Research Article

Journal of PeptideScience

Received: 14 January 2016 Revised: 16 February 2016 Accepted: 16 February 2016 Published online in Wiley Online Library

(wileyonlinelibrary.com) DOI 10.1002/psc.2873

Synthesis and conformational analysis of an expanded cyclic ketoxime-hexapeptide

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In this work the synthesis of a linear hexapeptide with a hydroxylamine functionality at the N-terminus and a ketone instead of the carboxylic acid at the C-terminus is described. Cyclization by ketoxime formation yields the 19-membered ring-expanded cyclic hexapeptide cyclo[Goly-Val-Ala-Pro-Leu-Kly] which adopts a main conformer with two intramolecular hydrogen bonds. The hydrolytic stability of a ketoxime lies between the inert amide and the labile imine. The substitution of an amide bond for an iminium bond transforms the irreversible macrocyclization into a reversible process, but macrocyclic imines are difficult to isolate because they are prone to hydrolysis. The enhanced chemical stability of the ketoxime justifies its application in ligation protocols. The detailed NMR analysis of a ketoxime linkage presented here identifies its local conformational preferences in a constrained peptide environment.

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Keywords: peptide; cyclization; NMR; oxime; conformation

Introduction

Several synthetic methods have been developed for the chemo- and regioselective ligation of unprotected peptides in high yields over the years. Common examples are disulfide formations, Michael additions with maleimides, alkylations with iodoacetamides and condensation reactions which form hydrazones, imines or oximes [1–7]. The oxime formation between aldehyde and hydroxylamine is the most widely used method in all these ligations because of the high stability at a physiological pH and the selectivity of the reaction [8,9]. Another advantage of this condensation reaction is the simple handling and the fast reaction rate at pH 5–6 [8,9]. The synthesis of oxime/ketoxime-ligated peptides is based on unnatural C-terminal α-oxo aldehyde peptides and pyruvic acid peptides, which can be synthesized via solid-phase peptide synthesis [6,10,11]. The advantages of the chemoselective ligation of unprotected peptides lead to a variety of improved methods, such as the α-ketoacid-hydroxylamine amide ligation, which was introduced in 2006 and further developed over the next few years [12–17].

The macrocyclization of peptides is an irreversible process which can be transformed into a reversible process by the substitution of an amide bond for an iminium bond. Through this substitution, the inherent cyclization tendency has already been analyzed for the peptides tyrocidine A, nostocyclopeptide and segetalin A by our group [18–20]. However, cyclic imino peptides are prone to hydrolysis and are, therefore, hard to isolate in pure form. By substituting the amide bond by an oxime linkage, the cyclic peptides become more stable and isolable, especially in the case of ketoximes [21]. Several approaches have been made to synthesize amino acid side chain aldehydes/ketones for the ligation or cyclization with hydroxylamines [22–27].

Here, we integrated the oxime bond into a common cyclic hexapeptide in order to study in detail whether it fits into a β-turn. The oxime, as a functional group in cyclic peptides, has the characteristic that the peptide backbone is expanded by one oxygen atom, which leads to uncommon 19-membered ring structures instead of cyclic 18-membered hexapeptides. We obtained the cyclic ketoxime peptide **2** cyclo[Goly-Val-Ala-Pro-Leu-Kly] from the linear precursor **1** (Figure 1) and analyzed the oxime *E/Z* and the proline *cis/trans* isomerism in solution by nuclear magnetic resonance (NMR) spectroscopy. The standard three-letter code Gly is supplemented to the four letter code Goly, which represents the expanded backbone. The six-letter code for unnatural dipeptides is adapted here for the four atom building block Goly [28]. Kly stands for keto glycine and is a blend of the ketone and the code Gly. The peptide **2** shown in Figure 1 can form four different structures, because of the *E/Z* isomerism of the ketoxime and the *cis/trans* isomerism of the proline.

Results and Discussion

We synthesized the linear peptide Boc-Goly-Val-Ala-Pro-Leu-Kly-Smc **1a** (Smc: semicarbazone) to perform the macrocyclization reaction. The N-terminal Boc-protected building block Goly represents the natural amino acid glycine, which is expanded through the formal insertion of an oxygen atom in the N—C bond. The synthesis was carried out in two steps starting with *N*-Boc-hydroxylamine (**3**), which forms the corresponding methyl ester **4**

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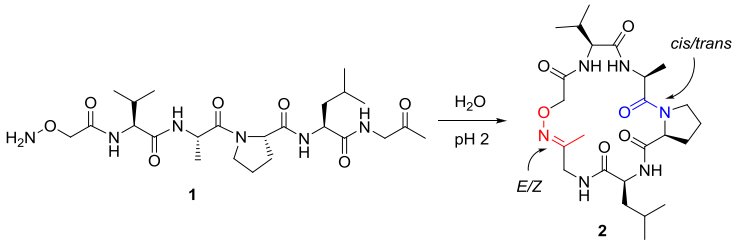
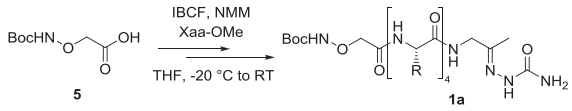


Figure 1. Condensation of the linear hexapeptide **1** to the cyclic ketoxime **2**. Based on the *E/Z* conformation (red) formed by the ketoxime and the *cis/trans*-isomerism (blue) of Pro[4], the formation of four different conformers is possible. The conformers formed are identified and analyzed via NMR spectroscopy. The peptide **2** is shown here as the *E-trans*-conformer.

in a nucleophilic substitution. After hydrolysis of the ester, the Boc-protected hydroxylamino acid **5** is obtained in good yield. The C-terminal ketone is synthesized in two steps from the natural amino acid glycine (**6**). In the first step, the corresponding glycine is transformed into the ketone **7** through a *Dakin-West* reaction, and the following cleavage of the acetyl groups yields the amino acetone hydrochloride. The ketone is protected as the semicarbazone **8** with semicarbazide (Scheme 1) for peptide synthesis. The synthesis of **8** has already been described in the literature [29].

The peptide **1a** was assembled stepwise from the N- to the C-terminus with the relatively reactive semicarbazone as a temporary protecting group. Because of this labile protecting group, the unconventional N- to C-terminus synthesis strategy was used, and the small amount of stereoisomers (less than 10%, estimated from NMR) during the synthesis were removed via HPLC. The synthesis started with Boc-Goly-OH, which was activated by isobutyl chloroformate (IBCF) as a mixed anhydride under basic conditions. The next amino acid valine was introduced as a methyl ester and, after isolation of the corresponding amide, the ester was saponified with LiOH in THF/H_2O (1:1). This procedure was very efficient, the side-products were removed easily, and it was repeated until the complete hexapeptide was obtained (Scheme 2). The protected linear peptides which contain proline show the expected two signal sets in the ^{1}H NMR with a *cis/trans* ratio at the proline amide bond of approximately 2:3.

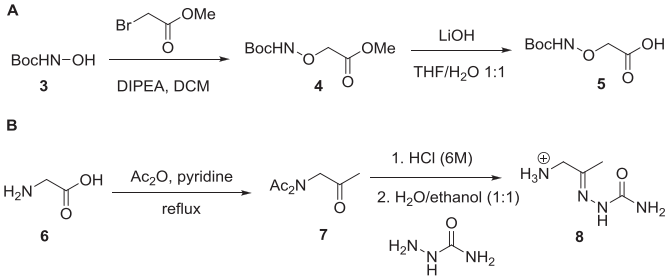
Before the final macrocyclization, the terminal protecting groups had to be cleaved and the peptide was purified by high performance liquid chromatography (HPLC). The acid labile protecting groups were cleaved with aqueous TFA (95%) for 1 h at 45 °C. The crude product was then dissolved in water and purified by preparative HPLC, and the peptide was cyclized directly. The synthesis was not optimized for maximum yield, and the 3% yield refers to the purest HPLC fraction which was used for NMR analysis to detect the *E/Z* and *cis/trans* isomers. For higher yields, the protecting group strategy of the ketone should be improved to avoid building



Scheme 2. Solution-phase synthesis of the protected peptide **1a**. The acid, in the first step Goly, was activated by IBCF under basic conditions at −20 °C. After the addition of the amino acid methyl ester, the amide formed was isolated and the ester cleaved for the next coupling. These steps were repeated until the protected peptide **1a** was obtained.

block **8** which has a low solubility. The isolated macrocyclic ketoxime peptide **2** was analyzed by 1D and 2D NMR spectroscopy. The ^{1}H NMR shows a dominating signal set instead of the possible four which would be expected for an equilibrium of several conformers.

The cross signals in red show the NH/NH ROE contacts of NH-Leu/Kly and NH-Val/Ala of the major conformer. The *trans* configuration in the case of the *cis/trans* isomerism of the Pro amide bond was assigned by the chemical shift of the β-CH_2 and γ-CH_2 methylene groups of the proline (δ = 28.8 and 25.5 ppm, respectively) for the major conformer. This method could not be used in this case based on the low intensity of the minor conformer. The chemical shift of the methyl group is characteristic of the assignment of the *E/Z* conformation of the ketoxime. We synthesized the model compound acetophenone *O*-benzyloxime as a mixture of the *E* and *Z* conformers to compare the chemical shifts of the *E* and *Z* isomer. The chemical shift of the methyl group was 12.6 and 21.3 ppm for the *E* conformer and the *Z* conformer, respectively, a difference of 8.7 ppm. We could compare the chemical shifts in both conformers because of the high intensity of the ketoxime methylgroups in peptide **2**. The methyl group has a chemical shift of 11.7 and 12.7 ppm for the major conformer and minor conformer, respectively, a difference of just 1.0 ppm. Based on these chemical shifts, the major conformer must be the *E*-ketoxime and the *trans*-proline, and the minor



Scheme 1. Synthesis of the N-terminal Boc-Goly-OH (**5**) and the C-terminal H_2N-Kly-Smc (**8**). **A)** Reagents and conditions: 1) Methylbromoacetate (2.0 eq) and DIPEA (2.0 eq), DCM, RT, 14 h. 2) LiOH (2.5 eq), THF/H_2O (1:1), 45 °C, 2 h. **B)** Reagents and conditions: 1) Ac_2O/pyridine (3:1), reflux, 4 h. 2) aq. HCl (6 M), reflux, 4 h. 3) Semicarbazide hydrochloride (1.31 eq), ethanol/H_2O (1:1), RT, 2 h.

conformer also contains the *E*-ketoxime, but the *cis*-proline (Figure 2).

The complete assignment of the major conformer was accomplished by 2D NMR spectroscopy (TOCSY, HSQC and ROESY). Only the NH signals could be assigned from the exchange peaks in the ROESY spectrum for the minor isomer. The temperature gradients of the amide protons of the major isomer identify two intramolecular hydrogen bonds (NH-Kly and NH-Ala) and one hydrogen bond for NH-Val for the minor isomer (Table 1).

The complete NOE list of the major isomer is contained in the SI. Two pairs of NH/NH contacts NH-Val/NH-Ala and NH-Leu/NH-Kly are characteristic for two β-turns. Further NOEs from the NH-αH sequential walk identify the βII turns. Even the transannular NOE between βCH_3-Ala and $\alpha H\text{-}Kly_{(proS)}$ is visible. The strong sequential NOE contact αH-Pro/NH-Leu and a medium NH-Leu/αH-Leu identify the βII turn, although the contribution from fast rotation about the central peptide bond $(i+1)$–$(i+2)$ about 180° – the so-called βI/II flip – cannot be excluded [30,31]. The structure in Figure 3A displays the β-turn type II with alanine, proline and leucine in the positions *i*, $i+1$ and $i+2$ of the turn. Pro is expected to occupy the $i+1$ position comparable to one of the first cyclic hexapeptides elucidated by NMR and other additional cyclic β,β-hexapeptides [32].

The structure calculations of **2** were carried out as described previously using the program package HyperChem with MM+ force field and without explicit water included [18,33,34]. Ten snapshots from a 100-ps molecular dynamics simulation (step size 1 fs, 300 K) were averaged, and the resulting structure was allowed to relax without restraints to verify its plausibility [35]. The NOE contacts of the minor conformer between NH-Ala/NH-Val and NH-Leu/αH-Ala indicate the formation of β-turn type VIa structure. In contrast to the major conformer, the sequence APL in this turn structure rotated clockwise by one position. Proline takes now the $i+2$ position in the turn and the central peptide bond $(i+1)$–$(i+2)$ changes from *trans* to *cis*. This confirms the assumption that the minor conformer gets structured by an *E*-ketoxime and *cis*-proline (Figures 3B and 4).

β-turns, as secondary structures, are stabilized by intramolecular hydrogen bonds, which are formed between the $i+4$ position and

Table 1. ^3J-NH/α couplings and temperature gradients of the amide protons of both conformers (major and minor) determined by ^{1}H NMR spectra at different temperatures (300, 310 and 320 K)

Amide proton	^3J-NH/α (Hz)	Major [ppb/K]	^3J-NH/α (Hz)	Minor [ppb/K]
NH-Leu	7.86	5.37	7.42	4.45
NH-Kly	8.70/2.88	2.54	7.56/4.11	6.55
NH-Val	8.28	7.64	8.97	0.79
NH-Ala	7.44	1.67	7.41	3.92

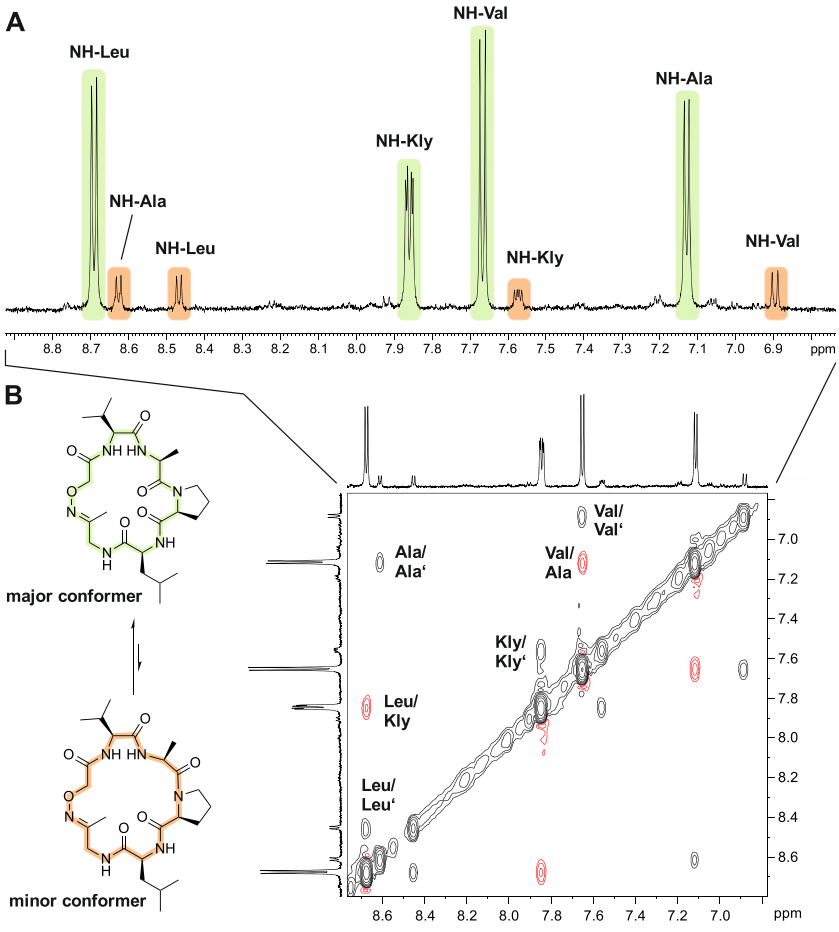


Figure 2. **A**) (600 MHz, 300 K, $DMSO_{d6}$) NH region of the ^{1}H NMR spectrum of peptide **2**. The equilibrium between both conformers – the major conformer (86%, green) and the minor conformer (14%, orange) – is visible from the exchange signals. **B**) The difference between both conformations is the Pro amide bond, which is *trans* for the major conformer and *cis* for the minor conformer. Only one conformer is populated by the *E/Z* isomerism of the ketoxime. The *E*-configured ketoxime was identified in both conformers. The ROE contacts in the expansion of the ROESY spectrum (mixing time 300 ms) are marked in red, and the black cross signals in black display the chemical exchange between both conformations.

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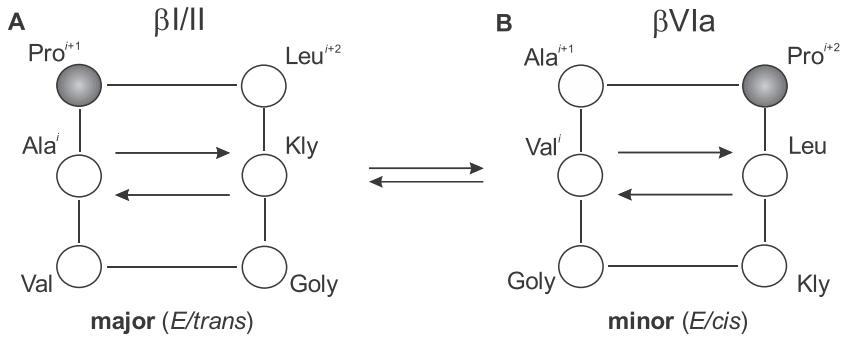

Figure 3. Illustration of the major conformer (A) and the minor conformer (B) as sphere models. Based on the *cis/trans* isomerism at the proline (grey) of both conformers, the APL sequence is located at different positions in the template. The hydrogen bonds are indicated by arrows.

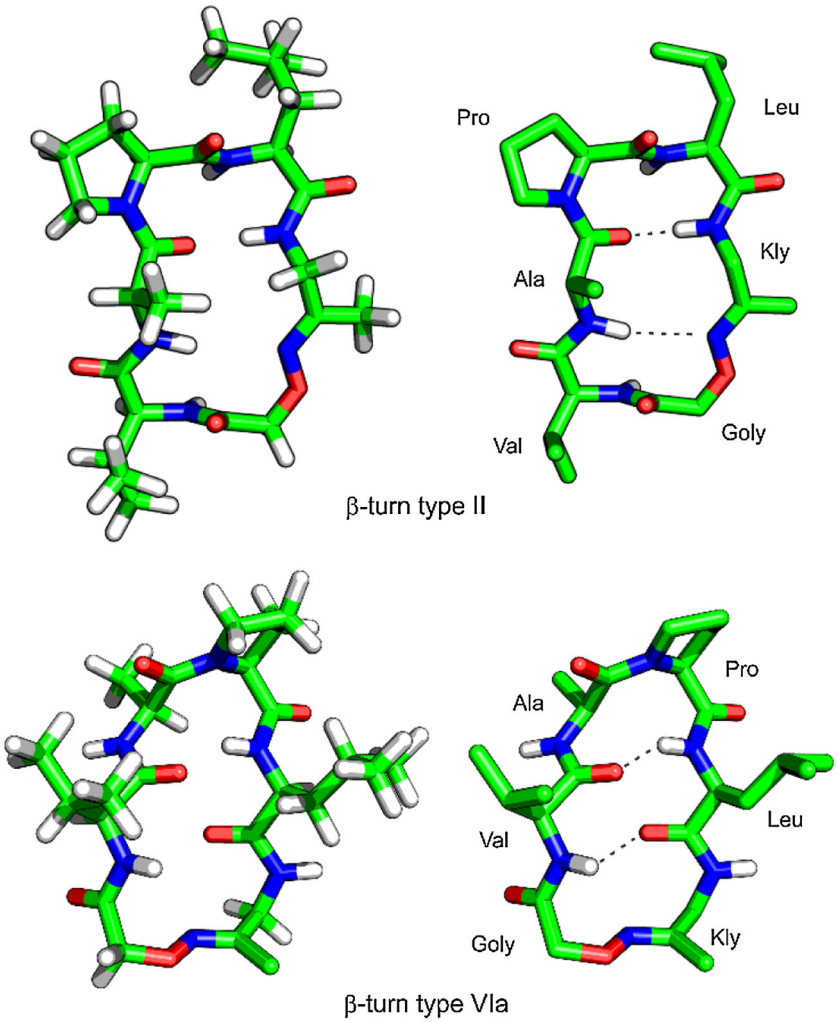

Figure 4. Illustration of the major conformer as a β-turn type II and the minor conformer as a β-turn type VIa. The NOE contacts served as distance restraints in a molecular dynamics simulation. In the major conformer the oxime nitrogen forms the electron donor for the intramolecular hydrogen bond with NH-Leu.

amino acid in the *i* position. In the case of the major conformer, these amino acids are Kly and Ala. The temperature gradients of the four amide protons support the structure proposed. NH-Ala possesses the lowest temperature dependency of the chemical shift, followed by the gradient of the NH-Kly. Both values are indicative of the hydrogen bond between each of them. The temperature gradients of NH-Leu indicate solvent-oriented NH-groups (Table 1). The determined torsion angels φ and ψ are listed in Table 2.

The black cross signals of the ROESY spectrum shown in Figure 2 can be further analyzed to determine the chemical exchange rate between both conformers. Based on the ratio determined, the activation energy which is necessary for the conformational change can be calculated. In our special case, the populations are unequal, and we have to consider the mole fractions $X_A = 0.86$ and $X_B = 0.14$ for the calculation of r [36,37]. Therefore, the intensities of the cross signals of both NH-Ala signals are determined and, with the mole fractions, a ratio of 13.33 was calculated. This ratio in combination

Table 2. Based on the illustration of the major conformer in Figure 4, we determined the ϕ and ψ torsion angels the β-turn type II (left) and compared them with the ideal values for a β-turn type II (right)

Amino acid	φ angle	ψ angle	φ angle (ideal)	ψ angle (ideal)
Goly	−171.5, -8.5	111.9	-60	120
Val	73.9	-3.5	80	0
Ala	-125.2	160.1	—	—
Pro	-46.8	100.9	-60	120
Leu	79.4	15.0	80	0
Kly	-116.9	-97.0	—	—

$$r = 4X_A X_B \frac{(I_{AA} + I_{BB})}{(I_{AB} + I_{BA})} - (X_A - X_B)^2 = 0.4816\frac{1.15}{0.04} - 0.5185 = 13.3275$$

$$k = \frac{1}{t_m} ln \frac{r+1}{r-1} = \frac{1}{300\ ms} ln \frac{13.3275+1}{13.3275-1} = 0.5012\ s^{-1}$$

$$\Delta G = T \cdot 0.0191 \left(10.32 + log\left(\frac{T}{k}\right)\right) = 75.1\ \frac{kJ}{mol}$$

Figure 5. Calculation of the activation energy of the conformational exchange [36,37]. The ratio r determined combined with the mixing time t_m of 300 ms lead to the constant $k = 0.5012\ s^{-1}$. An energy of 75.1 kJ/mol (ca. 18 kcal/mol) is required for the change between both conformers.

with the mixing time of the ROESY experiment of 300 ms leads to an equilibrium constant $k = 0.5012\ s^{-1}$. Based on this value, the activation energy of this process of 75.1 kJ/mol was determined (Figure 5). This value is comparable with the activation barrier of the *cis/trans* isomerism (75–85 kJ/mol) [38–40].

Conclusions

A linear peptide ketone with an N-terminal hydroxylamine was cyclized to a 19-membered macrocyclic peptide. Two-dimensional NMR spectroscopy identifies a minor conformer from the rotation about the Pro tertiary amide bond, but not around the oxime linkage. The facile macrocyclization process leads to a stable and isolable cyclic peptide, and this method could be used in other macrocyclizations where the oxime can be part of a β-turn structure. Broader application will be possible after integration of the oxime cyclization into solid-phase peptide synthesis, for which the C-terminal ketone must be covalently linked to a resin by a suitable linker. Instead of the flexible linkage expected, we identify a well-defined main conformer very similar to the canonical double β-turn cyclic hexapeptides. The oxime nitrogen serves as the electron donor in a hydrogen bond to the $i+2$ amide hydrogen atom in a βII-type reverse turn. Although the ketoxime linker expands the peptide backbone by the four atom building block Goly, the ketoxime is compatible with a hairpin conformation in the hexapeptide example presented here. This conformational preference here can serve as a template for the future design of fortunate ligation sites.

Experimental Section

General Information

The reactions were executed under a nitrogen atmosphere at ambient pressure. Thin-layer chromatography was performed on silica gel 60 F_{254} (*Merck KGaA*), and detection was carried out by fluorescence quenching under UV light (λ = 254 nm) or by staining with ninhydrin solution followed by heating to 500 °C. Flash chromatography was performed on silica gel 60 (0.040–0.063 mm) from *Merck KGaA*. Semi-preparative HPLC was performed on a *Dionex HPLC* system with a diode array detector using an *ACE HPLC* C18 RP, 7.75 × 150 mm, and analytical HPLC was performed on the same system using an *Intersil ODS-4*, C18 RP, 3.0 × 150 mm. The NMR spectra were recorded on *Bruker* AV 600 spectrometers. Chemical shifts (δ) are given in ppm referring to the solvent signal. The coupling constants are ^3J couplings, unless otherwise indicated. All mass spectra were recorded on a *Finnigan* LTQ-FT spectrometer.

Boc-2-(Aminooxy)methylacetate (4)

DIPEA (9.70 ml, 56.8 mmol, 2.00 eq) and methylbromoacetate (2.70 ml, 28.4 mmol, 1.00 eq) were added to a solution of *N*-Boc-hydroxylamine (**3**, 3.78 g, 28.4 mmol, 1.00 eq) in DCM (40 ml) at RT. After stirring for 14 h, the solvent was removed under reduced pressure, and a colorless solid was obtained. The residue was solved in EtOAc (100 ml), washed three times with 5% aq. $NaHCO_3$, twice with 10% aq. citric acid and three times with brine. The organic phase was dried over $MgSO_4$, filtered and concentrated *in vacuo* to afford **4** in 58% yield (3.37 g, 16.4 mmol) as a colorless oil. $R_f = 0.86$ (EtOAc/pentane 3:1); ^{1}H-NMR (300 MHz, $DMSO_{-d6}$, 300 K): δ = 10.17 (s, 1H, NH), 4.36 (s, 2H, CH_2), 3.67 (s, 3H, OCH_3), 1.40 (s, 9H, Boc) ppm; ^{13}C-NMR (75 MHz, $DMSO_{-d6}$, 300 K): δ = 169.1 (CO_{ester}), 156.3 ($CO_{carbamate}$), 80.1 ($Boc_{quat.}$), 72.0 (CH_2), 51.5 (OCH_3), 27.9 (Boc-CH_3) ppm; HRMS (ESI): calcd. for [$C_8H_{15}NO_5Na^+$]: 228.0842, found: 228.0843.

Boc-2-(Aminooxy)acetic Acid (5)

LiOH (0.96 g, 40.0 mmol, 2.47 eq) was added to a solution of Boc-2-(Aminooxy)methylacetate (**4**, 3.33 g, 16.2 mmol, 1.00 eq) in THF/H_2O (1:1, 100 ml) at RT. After stirring for 4 h at 45 °C, the solvent was removed under reduced pressure and the residue was diluted with aq. HCl (2 M) until the pH reached ~2. After extraction with EtOAc (200 ml), the organic phase was dried over $MgSO_4$, filtered and concentrated *in vacuo* to afford the acid **5** in 79% yield (2.43 g, 12.7 mmol) as a colorless solid. ^{1}H-NMR (300 MHz, $DMSO_{-d6}$, 300 K): δ = 12.80 (s, 1H, OH), 10.10 (s, 1H, NH), 4.26 (s, 2H, CH_2), 1.40 (s, 9H, Boc) ppm; ^{13}C-NMR (75 MHz, $DMSO_{-d6}$, 300 K): δ = 170.0 (CO_{acid}), 156.4 ($CO_{carbamate}$), 80.0 ($Boc_{quat.}$), 72.0 (CH_2), 28.0 (Boc-CH_3) ppm; HRMS (ESI): calcd. for [$C_7H_{13}NO_5Na^+$]: 214.0686, found: 214.0688.

Diacetyl Aminoacetone (7)

Based on a synthesis of Hepworth et al., glycine (**6**, 10.0 g, 0.13 mol, 1.00 eq) was solved in acetic anhydride (150 ml, 1.55 mol, 11.7 eq) and pyridine (65.0 ml, 0.80 mol, 6.00 eq), and this mixture was refluxed for 6 h. After stirring for 14 h at RT, the solvent was removed under reduced pressure and the residue was purified by vacuum distillation. The product **7** was obtained in 48% yield as a yellow oil (9.79 g, 62.3 mmol). Boiling point: 125 °C (1 mbar); ^{1}H-NMR (300 MHz, $DMSO_{-d6}$, 300 K): δ = 4.60 (s, 2H, CH_2), 2.26 (s, 6H, 2 x Ac), 2.16 (s, 3H, CH_3) ppm; ^{13}C-NMR (75 MHz, $DMSO_{-d6}$, 300 K): δ = 202.9 (CO_{ketone}), 172.6 (CO_{Ac}), 53.8 (CH_2), 26.9 (Ac), 25.8 (CH_3) ppm; HRMS (ESI): calcd. for [$C_7H_{11}NO_3Na^+$]: 180.0631, found: 180.0636 [29].

Aminoacetone-Semicarbazone Hydrochloride (8)

Based on a synthesis of Hepworth et al., the acetamide (**7**, 9.59 g, 61.0 mmol, 1.00 eq) was solved in aqueous HCl (6 M, 70 ml) and refluxed for 6 h. After stirring for 14 h at RT, the solvent was removed under reduced pressure and the dark red oil (6.95 g) obtained was used directly in the next step. The residue was solved in ethanol (45 ml) and a solution of semicarbazide hydrochloride (8.93 g, 80.0 mmol, 1.31 eq) in H_2O (20 ml) was added. The reaction was stirred for 2 h, and the colorless precipitate was filtered and washed with ethanol. The solid was purified by recrystallization (H_2O/ethanol 1:1), and the hydrochloride **8** (6.94 g, 41.7 mmol) was obtained in 68% yield as a colorless crystalline solid. Melting point: 193 °C; ^{1}H-NMR (300 MHz, DMSO$_{-d6}$, 300 K): δ = 9.35 (s, 1H, NH), 8.13 (s, 3H, NH_3^+), 6.78 (s, 1H, NH_2), 6.45 (s, 2H, NH_2), 3.32 (s, 2H, CH_2), 1.82 (s, 3H, CH_3) ppm; ^{13}C-NMR (75 MHz, DMSO$_{-d6}$, 300 K): δ = 157.3 (CO), 141.2 (C=N), 42.6 (CH_2), 14.6 (CH_3) ppm; HRMS (ESI): calcd. for [$C_4H_{10}N_4OH^+$]: 131.0927, found: 131.0929 [29].

Peptide Synthesis

The synthesis of peptide **2** was accomplished linearly in solution from the N-terminus to the C-terminus via IBCF coupling of the Boc-Goly-OH and the corresponding amino acid methyl ester. In general, the carboxylic acid (1.00 eq) was solved in THF (conc. = 0.2 M) and NMM (1.00 eq) was added for the peptide coupling. The solution was cooled to −20 °C, IBCF (1.00 eq) was added slowly, and the suspension was stirred for 10 min. The corresponding amine hydrochloride was solved in THF (10–20 ml), neutralized with NMM (1.00 eq) and added to the suspension. The reaction was stirred for 14 h at RT, and the solvent was removed under reduced pressure. The residue was solved in EtOAc (100 ml), washed three times with 5% aq. $NaHCO_3$, twice with 10% aq. citric acid and three times with brine. The organic phase was dried over $MgSO_4$, filtered and concentrated *in vacuo* to afford the corresponding peptide.

Ester Hydrolysis

For the hydrolysis of the peptide methyl esters the corresponding esters were solved in THF/H_2O (1:1, 20 ml) and LiOH (2.47 eq) was added. The solution was stirred at 45 °C until the reaction was complete. The solvent was removed under reduced pressure, and the residue was diluted with aq. HCl (2 M) until the pH reached ~2. After extraction with EtOAc (100 ml), the organic phase was dried over $MgSO_4$, filtered and concentrated *in vacuo* to afford the corresponding acid.

Boc-Goly-Val-OMe (9)

The peptide was synthesized as described above in two steps and was obtained in 69% yield as a colorless solid. R_f = 0.59 (EtOAc/pentane 3:1); ^{1}H-NMR (300 MHz, DMSO$_{-d6}$, 300 K): δ = 10.41 (s, 1H, Goly-NH), 8.36 (d, 1H, ^{3}J = 8.16 Hz, Val-NH), 4.26 (dd, 1H, ^{3}J = 6.32 Hz, ^{3}J = 8.45 Hz, Val-αH), 4.24 (d, 2H, ^{2}J = 15.88 Hz, Goly-αH), 3.64 (s, 3H, OCH_3), 2.02–2.13 (m, 1H, Val-bH), 1.42 (s, 9H, Boc), 0.91 (d, 3H, ^{3}J = 4.77 Hz, Val-γH), 0.88 (d, 3H, ^{3}J = 4.74 Hz, Val-γH) ppm; ^{13}C-NMR (75 MHz, DMSO$_{-d6}$, 300 K): δ = 171.5 (Val-CO), 168.5 (Goly-CO), 80.8 ($Boc_{quat.}$), 74.5 (Goly-αH), 57.0 (Val-αC), 51.7 (O-CH_3), 29.9 (Val-βC), 27.9 (Boc-CH_3), 18.8 (Val-γC), 18.0 (Val-γC) ppm; HRMS (ESI): calcd. for [$C_{13}H_{24}N_2O_6H^+$]: 305.1707, found: 305.1706.

Boc-Goly-Val-Ala-OMe (10)

The peptide was synthesized as described above in two steps and was obtained in 48% yield as a colorless solid. R_f = 0.33 (EtOAc/pentane 3:1); ^{1}H-NMR (300 MHz, DMSO$_{-d6}$, 300 K): δ = 10.32 (s, 1H, Goly-NH), 8.49 (d, 1H, ^{3}J = 6.60 Hz, Ala-NH), 7.97 (d, 1H, ^{3}J = 8.10 Hz, Val-NH), 4.28 (dd, 1H, ^{3}J = 7.11 Hz, ^{3}J = 9.20 Hz, Val-αH), 4.22–4.26 (m, 1H, Ala-αH), 4.19 (d, 2H, ^{2}J = 15.67 Hz, Goly-αH), 3.61 (s, 3H, OCH_3), 1.94–2.00 (m, 1H, Val-βH), 1.41 (s, 9H, Boc), 1.28 (d, 3H, ^{3}J = 6.60 Hz, Ala-βH), 0.89 (d, 3H, ^{3}J = 6.72 Hz, Val-γH), 0.91 (d, 3H, ^{3}J = 6.78 Hz, Val-γH) ppm; ^{13}C-NMR (75 MHz, DMSO$_{-d6}$, 300 K): δ = 172.8 (Ala-CO), 170.4 (Val-CO), 167.8 (Goly-CO), 156.9 (Boc-CO), 80.6 ($Boc_{quat.}$), 74.5 (Goly-αH), 56.7 (Val-αC), 51.7 (O-CH_3), 47.5 (Ala-αC), 30.8 (Val-βC), 27.9 (Boc-CH_3), 18.9 (Val-γC), 18.0 (Val-γC), 16.7 (Ala-βC) ppm; HRMS (ESI): calcd. for [$C_{16}H_{29}N_3O_7H^+$]: 376.3078, found: 376.3075.

Boc-Goly-Val-Ala-Pro-OMe (11)

The peptide was synthesized as described above in two steps and was obtained in 26% yield as a colorless oily solid. R_f = 0.14 (EtOAc); ^{1}H-NMR (300 MHz, DMSO$_{-d6}$, 300 K): δ = 10.33 (s, 1H, Goly-NH), 8.25 (d, 1H, ^{3}J = 6.69 Hz, Ala-NH), 7.97 (d, 1H, ^{3}J = 9.03 Hz, Val-NH), 4.47 (t, 1H, ^{3}J = 6.89 Hz, Pro-αC), 4.25–4.34 (m, 2H, Val-aH, Ala-aH), 4.19 (d, 2H, ^{2}J = 15.67 Hz, Goly-αH), 3.63–3.70 (m, 2H, Pro-dH), 3.60 (s, 3H, OCH_3), 2.12–2.23 (m, 1H, Val-βH), 1.78–1.95 (m, 4H, Pro-βH, Pro-γH), 1.41 (s, 9H, Boc), 1.20 (d, 3H, ^{3}J = 7.00 Hz, Ala-βH), 0.86 (d, 3H, ^{3}J = 6.78 Hz, Val-γH), 0.81 (d, 3H, ^{3}J = 6.75 Hz, Val-γH) ppm; ^{13}C-NMR (75 MHz, DMSO$_{-d6}$, 300 K): δ = 172.2 (CO), 170.5 (CO), 170.0 (CO), 167.7 (CO), 156.9 (Boc-CO), 80.6 ($Boc_{quat.}$), 74.5 (Goly-αH), 58.4 (Pro-αC), 56.8 (Val-αC), 51.7 (O-CH_3), 46.3 (Pro-δC), 46.1 (Ala-αC), 30.6 (Val-βC), 28.4 (Pro-βC), 27.9 (Boc-CH_3), 24.6 (Pro-γC), 19.0 (Val-γC), 18.0 (Val-γC), 16.4 (Ala-βC) ppm; HRMS (ESI): calcd. for [$C_{21}H_{36}N_4O_8Na^+$]: 492.2425, found: 492.2424.

Boc-Goly-Val-Ala-Pro-Leu-OMe (12)

The peptide was synthesized as described above in two steps and was obtained in 36% yield as a colorless oil. R_f = 0.40 (DCM/MeOH 20:1); ^{1}H-NMR (300 MHz, DMSO$_{-d6}$, 300 K): δ = 10.32 (s, 1H, Goly-NH), 8.35 (d, 1H, ^{3}J = 7.54 Hz, Ala-NH), 8.14 (d, 1H, ^{3}J = 7.51 Hz, Val-NH), 7.94 (d, 1H, ^{3}J = 8.62 Hz, Leu-NH), 4.44–4.59 (m, 1H, Ala-αH), 4.18–4.37 (m, 3H, Val-αH, Pro-αH, Leu-αH), 4.19 (d, 2H, ^{2}J = 15.64 Hz, Goly-αH), 3.60 (s, 3H, O-CH_3), 3.54–3.58 (m, 2H, Pro-δH), 1.78–2.07 (m, 5H, Pro-βH, Pro-γH, Val-βH), 1.60–1.68 (m, 1H, Leu-γH), 1.46–1.55 (m, 2H, Leu-βH), 1.41 (s, 9H, Boc-H), 1.17 (d, 3H, ^{3}J = 6.84 Hz, Ala-βH), 0.89 (d, 3H, ^{3}J = 6.51 Hz, Val-γH), 0.83 (d, 3H, ^{3}J = 6.42 Hz, Val-γH), 0.80–0.85 (m, 6H, Leu-δH) ppm; ^{13}C-NMR (75 MHz, DMSO$_{-d6}$, 300 K): δ = 173.2–167.7 (7 x CO), 156.9 (Boc-CO), 80.6 ($Boc_{quat.}$), 74.5 (Goly-αH), 56.8 (Val-αC), 51.7 (O-CH_3), 50.2 (Leu-αC), 46.6 (Pro-dC), 46.2 (Ala-αC), 39.5 (Leu-βC), 30.7 (Val-βC), 28.8 (Pro-βC), 27.9 (Boc-CH_3), 24.2 (Pro-γC), 24.1 (Leu-γC), 22.2 (Leu-δC), 21.3 (Leu-δC), 19.1 (Val-γC), 18.0 (Val-γC), 16.7 (Ala-βC) ppm; HRMS (ESI): calcd. for [$C_{27}H_{47}N_5O_9Na^+$]: 608.3266, found: 608.3272.

Cyclo-(Goly-Val-Ala-Pro-Leu-Kly) (2)

The peptide was synthesized as described above in two steps and directly treated with aqueous TFA (95%). The linear peptide was solved in MeCN/H_2O (1:1) and cyclized spontaneously. After purification via semi preparative HPLC the cyclic ketoxime peptide was obtained in 3% yield as a colorless solid. HPLC-R_t = 9.37 min (10%–90% MeCN in 20 min, flow: 0.6 ml/min); ^{1}H-NMR (600 MHz, DMSO$_{-d6}$, 300 K): δ = 8.67 (d, 1H, ^{3}J = 7.86 Hz, Leu-NH), 7.84 (dd, 1H, ^{3}J =

2.88 Hz, ^{3}J = 8.70 Hz, Kly-NH), 7.65 (d, 1H, ^{3}J = 8.28 Hz, Val-NH), 7.11 (d, 1H, ^{3}J = 7.44 Hz, Ala-NH), 4.58–4.60 (m, 1H, Ala-αH), 4.42 (d, 2H, ^{2}J = 14.41 Hz, Goly-αH), 4.23–4.26 (m, 2H, Pro-αH, Kly-αH), 4.15 (dd, 1H, ^{3}J = 5.37 Hz, ^{3}J = 8.25 Hz, Val-αH), 4.04–4.08 (m, Leu-αH), 3.71–3.75 (m, 1H, Pro-δH), 3.43–3.47 (m, 1H, Pro-δH), 3.33 (dd, 1H, ^{2}J = 15.36 Hz, ^{3}J = 3.16 Hz, Kly-αH), 2.18–2.24 (m, 1H, Val-βH), 2.02–2.11 (m, 2H, Pro-βH, Pro-γH), 1.84–1.88 (m, 1H, Pro-γH), 1.81 (s, 3H, Kly-CH_3), 1.78–1.79 (m, 1H, Pro-βH), 1.65–1.73 (m, 1H, Leu-γH), 1.50–1.73 (m, 2H, Leu-βH), 1.11 (d, 3H, ^{3}J = 6.84 Hz, Ala-βH), 0.89 (d, 3H, ^{3}J = 6.51 Hz, Val-γH), 0.88 (d, 3H, ^{3}J = 7.14 Hz, Val-γH), 0.82 (d, 3H, ^{3}J = 7.14 Hz, Val-γH), 0.81–0.90 (m, 6H, Leu-δH) ppm; ^{13}C-NMR (75 MHz, $DMSO_{-d6}$, 300 K): δ = 72.5 (Goly-αH), 59.9 (Pro-αC), 57.7 (Val-αC), 51.5 (Leu-αC), 46.8 (Pro-δC), 45.9 (Ala-αC), 42.0 (Kly-αC), 38.7 (Leu-βC), 28.7 (Val-βC), 28.1 (Pro-βC), 24.9 (Pro-γC), 24.2 (Leu-γC), 22.9 (Leu-δC), 20.6 (Leu-δC), 19.0 (Val-γC), 17.4 (Ala-βC) 17.3 (Val-γC), 11.7 (Kly-CH_3) ppm; HRMS (ESI): calcd. for [$C_{24}H_{40}N_6O_6Na^+$]: 531.2902, found: 531.2910.

Acknowledgements

We thank the Evonik-Stiftung for a Werner-Schwarze scholarship and financial support.

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