion-exchange chromatography (Dowex-H^+ 50 × 8) and recrystallization from MeOH. The mother liquid was evaporated and recrystallized from MeOH/hexane to give the white microcrystalline solid **3** (4.86 g, 63 %). Mp: 231.0 – 232.5 °C; $[\alpha]_D^{RT} = +29.3$ ($c = 0.9$, H_2O); IR (KBr): $\tilde{\nu} = 2954$, 2180, 1644, 1556, 1469, 1402, 1312 cm^{-1}; ^1H NMR (400 MHz, D_2O): $\delta = 0.95$ (d, $J = 6.8$ Hz; Me), 0.97 (d, $J = 6.9$ Hz; Me), 1.47 – 1.51 (m; 2 CH), 1.68 – 1.78 (m; CH), 2.28 (dd, $J = 16.7$, 9.1 Hz, 1 H; CH_2CO), 2.47 (dd, $J = 16.7$, 3.8 Hz, 1 H; CH_2CO), 3.37 – 3.45 (m; CHN); ^{13}C NMR (75 MHz, D_2O): $\delta = 22.3$, 22.8, 25.3, 39.1, 43.0, 48.9, 177.7; MS (ESI, positive) (%): 168 (96) $[(M+Na)]^+$, 146 (100) $[(M+H)]^+$; MS (ESI, negative) (%): 144 (100) $[(M-H)]^-$; elemental analysis: calcd for $C_7H_{15}NO_2$ (145.2): C 57.90, H 10.41, N 9.65; found: C 57.85, H 10.42, N 9.54.

(3*S*,4*S*)-3-Amino-4-methyl-hexanoic acid (H-(*S*)-β^3-HIle-OH (**4**)): Boc-(*S*)-β^3-HIle-OH (8.73 g, 35.6 mmol) was deprotected in CH_2Cl_2 (71 mL) according to GP 1 with stirring for 1.5 h. The resulting TFA salt was purified by ion-exchange chromatography (Dowex-H^+ 50 × 8) and recrystallization from MeOH. The mother liquid was evaporated and recrystallized from MeOH/hexane to give **4** (4.07 g, 79 %) as colorless crystals. Mp: 209.5 – 211.0 °C; $[\alpha]_D^{RT} = +43.3$ ($c = 1.0$, H_2O); IR (KBr): $\tilde{\nu} = 2968$, 2926, 2135, 1624, 1542, 1459, 1415, 1401 cm^{-1}; ^1H NMR (400 MHz, D_2O): $\delta = 0.95 – 0.98$ (m; 2 Me), 1.18 – 1.29 (m; CH), 1.46 – 1.56 (m; CH) 1.64 – 1.74 (m; CH), 2.27 (dd, $J = 16.8$, 10.5 Hz, 1 H; CH_2CO), 2.42 (dd, $J = 16.8$, 3.4 Hz, 1 H; CH_2CO), 3.29-3.34 (m; CHN); ^{13}C NMR (100 MHz, D_2O): $\delta = 11.7$, 14.3, 26.6, 35.6, 38.4, 54.8, 178.2; MS (ESI, positive) (%): 168 (100) $[(M+Na)]^+$, 146 (92) $[(M[\alpha]+H)]^+$; MS (ESI, negative) (%): 144 (100) $[(M-H)]^-$; elemental analysis: calcd for $C_7H_{15}NO_2$ (145.2): C 57.90, H 10.41, N 9.65; found: C 57.77, H 10.25, N 9.60.

(3*S*)-3-[(Acetyl)amino]-butanoic acid (Ac-(*S*)-β^3-HAla-OH (**5**)): According to GP 3, a solution of **1** (0.99 g, 9.64 mmol) in CH_2Cl_2 (24 mL) was treated with Et_3N (0.27 mL, 1.94 mmol) and Ac_2O (1.09 mL, 11.5 mmol) and heated for 16 h to yield a colorless oil, which was a 5:95 mixture of compound **5** and Ac-(*S*)-β^3-HAla-(*S*)-β^3-HAla-OH (0.18 g, 13 %), as determined by ^1H NMR spectroscopy (400 MHz) and mass spectrometry. $[\alpha]_D^{RT} = -38.0$ ($c = 1.3$, $CHCl_3$); IR ($CHCl_3$): $\tilde{\nu} = 3435$, 3007, 1713, 1661, 1517, 1448, 1374 cm^{-1}; ^1H NMR (400 MHz, CD_3OD): $\delta = 1.18$ (d, $J = 6.7$ Hz; Me), 1.90 (s; Ac), 2.39 (dd, $J = 15.5$, 7.1 Hz, 1 H; CH_2CO), 2.51 (dd, $J = 15.5$, 6.5 Hz, 1 H; CH_2CO), 4.19 – 4.28 (m; CHN); ^{13}C NMR (100 MHz, CD_3OD): $\delta = 20.4$, 22.7, 41.5, 43.8, 172.5, 174.8; MS (ESI, positive) (%): 168 (100) $[(M+Na)]^+$; MS (ESI, negative) (%): 144 (100) $[(M-H)]^-$; elemental analysis: calcd for $C_6H_{11}NO_3$ (145.2): calcd: C 49.65, H 7.64, N 9.65; found: C 49.55, H 7.64, N 9.80.

(3*R*)-3-[(Acetyl)amino]-4-methylpentanoic acid (Ac-(*R*)-β^3-HVal-OH (**6**)): According to GP 3, a solution of **2** (1.00 g, 7.60 mmol) in CH_2Cl_2 (19 mL) was treated with Et_3N (0.21 mL, 1.51 mmol) and Ac_2O (0.86 mL, 9.10 mmol) and heated for 21 h to yield a colorless crystalline solid, which was a 9:1 mixture of **6** and Ac-(*R*)-β^3-HVal-(*R*)-β^3-HVal-OH (0.43 g, 25 %), as determined by ^1H NMR spectroscopy (400 MHz) and mass spectrometry. $[\alpha]_D^{RT} = -46.7$ ($c = 0.9$, $CHCl_3$); IR ($CHCl_3$): $\tilde{\nu} = 3434$, 2967, 2875, 1713, 1663, 1514, 1373 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): $\delta = 0.93$ (d, $J = 6.8$ Hz; Me), 0.94 (d, $J = 6.8$ Hz; Me), 1.83 – 1.93 (m; CH), 2.01 (s; Ac), 2.47 – 2.59 (m; CH_2CO), 4.03 – 4.10 (m; CHN), 6.63 (d, $J = 9.4$ Hz; NH), 10.85 (br s; OH); ^{13}H NMR (100 MHz, $CDCl_3$): $\delta = 18.9$, 19.2, 23.1, 31.5, 36.5, 51.9, 171.1, 175.5; MS (ESI, positive) (%): 196 (100) $[(M+Na)]^+$; MS (ESI, negative) (%): 172 (100) $[(M-H)]^-$; elemental analysis: calcd for $C_8H_{15}NO_3$ (173.2): C 55.47, H 8.73, N 8.09; found: C 55.42, H 8.70, N 8.17.

(3*S*)-3-[(Acetyl)amino]-5-methylhexanoic acid (Ac-(*S*)-β^3-HLeu-OH (**7**)): According to GP 3, a solution of **3** (1.00 g, 6.86 mmol) in CH_2Cl_2 (17 mL) was treated with Et_3N (0.19 mL, 1.36 mmol) and Ac_2O (0.78 mL, 8.25 mmol) and heated for 21 h to yield a 94:6 mixture of **7** and Ac-(*S*)-β^3-HLeu-(*S*)-β^3-HLeu-OH (0.56 g, 44 %), as determined by ^1H NMR spectroscopy (400 MHz) and mass spectrometry. Compound **7** was distilled for analytical purposes (bulb-to-bulb distillation, oven temperature = 150 °C, pressure = 0.25 Torr). Ac-(*S*)-β^3-HLeu-(*S*)-β^3-HLeu-OH was not detected in the ^1H NMR spectrum (400 MHz) or in the mass spectra of distilled **7**, a colorless highly viscous oil. $[\alpha]_D^{RT} = -47.6$ ($c = 1.0$, $CHCl_3$); IR ($CHCl_3$): $\tilde{\nu} = 3432$, 3007, 2960, 2871, 1711, 1664, 1517, 1468, 1438, 1370m cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): $\delta = 0.91$ (d, $J = 6.5$ Hz; Me), 0.92 (d, $J = 6.7$ Hz; Me), 1.27 – 1.34 (m; CH), 1.42 – 1.49 (m; CH), 1.55 – 1.67 (m; CH), 1.91 (s; Ac), 2.41 (dd, $J = 18.3$, 6.6 Hz, 1 H; CH_2CO), 2.42 (dd, $J = 18.3$, 6.6 Hz, 1 H; CH_2CO), 4.25-4.32 (m; CHN); ^{13}H NMR (100 MHz, $CDCl_3$): $\delta = 22.2$, 22.7, 23.6, 26.1, 41.0, 44.6, 46.1, 172.7, 174.9; MS (ESI, positive) (%): 242 (34) $[(M+2Na-H)]^+$, 226 (57) $[(M+K)]^+$, 210 (100) $[(M+Na)]^+$; MS (ESI, negative) (%): 186 (100) $[(M-H)]^-$; elemental analysis: calcd for $C_9H_{17}NO_3$ (187.2): C 57.73, H 9.15, N 7.48; found: C 57.77, H 9.01, N 7.49.

(3*S*,4*S*)-3-[(Acetyl)amino]-4-methylhexanoic acid (Ac-(*S*)-β^3-HIle-OH (**8**)): According to GP 3, a solution of **4** (0.97 g, 6.66 mmol) in CH_2Cl_2 (17 mL) was treated with Et_3N (0.18 mL, 1.29 mmol) and Ac_2O (0.76 mL, 8.04 mmol) and heated for 21 h to yield a 91:9 mixture of **8** and Ac-(*S*)-β^3-HIle-(*S*)-β^3-HIle-OH (0.57 g, 46 %), as determined by ^1H NMR spectroscopy (400 MHz) and mass spectrometry. The product was a colorless crystalline solid. M.p.: 90.5 – 92.5 °C. $[\alpha]_D^{RT} = -41.4$ ($c = 1.0$, $CDCl_3$); IR ($CDCl_3$): $\tilde{\nu} = 3432$, 2968, 2933, 2878, 1715, 1661, 1515, 1411, 1374 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): $\delta = 0.85 – 0.96$ (m; 2 Me), 1.05 – 1.17 (m; CH), 1.42 – 1.55 (m; CH), 1.60 – 1.71 (m; CH), 2.01 (s; Ac), 2.50 (dd, $J = 15.6$, 6.8 Hz, 1 H; CH_2CO), 2.56 (dd, $J = 15.6$, 4.4 Hz, 1 H; CH_2CO), 4.10 – 4.17 (m; CHN), 6.37 (d, $J = 9.3$ Hz; NH), 9.48 (br s; OH); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 11.3$, 15.3, 23.2, 25.7, 36.1, 37.8, 50.7, 170.8, 175.7; MS (ESI, positive) (%): 226 (8) $[(M+K)]^+$, 210 (100) $[(M+Na)]^+$, 188 (6) $[(M+H)]^+$; MS (ESI, negative) (%): 186 (100) $[(M-H)]^-$; elemental analysis: calcd for $C_9H_{17}NO_3$ (187.2): C 57.73, H 9.15, N 7.48; found: C 57.90, H 8.99, N 7.62.

(*S*)-β^3-Homoalanyl-(*S*)-β^3-homoalanine (H-(*S*)-β^3-HAla-(*S*)-β^3-HAla-OH (**9**)): In a procedure similar to that described in ref. [21], a solution of Boc-(*S*)-β^3-HAla-(*S*)-β^3-HAla-OMe (0.59 g, 1.96 mmol) in MeOH (1.6 mL) and THF (0.5 mL) was treated with 0.75 N aq NaOH (3.13 mL, 2.35 mmol) at RT. After 23 h, CH_2Cl_2 was added and the mixture was extracted with saturated aq $NaHCO_3$. The aqueous phase was adjusted to pH 1 – 2 with 6 N HCl and extracted with AcOEt. The AcOEt phase was dried ($MgSO_4$) and evaporated. A solution of the residue in CH_2Cl_2 (4 mL) was treated according to GP 1 for 2 h. Purification by ion-exchange chromatography (Dowex-H^+ 50 × 8) yielded the white amorphous solid **9** (0.26 g, 70 %). Mp: 230.0 – 231.0 °C. $[\alpha]_D^{RT} = +6.4$ ($c = 0.4$, H_2O); IR (KBr): $\tilde{\nu} = 3321$, 2965, 2199, 1645, 1585, 1545, 1454, 1407, 1340 cm^{-1}; ^1H NMR (500 MHz, D_2O): $\delta = 1.13$ (d, $J = 6.6$ Hz; Me), 1.29 (d, $J = 6.7$ Hz; Me), 2.24 – 2.41 (m; CH_2CO), 2.49 – 2.59 (m; CH_2CO), 3.65 – 3.71 (m; CHN), 4.10 – 4.24 (m; CHN); ^{13}C NMR (125 MHz, D_2O): $\delta = 20.2$, 22.3, 41.9, 46.6, 46.6, 47.7, 173.3, 182.6; MS (FAB) (%): 377 (5) $[(2M+H)]^+$, 274 (31), 245 (12), 211 (7) $[(M+Na)]^+$, 190 (12), 189 (100) $[(M+1)]^+$, 176 (13), 167 (11), 166 (14), 165 (15); elemental analysis: calcd for $C_8H_{16}N_2O_3$ (188.2) C 51.05, H 8.57, N 14.88; found: C 50.89, H 8.36, N 14.96.

(*S*)-β^3-Homoalanyl-(*S*)-β^3-homoleucine (H-(*S*)-β^3-HAla-(*S*)-β^3-HLeu-OH (**10**)): A solution of Boc-(*S*)-β^3-HAla-(*S*)-β^3-HLeu-OMe (0.42 g, 1.21 mmol) in MeOH (1 mL) was treated with 0.75 N aq NaOH (2 mL, 1.50 mmol) at RT. After 2.5 h, MeOH (1 mL) and 0.75 N aq NaOH (2 mL, 1.50 mmol) were added to the mixture, then after 6.5 h, the mixture was cooled to 0 °C (ice bath), adjusted to pH 2 – 3 with 1 N HCl, and extracted with AcOEt (5 ×). The organic phase was dried ($MgSO_4$) and evaporated and a solution of the residue in CH_2Cl_2 (2.4 mL) was treated according to GP 1 for 1.5 h. Purification by ion-exchange

chromatography (Dowex-H^+ 50×8) yielded the white solid **10** (0.25 g, 89%). Mp: 236.0 – 237.0 °C. $[\alpha]_D^{RT} = +2.8$ ($c = 0.4$, MeOH); IR (KBr): $\tilde{\nu} = 3250$, 3094, 2957, 1644, 1571, 1472, 1451, 1399 cm^{-1}; ^1H NMR (400 MHz, CD_3OD): $\delta = 0.92$ (d, $J = 6.7$ Hz; Me), 0.92 (d, $J = 6.5$ Hz; Me), 1.28 – 1.35 (m; CH), 1.31 (d, $J = 6.7$ Hz; Me), 1.40 – 1.48 (m; CH), 1.57 – 1.67 (m; CH), 2.22 (dd, $J = 13.8$, 8.0 Hz, 1H; CH_2CO), 2.32 (dd, $J = 13.8$, 5.3 Hz, 1H; CH_2CO), 2.44 (dd, $J = 15.6$, 8.6 Hz, 1H; CH_2CO), 2.49 (dd, $J = 15.6$, 4.8 Hz, 1H; CH_2CO), 3.57 – 3.66 (m; CHN), 4.28 – 4.35 (m; CHN); ^{13}C NMR (100 MHz, CD_3OD): $\delta = 19.1$, 22.5, 23.6, 26.2, 40.6, 44.7, 45.3, 46.5, 47.3, 171.0, 179.8; MS (FAB) (%): 461 (18) $[(2M+H)]^+$, 253 (19) $[(M+Na)]^+$, 232 (14), 231 (100) $[M+1]^+$.

Alanyl-(*S*)-β^3-homoalanine (H-Ala-(*S*)-β^3-HAla-OH (**11**)): A procedure similar to that described in ref. [21] was used. A solution of Boc-Ala-(*S*)-β^3-HAla-OMe (0.59 g, 2.03 mmol) in MeOH (1.7 mL) and THF (0.5 mL) was treated with 0.75 N aq NaOH (3.25 mL, 2.44 mmol) at RT. After 24 h, CH_2Cl_2 was added and the mixture was extracted with a saturated aq $NaHCO_3$ solution. The aqueous phase was adjusted to pH 1 – 2 with 6 N HCl and extracted with AcOEt. The AcOEt phase was dried ($MgSO_4$) and evaporated. The residue was deprotected in CH_2Cl_2 (4 mL) according to GP 1 for 2 h. Purification by ion-exchange chromatography (Dowex-H^+ 50+8) yielded **11** (0.19 g, 54%) as a white solid. Mp: 220.0 – 221.0 °C. $[\alpha]_D^{RT} = +7.0$ ($c = 0.4$, H_2O); IR (KBr): $\tilde{\nu} = 3227$, 2964, 1681, 1546, 1448, 1398, 1320 cm^{-1}; ^1H NMR (500 MHz, D_2O): $\delta = 1.15$ (d, $J = 6.7$ Hz; Me), 1.46 (d, $J = 7.1$ Hz; Me), 2.31 (dd, $J = 14.3$, 6.8 Hz, 1H; CH_2CO), 2.35 (*dd*, $J = 14.3$, 7.2 Hz, 1H; CH_2CO), 3.94 (q, $J = 7.1$; CHN), 4.14 – 4.21 (m; CHN); ^{13}C NMR (125 MHz, D_2O): $\delta =$ 19.4, 22.2, 46.6, 46.8, 51.9, 172.3, 182.3; MS (FAB) (%): 197 (5) $[(M+Na)]^+$, 195 (18), 190 (17), 189 (23), 180 (13), 178 (20), 177 (11), 175 (36) $[(M+H)]^+$, 165 (50), 164 (15), 163 (15), 155 (17), 154 (48), 153 (26), 152 (63), 151 (18), 150 (18), 142 (14), 141 (22), 140 (11), 139 (35), 138 (33), 137 (87), 136 (100), 135 (21); elemental analysis: calcd for $C_7H_{14}N_2O_3$ (174.2): C 48.26, H 8.10, N 16.08; found: C 48.28, H 7.98, N 16.09.

(*S*)-β^3-homoalanyl-alanine (H-(*S*)-β^3HAla-Ala-OH (**12**)): Boc-(*S*)-β^3-HAla-Ala-OBn (0.13 g, 0.35 mmol) was dissolved in MeOH (8.6 mL) and a catalytic amount of Pd/C (10%) was added. The apparatus was evacuated and flushed three times with H_2, and the mixture was stirred under H_2 for 30 h. Subsequent filtration through Celite was followed by concentration under reduced pressure. The residue was Boc deprotected in CH_2Cl_2 (0.7 mL) according to GP 1 for 2 h. Purification by ion-exchange chromatography (Dowex-H^+ 50+8) yielded the white powder **12** (30 mg, 47%). Mp: > 200 °C (dec); $[\alpha]_D^{RT} = +6.3$ ($c = 0.25$, AcOH); IR (KBr): $\tilde{\nu} = 3244$, 3074, 2979, 1646, 1566, 1566, 1499, 1458, 1399 cm^{-1}; ^1H NMR (300 MHz, CD_3CO_2D): 1.40 (d, $J = 6.9$ Hz; Me), 1.47 (d, $J = 7.2$ Hz; Me), 2.71 – 2.87 (m; CH_2); 3.81 – 3.90 (m; CH); 4.53 – 4.60 (m; CH); ^{13}C NMR (75 MHz, CD_3CO_2D): 17.1, 18.3, 39.3, 46.8, 49.4, 172.5; MS (ESI, positive) (%): 197 (100) $[(M+Na)]^+$; MS (ESI, negative) (%): 174 (100) $[(M-H)]^-$.

(*R*)-β^3-Homovalyl-(*S*)-β^3-Homoalanyl-(*S*)-β^3-homoleucine (H-(*R*)-β^3-HVal-(*S*)-β^3-HAla-(*S*)-β^3-HLeu-OH (**14**)): Boc-(*R*)-β^3-HVal-(*S*)-β^3-HAla-(*S*)-β^3-HLeu-OH (0.47 g, 1.1 mmol) was deprotected in $CHCl_3$ (2.1 mL) according to GP 1 for 1.75 h. The residue was purified by ion-exchange chromatography (Dowex-H^+ 50×8) to yield **14** (0.27 g, 74%), a white amorphous solid. Mp: 288.5 – 289.5 °C; $[\alpha]_D^{RT} = -7.1$ ($c = 1.0$, H_2O); IR (KBr): $\tilde{\nu} = 3263$, 2968, 1725, 1655, 1555, 1485, 1429, 1386 cm^{-1}; ^1H NMR (400 MHz, D_2O): $\delta = 0.79$ (d, $J = 6.5$ Hz; Me), 0.81 (d, $J = 6.6$ Hz; Me), 0.90 (d, $J = 6.9$ Hz; Me), 0.91 (d, $J = 6.9$ Hz; Me), 1.08 (d, $J = 6.7$ Hz; Me), 1.21 – 1.28 (m; CH), 1.35 – 1.44 (m; CH), 1.45 – 1.54 (m; CH), 1.82 – 1.94 (m; CH), 2.30 – 2.59 (m; 3 CH_2CO), 3.34 – 3.38 (m; CHN), 4.09 – 4.21 (m; 2 CHN), 7.99 (d, $J = 9.3$; NH); ^3C NMR (100 MHz, D_2O): $\delta = 19.7$, 19.9, 21.9, 23.6, 25.0, 27.0, 32.6, 37.1, 42.7, 44.7, 45.3, 46.1, 47.7, 56.9, 173.9, 175.2, 178.5; MS (FAB) (%): 709 (10), 688 (13), 687 (38) $[(2M+H)]^+$, 366 (38) $[(M+Na)]^+$, 345 (34), 344 (100) $[(M+1)]^+$.

Valyl-alanyl-leucine hydrochloride (HCl·H-Val-Ala-Leu-OH (**15**)): Following a procedure described in ref. [21] a solution of Boc-Val-Ala-Leu-OMe (1.30 g, 3.14 mmol) in MeOH (2.62 mL) was treated with 0.75 N aq NaOH (5.02 mL) at RT. After 4 h, the mixture was adjusted to pH 2 – 3 with 1 N HCl and extracted with AcOEt. The organic layer was dried ($MgSO_4$) and evaporated. According to a procedure described in ref. [22] the Boc-protected peptide acid was dissolved in saturated HCl/dioxane (12.6 mL) at 0 °C (ice bath). The mixture was allowed to warm to RT and stirring was continued for 1.5 h. The solvent was removed under reduced pressure to yield compound **15**. Mp: 130.0 – 133.0 °C; IR (KBr): $\tilde{\nu} = 3084$, 2977, 2810, 1700, 1675, 1652, 1551, 1388 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): $\delta = 0.91$ (d, $J = 6.5$ Hz; Me), 0.96 (d, $J = 6.5$ Hz; Me), 1.05 (d, $J = 7.0$ Hz; Me), 1.09 (d, $J = 7.0$ Hz; Me), 1.40 (d, $J = 7.1$ Hz; Me), 1.65 (d, $J = 6.6$ Hz; CH_2), 1.80 – 1.70 (m; CH), 2.25 – 2.15 (m; CH), 3.71 (d, $J = 5.6$ Hz; CHN), 4.50 – 4.40 (m; 2 CHN), 8.28 (d, $J = 8.1$ Hz; NH); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 17.9$, 18.0, 18.9, 21.8, 23.4, 25.9, 31.6, 41.7, 49.4, 52.0, 59.5, 169.1, 174.4, 175.8; MS (HR-MALDI) (%): 324.1974 (36), 302.2147 (6).

Biodegradation of β-Peptides:

Enrichment experiments with tripeptide **14**: For initial experiments, 500 g of solid material from each of the two soil samples were suspended in nondistilled fresh water and stirred vigorously for 10 min. After standing for 5 min at RT, samples of the turbid supernatant liquids (5 mL) were added to a mineral medium (42 mL, 1/3 of the scale described above) that contained tripeptide **14** (79 mg, 0.23 mmol, $c = 5$ mmol L^{-1}) as the sole carbon source. A sample was taken directly from the solution that originated from the aeration tank without further workup. After initial growth and mixing experiments, the suspensions A1, B1, and C1 were inoculated with samples A, B, and C of the fastest growing microorganisms and incubated in 250 mL Erlenmeyer flasks at RT. on a rotary shaker (120 rpm) for 25 days. Subsequent to these initial tests, buffered solutions of tripeptide **14** (238 mg, 0.69 mmol, $c \approx 5$ mmol L^{-1}) were inoculated with samples of the turbid mixtures from the triad of preceding experiments A1, B1, and C1 (5 vol%). These new incubation mixtures A2, B2, and C2 were shaken in 250 mL Erlenmayer flasks at RT for 62 days. Samples were taken out after 25, 42, and 62 days and analyzed by UV/Vis spectroscopy and reversed-phase HPLC. Microorganisms from experiment C were chosen for further growth experiments. Inoculation of buffered solutions of tripeptide **14** in mineral medium with samples (5 vol%) taken from the preceding experiment C(n-1) ($n = 1,2,3\ldots$) and incubation of solution C(n) at RT on a rotary shaker was repeated continuously over a period of 6 months. Solutions C(n-1) used for inoculation of solutions C(n) were taken from the exponential growth phase of experiment C(n-1). Growth of microorganisms was monitored by UV/Vis analysis of the turbidity (optical density).

Isolation of dipeptide **10** as a metabolite by the degradation of tripeptide **14**: A sample was taken of microorganisms from experiment C (10 mL) in the exponential-growth phase, filtered, and evaporated under reduced pressure to an end volume of approximately 2 mL. Isolation and purification were performed by preparatory reversed-phase HPLC on a Macherey-Nagel C_8 column/Nucleosil 100-7 C_8 (250×21 mm) by using a gradient of A and B (A: 0.1% TFA in H_2O, B: MeCN; 1% B for 5 min, increased to 8% B within 5 min then to 35% B within 25 min) at a flow rate of 4 mL min^{-1} with UV detection at 220 nm. Lyophilization of the peak fractions afforded the TFA salt of **10** (4 mg). ^1H NMR and MS data were in agreement with those of the prepared sample of compound **10**.

Degradation experiments with β-peptides and β-amino acids: β-peptides **10** and **14** and β-amino acids **1** – **3** were dissolved in mineral medium ($c \approx 5$ mmol L^{-1}), filtered through a sterile filter (Millipore Millex-GP 0.22 μm filters) and stored in sterile glass bottles. For degradation experiments with microorganisms from experiment C the following scales of peptide and amino acid solution were chosen: **14** (0.24 g, 0.69 mmol in 126 mL mineral medium; 0.12 g, 0.35 mmol in 63 mL), **10** (48 mg, 0.21 mmol in 42 mL), **1** (22 mg, 0.21 mmol in 42 mL), **2** (27 mg, 0.21 mmol in 42 mL) and **3** (30 mg, 0.21 mmol in 42 mL). The sterile homogeneous solutions were inoculated with a suspension of microorganisms (5 vol%) and incubated at RT on a rotary shaker (160 rpm) until turbidity reached a maximum, which indicated complete consumption of the peptide or amino acid. Samples were taken out regularly, analyzed by UV/Vis spectroscopy, stored at $-18\,^{\circ}$C, and analyzed by HPLC.

Degradation experiments with β-peptide **14** and α-peptide **15**: In two parallel experiments α-peptide **15** and a 1:1 mixture of β-peptide **14** and α-peptide **15** were dissolved in mineral medium (overall concentration about 5 mmol L^{-}), filtered through a sterile filter, and stored in sterile glass bottles. The following scales of α- and β-peptide solutions were chosen: 1) α-tripeptide **15** (0.11 g, 0.32 mmol) in 63 mL mineral medium; 2) α-tripeptide **15** (53 mg, 0.16 mmol) and β-tripeptide **14** (59 mg, 0.17 mmol) in 63 mL mineral medium. The sterile homogeneous solutions were inoculated with a suspension of microorganisms (5 vol%) and incubated at RT on a rotary shaker (160 rpm) until turbidity reached a maximum, which indicated complete consumption of the peptide or amino acid. Samples were taken out regularly, analyzed by UV/Vis spectroscopy, stored at $-18\,^{\circ}$C and analyzed by HPLC.

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