Supporting Information
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Supporting Information

Azadipeptide Nitriles – Highly Potent and Proteolytically Stable Inhibitors of Papain-like Cysteine Proteases

Reik Löser, Maxim Frizler, Klaus Schilling, and Michael Gütschow

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Experimental Section

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Figure S1. (A) Congruent with Figure 1 of the publication. Monitoring of the human cathepsin L-catalyzed hydrolysis of Z-Phe-Arg-NHMec (10 µM) in the presence of increasing concentrations of the azadipeptide nitrile \textbf{6} (○, 0; ●, 3 nM; ●, 4 nM; ●, 8 nM; ●, 9 nM; ●, 10 nM; ●, 20 nM; ●, 30 nM; ●, 50 nM; ●, 100 nM). The reaction (100 mM sodium phosphate pH 6.0, 100 mM NaCl, 5 mM EDTA, 0.01 % Brij 35, 25 µM DTT, 1 % DMSO, 37 °C) was initiated by addition of the enzyme. Fluorescence emission at 440 nm (I = fluorescence intensity) was measured after excitation at 360 nm. Fluorescence units (FU) were corrected for background fluorescence. (B) Plot of the rates of hydrolysis of Z-Phe-Arg-NHMec versus concentrations of \textbf{6}. Non-linear regression gave an apparent inhibition constant \(K'_i = (1+|[S]/K_m) K_i = 0.60 \pm 0.08\) nM. Inset: Plot of the \(k_{obs}\) values versus concentrations of \textbf{6}. The linear dependence indicates a one-step mechanism for the enzyme-inhibitor interaction. Linear regression gave an apparent second-order rate constant \(k_{on}' = k_{on} / (1 + [S]/K_m) = (910 \pm 10) \times 10^3 \text{ M}^{-1}\text{s}^{-1}\). From the corresponding \(k_{on}\) value, a first-order rate constant \(k_{off} = k_{on} K_i = (0.54 \pm 0.07) \times 10^3 \text{ s}^{-1}\) was calculated.
Figure S2. (A) Monitoring of the human cathepsin L-catalyzed hydrolysis of Z-Phe-Arg-NHMec (10 µM) in the presence of increasing concentrations of the dipeptide nitrile 12 (●, 0; ○, 1 µM; ●, 2 µM; ●, 3 µM; ●, 4 µM; ●, 5 µM; ●, 7 µM; ●, 10 µM). The reaction (100 mM sodium phosphate pH 6.0, 100 mM NaCl, 5 mM EDTA, 0.01 % Brij 35, 25 µM DTT, 1 % DMSO, 37 °C) was initiated by addition of the enzyme. Fluorescence emission at 440 nm (I = fluorescence intensity) was measured after excitation at 360 nm. Fluorescence units (FU) were corrected for background fluorescence. (B) Plot of the rates of hydrolysis of Z-Phe-Arg-NHMec versus concentrations of 12. Non-linear regression gave an apparent inhibition constant $K_i' = (1 + [S] / K_m) K_i = 2.3 \pm 0.2 \mu$M.
Figure S3. Reactivation of cathepsin L and papain, respectively, inhibited by the azadipeptide nitrile 6. Fluorescence emission at 440 nm (I = fluorescence intensity) was measured after excitation at 360 nm. Fluorescence units (FU) were corrected for background fluorescence. (A) Reactivation of cathepsin L. The cathepsin L-catalyzed hydrolysis of Z-Phe-Arg-NHMec (10 µM) was monitored in 0.1 M sodium phosphate pH 6.0, 5 mM EDTA, 0.1 M sodium chloride and 0.01 % Brij 35 with final concentrations of 10 pg/mL cathepsin L, 20 pM 6, 1.02 % DMSO, 0.5 µM DTT. In the reactivation experiment, cathepsin L (1 ng/mL) was preincubated with 6 (2 nM) for 30 min at 25 °C. In the control experiment, the preincubation was done in the absence of the inhibitor. The initial rates were clearly different, 1.3 ± 0.1 FU min⁻¹ and 9.6 ± 0.1 FU min⁻¹ for reactivation (●) and control (○), respectively. The final rates were similar, 2.2 ± 0.1 FU min⁻¹ and 1.6 ± 0.1 FU min⁻¹ for reactivation and control, respectively. The first-order rate constant of reactivation \( k_{\text{obs}} = (1.4 ± 0.1) \times 10^{-3} \text{ s}^{-1} \) was in the same range as the \( k_{\text{off}} \) value \((0.54 \times 10^{-3} \text{ s}^{-1})\) calculated from the data of the association experiments (Figure S1). (B) Reactivation of papain. The papain-catalyzed hydrolysis of Z-Phe-Arg-NHMec (10 µM) was monitored in 0.1 M sodium phosphate pH 6.5, 2.5 mM EDTA with final concentrations of 1 ng/mL papain, 50 pM 6, 1.4 % DMSO, 1.5 µM DTT. In the reactivation experiment, papain (0.2 µg/mL) was preincubated with 6 (10 nM) for 30 min at 25 °C. In the control experiment, the preincubation was done in the absence of the inhibitor. The initial rates were clearly different, 0.62 ± 0.01 FU min⁻¹ and 5.8 ± 0.1 FU min⁻¹ for reactivation (●) and control (○), respectively. The final rates were similar, 1.3 ± 0.1 FU min⁻¹ and 0.95 ± 0.01 FU min⁻¹ for reactivation and control, respectively. The first-order rate constant of reactivation \( k_{\text{obs}} = (1.1 ± 0.1) \times 10^{-3} \text{ s}^{-1} \) was in the same range as the \( k_{\text{off}} \) value \((0.40 \times 10^{-3} \text{ s}^{-1})\) calculated from the data of the association experiments.
Figure S4. (A) Congruent with Figure 2 of the publication. Time course of the chymotrypsin-catalyzed degradation of the azadipeptide nitrile 6 (•) and the dipeptide nitriles 12 (•) and 14 (•). Mixtures of the corresponding nitrile (600 µM) and chymotrypsin (100 µg/mL) in 20 mM Tris-HCl pH 8.4, 150 mM NaCl, 10 % acetonitrile were kept at 25 °C and 20 µL aliquots were injected into the HPLC. The pseudo first-order rate constant for the decay of 12 obtained by using the equation $c = c_0 \exp(-kt)$ was $0.015 \pm 0.001 \text{ min}^{-1}$, which corresponds to a half-life of 45 min. Linear regression of the data points for the hydrolysis of 14 gave an initial rate of $0.48 \pm 0.03 \text{ µM min}^{-1}$, which corresponds to a half-life of 14 h, assuming a pseudo first-order kinetics. (B) Time course of the chymotrypsin-catalyzed degradation of 12 (•) to form Z-Phe-OH (•). From the time course of the chymotrypsin-catalyzed hydrolysis of 12 and from the formation of Z-Phe-OH the same pseudo first-order rate constants of $0.015 \pm 0.001 \text{ min}^{-1}$ were calculated by using the equation $c = c_0 \exp(-kt)$. This corresponds to a half-life of 45 min. The control reaction in the absence of chymotrypsin is also shown (•).
Figure S5. Time course of the chymotrypsin-catalyzed cleavage of 6 (●) to form Z-Phe-OH (●). A mixture of 6 (600 µM) and chymotrypsin (1000 µg/mL) in 20 mM Tris-HCl pH 8.4, 150 mM NaCl, 10 % acetonitrile was kept at 25 °C and 20 µL aliquots were injected into the HPLC. Non-linear regression of the data points for the hydrolysis of 6 and the formation of Z-Phe-OH according to equation (3) gave steady-state rates of 0.57 ± 0.01 µM min⁻¹ and 0.51 ± 0.01 µM min⁻¹, respectively.
Scheme S1. The lower part is congruent with Scheme 2 of the publication. Synthesis of hydrazides 1-3 and azadipeptide nitriles 6 and 7. Reagents and conditions: a) HCl/MeOH, reflux; b) N$_2$H$_4$ × H$_2$O, MeOH, RT; c) BrCN, NaOAc, MeOH, RT; d) 1. N-methylmorpholine, isobutylchloroformate, THF, 2. methylhydrazine, –25 °C to RT; e) 1. N-methylmorpholine, isobutylchloroformate, THF, 2. dimethylhydrazine dihydrochloride, H$_2$O, 1 M NaOH, –25 °C to RT.
Scheme S2. Congruent with Scheme 3 of the publication. Synthesis of azadipeptide nitriles 8-10. Reagents and conditions: a) RCHO, THF, RT; b) 1. (CH$_3$)$_2$NH · BH$_3$, pTsOH, CH$_2$Cl$_2$, 4 °C; 2. 1.5 M NaOH, RT; c) BrCN, NaOAc, MeOH, RT.
Experimental Section

**General Methods and Materials.** Melting points were determined on a Büchi 510 oil bath apparatus and are not corrected. Thin layer chromatography was performed on Merck aluminium sheets. Preparative column chromatography was performed on silica gel 60 (Fluka) 70-230 mesh. Optical rotations were determined on a Perkin-Elmer 241 polarimeter. $^1$H NMR spectra (500 MHz) and $^{13}$C NMR spectra (125 MHz) were recorded on a Bruker Avance 500. Mass spectra were obtained on an API 2000 spectrometer from Applied Biosystems (ESI, sprayed from a $10^{-5}$ M solution in 2mM NH$_4$OAc/MeOH 1:1; volumetric flow rate 10 µL/min) and on a A.E.I. MS-50 spectrometer (EI, 70 eV). IR spectra were recorded on a Bruker Tensor 27 FT-IR spectrometer. Enzymatic activity of papain and cathepsins was measured spectrophotometrically at a Varian Cary Bio 50 UV/Vis spectrophotometer or fluorometrically at a Perkin Elmer luminescence spectrometer LS 50 B, respectively. Reactivation experiments were performed at a Perkin Elmer luminescence spectrometer LS 55. Analytical HPLC as performed on a Dionex HPLC system, equipped with a P580 A LPG gradient pump, a manual injection valve (20 µL), an UV/VIS detector UVD 170, and a Hypersil NC-04 column (RP-18, 5 µm, 250×4.60 mm). Papain was purchased from Sigma-Aldrich, Steinheim, Germany. Recombinant human cathepsin L, His Tag, and recombinant human cathepsin S were purchased from Calbiochem, Darmstadt, Germany. Recombinant human cathepsin K (expressed in *Pichia pastoris*) was a gift of D. Brömme.\(^1\) α-Chymotrypsin (bovine pancreas) was purchased from Fluka, Buchs, Switzerland. The substrates, Z-Phe-Arg-NHNp, Z-Phe-Arg-NHMec, Z-Val-Val-Arg-NHMec, Z-Leu-Arg-NHMec, and Suc-Ala-Ala-Pro-Phe-NHNp were from Bachem, Bubendorf, Switzerland. The amino acid derivatives were purchased from Novabiochem, Läufelfingen, Switzerland; Bachem, Bubendorf, Switzerland; and Fluka, Deisenhofen, Germany. DTT (= (±)-threo-2,3-dihydroxy-1,4-butanedithiol), Brij 35 P and Triton X-100 were obtained from Fluka, Deisenhofen, Germany; CHAPS (= 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) was from Sigma, Germany. Mathematical data analyses were done with the programs Grafit 4 (Erithacus Software) and GraphPad Prism 4 (GraphPad Software). N-(Benzyloxy carbonyl)-phenylalanlyglycine-nitrile (11) was prepared as described.\(^2\)

**Inhibition Assays. Cathepsins.** Enzyme activities were calculated from kinetic measurements performed by fluorimetric detection of the product AMC at 37 °C in a stirred cuvette. The wavelengths for excitation and emission were 360 nm and 440 nm, respectively. The reaction volume of the assay was 2 mL. To assay cathepsin L, Z-Phe-Arg-NHMec was
used as substrate at a concentration of 10 µM (= 7.07 $K_m$)\(^{[3]}\) in 100 mM sodium phosphate pH 6.0, 100 mM NaCl, 5 mM EDTA, 0.01 % Brij 35, 25 µM DTT and 1 % DMSO. For cathepsin S, Z-Val-Val-Arg-NHMe was chosen as substrate at a concentration of 40 µM (= 2.08 $K_m$)\(^{[2]}\) in 50 mM potassium phosphate pH 6.5, 50 mM NaCl, 2 mM EDTA, 0.01 % Triton X-100, 25 µM DTT and 1 % DMSO. In the cathepsin K assay, Z-Leu-Arg-NHMe was used as substrate at a concentration of 20 µM (= 3.23 $K_m$)\(^{[2]}\) in 100 mM sodium citrate pH 5.0, 100 mM NaCl, 1 mM EDTA, 0.01 % CHAPS, 25 µM DTT and 1 % DMSO. Stock solutions of the substrates and the inhibitors were prepared in DMSO. The enzyme dilutions were daily prepared from a stock with the corresponding assay medium without DMSO, containing 5 mM DTT (the complete amount of DTT required for the final concentration noted above), and kept on ice. After thermal equilibration, 10 µL of the enzyme solution were added and product formation was monitored over 5 min. The inhibition constants were calculated from measurements at ten to twelve different inhibitor concentrations and two or three controls in the absence of the inhibitor.

**Papain.** Enzyme activities were determined by spectrophotometric detection of the product $p$-nitroaniline (pNA) at 25 °C in a multi-cell holder (final volume 1 mL) at a wavelength of 405 nm. A 4 mM stock solution of the chromogenic substrate Z-Phe-Arg-NHNp was prepared in DMSO, the final concentration was 200 µM (= 0.207 $K_m$)\(^{[2]}\). The assay medium was 0.1 M sodium phosphate pH 6.5, 2.5 mM EDTA, 300 µM DTT and 12 % DMSO. Stock solutions of the inhibitors were prepared in DMSO. In the absence of inhibitor, 70 µL of DMSO were added to the cuvette. A papain stock solution was prepared in 1 mM HCl. For daily activation, the papain stock solution was diluted 1:100 in 0.1 M sodium phosphate pH 6.5, 2.5 mM EDTA, 15 mM DTT and incubated at 25 °C for 1 h. The activated enzyme was kept on ice. After thermal equilibration, the reaction was initiated by addition of the enzyme (20 µL), its final concentration catalyzed the conversion of the substrate with a rate of 1-2 µM/min. Progress curves were monitored over 10 min. Rates were determined for seven or eight different inhibitor concentrations in duplicate and two measurements in the absence of the inhibitor.

**Determination of Kinetic Parameters for the Inhibition of Cysteine Proteases.** The progress curves of the cysteine protease-catalyzed reactions in the presence of the dipeptide nitriles 11-14 were linear. The apparent inhibition constant $K_i'$ was determined by fitting

\[
v = v_0/(1+[I]/K_i')
\]  
\(1\)
to the experimental data, where \( v \) is the rate, \( v_0 \) is the rate in absence of inhibitor, and \([I]\) is the inhibitor concentration. The true inhibition constant \( K_i \) was calculated by correction of \( K_i' \) according to

\[
K_i = K_i'/(1+[S]/K_m)
\]  

(2)

where \([S]\) is the substrate concentration and \(K_m\) is the Michaelis constant.

Progress curves of the reactions of cysteine proteases in the presence of azadipeptide nitriles 6-10 were analyzed by non-linear regression using equation (3),

\[
[P] = v_s t + (v_i - v_s)(1 - \exp(-k_{obs} t))/k_{obs} + d
\]

(3)

where \([P]\) is the product concentration, \(v_s\) is the steady state rate, \(v_i\) is the initial rate, \(k_{obs}\) is the observed pseudo first-order rate constant and \(d\) is the offset. To obtain \(K_i'\) values, steady state rates together with the rate in the absence of the inhibitor were fitted according to equation (1), and \(K_i\) was calculated from equation (2). The apparent second-order rate constant \(k_{on}'\) was obtained by linear regression according to equation (4).

\[
k_{obs} = k_{on}' [I] + k_{off}
\]

(4)

The true rate constant \(k_{on}\) was calculated by correction of \(k_{on}'\) according to equation (5).

\[
k_{on} = k_{on}'(1+[S]/K_m)
\]

(5)

The first-order rate constant \(k_{off}\) for the dissociation of the enzyme-inhibitor complex was calculated according to equation (6).

\[
k_{off} = k_{on} K_i
\]

(6)

**Reactivation of Cathepsin L.** Twenty microliters of a solution of 6 (100 nM in DMSO) were added to 970 \( \mu \)L of 0.1 M sodium phosphate pH 6.0, 5 mM EDTA, 0.1 M sodium chloride and 0.01 % Brij 35. A cathepsin L stock solution (50 \( \mu \)g/mL in 20 mM sodium acetate pH 5.0, 100 mM sodium chloride, 10 mM trehalose, 1 mM EDTA and 50 % glycerol) was diluted 1:100 in 0.1 M sodium phosphate pH 6.0, 5 mM EDTA, 0.1 M sodium chloride,
0.01 % Brij 35, 5 mM DTT and kept at 25 °C for 30 min. After activation, the enzyme solution was diluted 1:5 with the same buffer. Ten microliters of the resulting enzyme dilution were added to the solution of 6. After preincubation for 30 min at 25 °C, 10 µL of the solution were added into a cuvette containing 980 µL of 0.1 M sodium phosphate pH 6.0, 5 mM EDTA, 0.1 M sodium chloride and 0.01 % Brij 35. The reaction was immediately initiated by addition of 10 µL of a solution of Z-Phe-Arg-NHMec (1 mM in DMSO). The reaction volume of the reactivation experiment was 1 mL. Final concentrations were as follows, 10 pg/mL of cathepsin L, 10 µM Z-Phe-Arg-NHMec, 20 pM of 6, 1.02 % DMSO and 0.5 µM DTT. The cathepsin L activity was followed for 70 min by fluorimetric detection of the product AMC. The wavelengths for excitation and emission were 360 nm and 490 nm, respectively. In the control measurement, the solution of 6 was replaced by DMSO. The reactivation was analyzed by fitting the data to equation (3) to determine \( k_{\text{obs}} \), the first-order rate constant of reactivation, \( v_i \), the initial rate, and \( v_s \), the final rate. The control reaction was analyzed by fitting the data to equation (3) to determine \( v_i \), the initial rate, and \( v_s \), the final rate.

**Reactivation of Papain.** Ten microliters of a solution of 6 (1 µM in DMSO) were added to 900 µL of 0.1 M sodium phosphate pH 6.5, 2.5 mM EDTA and 70 µL of DMSO. A papain stock solution (1 mg/mL in 1 mM HCl) was diluted 1:100 in 0.1 M sodium phosphate pH 6.5, 2.5 mM EDTA, 15 mM DTT and kept at 25 °C for 1 h. Twenty microliters of the activated enzyme solution were added to the solution of 6. After preincubation for 30 min at 25 °C, 5 µL of the solution were added into a cuvette containing 985 µL of 0.1 M sodium phosphate pH 6.5, 2.5 mM EDTA. The reaction was immediately initiated by addition of 10 µL of a solution of Z-Phe-Arg-NHMec (1 mM in DMSO). The reaction volume of the reactivation experiment was 1 mL. Final concentrations were as follows, 1 ng/mL of papain, 10 µM Z-Phe-Arg-NHMec, 50 pM of 6, 1.4 % DMSO, 1.5 µM DTT. The papain activity was followed for 100 min by fluorimetric detection of the product AMC as described above. In the control measurement, the solution of 6 was replaced by DMSO. The papain reactivation experiment was analyzed by the same mathematical method used for the cathepsin L reactivation experiment.

**Inhibition of Chymotrypsin.** Chymotrypsin activity was determined in the presence of compounds 6, 12, and 14. The assay was performed at 25 °C in a multi-cell holder at a wavelength of 405 nm in the presence of 9 % acetonitrile and 1 % DMSO (final volume 1 mL). The assay buffer was 20 mM Tris-HCl pH 8.4, 150 mM NaCl. A 20 mM stock solution of the chromogenic substrate Suc-Ala-Ala-Pro-Phe-NHNP was prepared in DMSO, the final concentration was 200 µM. Stock solutions of the inhibitors were prepared in acetonitrile. In
the absence of inhibitor, 90 µL of acetonitrile were added to the cuvette. A chymotrypsin solution (10 µg/ml) was prepared in 1 mM HCl and diluted with assay buffer. The reaction was initiated by addition of 40 µL of a chymotrypsin solution (250 ng/mL). Progress curves were monitored over 12 min. Rates were determined in duplicate for six different inhibitor concentrations (compound 6) or at 600 µM inhibitor concentration (compounds 12 and 14).

**Analysis of the Proteolytic Stability of Selected Inhibitors.** Separate solutions of compounds 6, 12, 14, and Z-Phe-OH in 20 mM Tris-HCl pH 8.4, 150 mM NaCl, 10 % acetonitrile were prepared at the following concentrations, 600, 500, 400, 300, 200, 100, 50, 25 µM. Solutions of compounds 6, 12, and Z-Phe-OH were kept in glass vials, whereas those of 14 were kept in quartz vials. Aliquots were injected into the HPLC, and isocratic elution conditions (water : acetonitrile : trifluoroacetic acid, 1 : 1 : 0.0016, v/v) and a flow rate of 1.5 mL/min were used. Detection was performed at a wavelength of 214 nm. Calibration curves were generated and linear regression analysis was carried to give the following slopes and standard errors, 0.0507 ± 0.0006 mAU min µM⁻¹ (6), 0.0436 ± 0.0004 mAU min µM⁻¹ (12), 0.0606 ± 0.0012 mAU min µM⁻¹ (14), 0.0394 ± 0.0010 mAU min µM⁻¹ (Z-Phe-OH). The compounds had the following retention times, 5.2 min (6), 4.3 min (12), 6.0 min (14), 3.7 min (Z-Phe-OH).

To determine the proteolytic stability towards chymotrypsin, to 800 µL of 20 mM Tris-HCl pH 8.4, 150 mM NaCl, 40 µL of acetonitrile and 60 µL of a 10 mM solution of the compounds 6, 12 and 14 in acetonitrile were added. At 25 °C, solutions of 6 and 12 were kept in glass vials, whereas those of 14 was kept in a quartz vial. Reactions were initiated by addition of 100 µL of 1 mg/mL solution of chymotrypsin. Final concentrations were as follows, 600 µM of each compound, 10 % acetonitrile, 100 µg/mL chymotrypsin. Aliquots were injected into the HPLC in 15 min intervals over 195 min. The HPLC conditions noted above were applied. In a second experiment, incubations of 6 were performed similarly, with a final chymotrypsin concentration of 1 mg/mL. Aliquots were injected in 30 min intervals over 360 min. The chymotryptic cleavage was analyzed by using the calibration curves.
N-(Benzyloxy carbonyl)-phenyl alanine methyl ester

On an ice-bath, acetyl chloride (0.62 mL, 8.7 mmol) was dropped to MeOH (10 mL). After 15 min, N-(benzyloxy carbonyl)-phenylalanine (2.0 g, 6.7 mmol) was added in portions. The solution was refluxed for 2 h and stirred for additional 2 h at room temperature. The solvent was evaporated, and the residue was dissolved in ethyl acetate (60 mL), washed with 10 % NaHSO₄ (10 mL), H₂O (10 mL), sat. NaHCO₃ (2 × 10 mL) and brine (10 mL) and dried (Na₂SO₄). The solvent was removed under reduced pressure to obtain N-(benzyloxy carbonyl)-phenylalanine methyl ester[4] (1.95 g, 93 %) as a colorless oil: ¹H NMR (500 MHz, CDCl₃) δ 3.07 (dd, ²J = 13.9 Hz, ³J = 6.0 Hz, 1H, PhCHHCH), 3.13 (dd, ²J = 13.9 Hz, ³J = 5.7 Hz, 1H, PhCHHCH), 3.70 (s, 3H, OCH₃), 4.62-4.68 (m, 1H, NHCHCO), 5.06 (d, ²J = 12.3 Hz, 1H, PhCHHO), 5.10 (d, ²J = 12.3 Hz, 1H, PhCHHCO), 5.22 (d, ³J = 7.9 Hz, 1H, NHCHCO), 7.06-7.10 (m, 2H, 2’-H, 6’-H), 7.20-7.37 (m, 8H, H_arom), ¹³C NMR (125 MHz, CDCl₃) δ 38.19 (PhCH₂CH), 52.28 (OCH₃), 54.77 (NHCHCO), 66.93 (PhCH₂O), 127.11 (C-4’), 128.05 (C-2”, C-6”), 128.15 (C-4’), 128.49, 128.57, 129.23 (C-2’, C-6’, C-3’, C-5’, C-3”, C-5”), 135.66, 136.22 (C-1’, C-1”), 155.59 (OCONH), 171.93 (CHCOOCH₃). C₁₈H₁₉NO₄ (313.35 g/mol).
**N-(Benzyloxycarbonyl)-phenylalanine hydrazide (I)**

Hydrazine hydrate (1.51 mL, 31.1 mmol) was added to a solution of **N**-(benzyloxycarbonyl)-phenylalanine methyl ester (1.90 g, 6.1 mmol) in MeOH (10 mL). A white product precipitated within 20 min. H$_2$O (20 ml) was added after 1 h, the precipitate was isolated by suction filtration, washed with H$_2$O, and dried in a desiccator over P$_4$O$_{10}$ to yield I (1.83 g, 96 %): mp 164-165 °C (lit.$^5$ mp 165 °C); $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 2.99-3.09 (m, 2H, PhCH$_2$CH), 3.40 (br s, 2H, NHN$_2$H$_2$), 4.30-4.39 (m, 1H, NHCHCO), 5.03 (d, $^2$J = 12.3 Hz, 1H, PhCHHO), 5.06 (d, $^2$J = 12.6 Hz, 1H, PhCHHO), 5.33 (d, $^3$J = 6.0 Hz, 1H, NHCHCO), 7.10-7.17 (m, 3H, 2'-H, 6'-H, CONH$_2$), 7.20-7.36 (m, 8H, H$_{arom}$); $^{13}$C NMR (125 MHz, CDCl$_3$): $\delta$ 38.41 (PhCH$_2$CH), 55.09 (NHCHCO), 67.23 (PhCH$_2$O), 127.22 (C-4'), 128.09 (C-2'', C-6''), 128.31 (C-4''), 128.57, 128.81, 129.14 (C-2', C-6', C-3', C-5', C-3'', C-5''), 135.93, 136.04 (C-1', C-1''), 155.91 (OCONH), 171.53 (CHCONH$_2$). Anal. C$_{17}$H$_{19}$N$_3$O$_3$ (313.35 g/mol) calcd C 65.16, H 6.11, N 13.41; found C 65.04, H 6.32, N 13.39.
\[
N-(\text{Benzyloxycarbonyl})-\text{phenylalanine 2-methylhydrazide (2)}
\]

\[
\begin{align*}
\text{N} & - (\text{Benzyloxycarbonyl}) - \text{phenylalanine (2.0 g, 6.68 mmol) was dissolved in THF (15 mL) and} \\
& \text{cooled to –25 °C. To the stirred solution, } N-\text{methylmorpholine (0.74 mL, 6.68 mmol) and} \\
& \text{isobutylchloroformate (0.88 mL, 6.68 mmol) were added consecutively. After precipitation of} \\
& N-\text{methylmorpholine hydrochloride, methyl hydrazine (1.78 mL, 33.4 mol) was added and the} \\
& \text{mixture was allowed to warm to room temperature within 30 min. It was stirred for additional} \\
& \text{90 min, and the solvent was removed under reduced pressure. The residue was dissolved in} \\
& \text{ethyl acetate (60 mL) and the solution was washed with H}_2\text{O (1 × 15 mL), sat. NaHCO}_3 \text{ (1 ×} \\
& \text{15 mL) and brine (1 × 15 mL). The solvent was dried (Na}_2\text{SO}_4 \text{) and evaporated. The resulting} \\
& \text{mixture of the regioisomers 2 and 3 was fractioned on silica gel using CH}_2\text{Cl}_2 / \text{MeOH (40:1)} \\
& \text{as eluent. Compound 2 (0.54 g, 29 %) was obtained as the second main fraction: mp 138-139} \\
& \text{°C (lit.[6] mp 138-140 °C); } ^1\text{H NMR (500 MHz, DMSO-}d_6) \delta 2.39 \text{ (s, 3H, CH}_3\text{NH), 2.79 (dd,} \\
& ^2J = 13.6 \text{ Hz, } ^3J = 9.8 \text{ Hz, 1H, PhCHHCH), 2.92 (dd, } ^2J = 13.6 \text{ Hz, } ^3J = 5.0 \text{ Hz, 1H,} \\
& \text{PhCHHCH), 3.30 (br s, 1H, NHNHCH}_3, 4.12-4.20 \text{ (m, 1H, NHCHCO), 4.94 (s, 2H,} \\
& \text{PhCH}_2\text{CO), 7.15-7.35 \text{ (m, 10H, H}_\text{arom}), 7.52 \text{ (d, } ^3J = 8.5 \text{ Hz, 1H, NHCHCO), 8.46 (s, 1H,} \\
& \text{CONHCH); } ^13\text{C NMR (125 MHz, DMSO-}d_6) \delta 37.74, 38.16 \text{ (PhCH}_2\text{CH, CH}_3\text{NH), 54.97} \\
& \text{(NHCHCO), 65.37 (PhCH}_2\text{O), 126.43 (C-4'), 127.59 (C-2''', C-6''), 127.80 (C-4'''), 128.18,} \\
& 128.40, 129.35 \text{ (C-2', C-6', C-3', C-5', C-3''', C-5'''}, 137.16, 137.89 \text{ (C-1', C-1'''}, 155.86 \text{ (OCONH),} \\
& 170.06 \text{ (CHCONHNH). Anal. C}_{18}\text{H}_{21}\text{N}_3\text{O}_3 \text{ (327.38 g/mol) calcd C 66.04, H 6.47,} \\
& \text{N 12.84; found C 66.14, H 6.74, N 12.71.}
\end{align*}
\]
Compound 3 was prepared by the aforementioned procedure and was isolated as the first main fraction of the column chromatography (1.78 g, 54 %): mp 121-122 °C (lit.[] mp 122-124 °C); $^1$H NMR (500 MHz, CDCl$_3$) mixture of cis/trans rotamers δ 2.85-3.01 (m), 3.04 (s) (Σ 5H, PhCH$_2$CH, CH$_3$N), 3.87 (br s, 2H, CONNH$_2$), 4.75-4.85 (m), 4.97-5.09 (m), 5.39-5.60 (m), 5.79 (d, $^3$J = 8.5 Hz) (Σ 4H, NHCHCO, PhCH$_2$O, NHCHCO), 7.05-7.35 (m, 10 H, H$_{arom}$); $^{13}$C NMR (125 MHz, CDCl$_3$) mixture of cis/trans rotamers, w = weak (refers to minor rotamer), i = intensive (refers to major rotamer) δ 37.42 (w), 38.56 (i) (PhCH$_2$CH), 39.47 (w), 39.95 (i) (CH$_3$N), 51.39 (i), 52.10 (w) (NHCHCO), 66.60 (i), 66.94 (w) (PhCH$_2$O), 126.72 (i), 127.16 (w) (C-4’), 127.91 (w), 127.96 (i) (C-2’, C-6’’), 128.11 (w), 128.18 (i), 128.42 (i), 128.47 (w) (C-2’, C-6’, C-3’, C-5’), 128.64 (i), 128.70 (w) (C-4’’), 129.28 (w), 129.45 (i) (C-3’’, C-5’’), 135.74 (w), 136.18 (w), 136.51 (i), 136.82 (i) (C-1’, C-1’’), 155.65 (w), 155.70 (i) (OCONH) 169.80 (w), 173.49 (i) (CHCONN$_2$). Anal. C$_{18}$H$_{21}$N$_3$O$_3$ (327.38 g/mol) calcd C 66.04, H 6.47, N 12.84; found C 66.24, H 6.81, N 12.53.
N-(Benzyloxy carbonyl)-phenylalanine 1,2-dimethylhydrazide (4)

N-(Benzyloxy carbonyl)-phenylalanine (2.0 g, 6.68 mmol) was dissolved in THF (15 mL) and cooled to –25 °C. To the stirred solution, N-methylmorpholine (0.74 mL, 6.68 mmol) and isobutylchloroformate (0.88 mL, 6.68 mmol) were added consecutively. 1,2-Dimethylhydrazine dihydrochloride (4.44 g, 33.4 mmol) was dissolved in H₂O (1 mL), and 5 M NaOH (13.4 mL) was added under ice-cooling. This solution was given to the reaction mixture when the precipitation of N-methylmorpholine hydrochloride occurred. It was allowed to warm to room temperature within 30 min and stirred for additional 90 min. After evaporation of the solvent, the resulting aqueous residue was extracted with ethyl acetate (1 × 40 mL, 3 × 10 mL). The combined organic layers were washed with H₂O (1 × 15 mL), sat. NaHCO₃ (2 × 15 mL), H₂O (1 × 15 mL), and brine (1 × 15 mL). The solvent was dried (Na₂SO₄) and evaporated to obtain a colorless oil which slowly solidified at room temperature (2.22 g, 97 %): mp 65-70 °C; ¹H NMR (500 MHz, CDCl₃) δ 2.45 (s, 3H, CH₃NH), 2.90 (dd, ²J = 13.3 Hz, ³J = 6.6 Hz, PhCHHCH), 2.98 (dd, ²J = 13.6 Hz, ³J = 7.0 Hz, 1H, PhCHHCH), 3.01 (s, 3H, CH₃N), 5.01 (d, ²J = 12.6 Hz, 1H, PhCHH), 5.06 (d, ²J = 12.3 Hz, 1H, PhCHH), 5.40-5.53 (m, 2H, NHCO, NHCH), 7.11-7.37 (m, 10H, H arom); ¹³C NMR (125 MHz, CDCl₃) δ 31.75, 35.39, 39.62 (CH₃NH, PhCH₂CH, CH₃N), 51.51 (NHCO), 66.57 (PhCH₂O), 126.66 (C-4'), 127.91 (C-2'', C-6''), 127.95 (C-4''), 128.21, 128.42, 129.46 (C-2', C-6', C-3', C-5', C-3'', C-5''), 136.51, 136.84 (C-1', C-1''), 155.72 (OCONH), 173.69 (OCONNH). Anal. C₁₉H₂₃N₃O₃ (341.40 g/mol) calcd C 66.84, H 6.79, N 12.31; found C 66.93, H 6.83, N 12.14.
**N-(Benzyloxycarbonyl)-leucine 1,2-dimethylhydrazide (5)**

![Chemical structure of N-(Benzyloxycarbonyl)-leucine 1,2-dimethylhydrazide](image)

In a separating funnel, N-(benzyloxycarbonyl)-leucine dicyclohexylamine salt (2.0 g, 4.4 mmol) was suspended in ethyl acetate (40 mL), and ice-cold 1 M H$_2$SO$_4$ (12 mL) was added. The aqueous layer was diluted with H$_2$O (15 mL) and extracted with ethyl acetate (2 × 20 mL). The combined organic layers were washed with H$_2$O (1 × 15 mL), brine (1 × 10 mL), dried (Na$_2$SO$_4$) and evaporated. The obtained N-(benzyloxycarbonyl)-leucin (1.3 g, 4.90 mmol) was dissolved in THF (10 mL) and cooled to –25 °C. To the stirred solution, N-methylmorpholine (0.54 mL, 4.90 mmol) and isobutyl chloroformate (0.64 mL, 4.90 mmol) were added consecutively. 1,2-Dimethylhydrazine dihydrochloride (3.26 g, 24.5 mmol) was dissolved in H$_2$O (1 mL), and 5 M NaOH (10 mL) was added under ice-cooling. This solution was given to the reaction mixture when the precipitation of N-methylmorpholine hydrochloride occurred. It was allowed to warm to room temperature within 30 min and stirred for additional 90 min. After evaporation of the solvent, the residue was suspended in H$_2$O (5 mL) and extracted with ethyl acetate (1 × 30 mL, 3 × 10 mL). The combined organic layers were washed with H$_2$O (2 × 15 mL), sat. NaHCO$_3$ (1 × 15 mL), H$_2$O (1 × 15 mL), and brine (1 × 15 mL). The solvent was dried (Na$_2$SO$_4$) and removed under reduced pressure. The oily residue was purified by column chromatography on silica gel with petroleum ether / ethyl acetate (1:1) as eluent to obtain 5 (1.13 g, 75 %) as a colorless oil: $^1$H NMR (500 MHz, CDCl$_3$) δ 0.90 (d, $^3J = 6.7$ Hz, 3H, CH$_3$CHCH$_3$), 0.96 (d, $^3J = 6.6$ Hz, 3H, (CH$_3$CHCH$_3$)$_2$, 1.37-1.45 (m, 1H, (CH$_3$)$_2$CHCH$_2$), 1.61-1.76 (m, 2H, CHCH$_2$CH), 2.66 (d, $^3J = 2.2$ Hz, 3H, CH$_3$NH), 3.07 (s, 3H, CH$_3$N), 3.34 (br s, 1H, NNHCH$_3$), 5.04 (d, $^2J = 12.3$ Hz, 1H, PhCHHO), 5.08 (d, $^2J = 12.3$ Hz, 1H, PhCHHO), 5.20 (td, $^3J = 10.0$ Hz, $^3J = 4.1$ Hz, 1H, NHCHCO), 5.37 (d, $^3J = 9.5$ Hz, 1H, NHCHCO), 7.25-7.34 (m, 5H, H$_{arom}$); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 21.61 (CH$_3$CHCH$_3$), 23.47 (CH$_3$CHCH$_3$), 24.82 ((CH$_3$)$_2$CHCH$_2$), 31.88 (CH$_3$N), 35.62 (CH$_3$NH), 42.73 (CHCH$_2$CH), 49.37 (NHCHCO), 66.62 (PhCH$_2$O), 127.96 (C-2’, C-6’), 128.10 (C-4’), 128.43 (C-3’, C-4’), 136.55 (C-1’), 156.24 (OCONH), 175.19 (CHCON). C$_{16}$H$_{25}$N$_3$O$_3$ (307.39 g/mol).
N-(Benzyloxycarbonyl)-phenylalanyl-methylazaalanine-nitrile (6)

Sodium acetate (0.34 g, 4.09 mmol) and cyanogen bromide (0.51 g, 4.83 mmol) were added to a solution of N-(benzyloxycarbonyl)-phenylalanine 1,2-dimethylhydrazide (4; 0.50 g, 1.46 mmol) in MeOH (20 mL). The mixture was stirred at room temperature for 5 h and the solvent was removed under reduced pressure. The residue was suspended in H$_2$O (10 mL), a pH of 1-2 was adjusted (10 % KHSO$_4$), and it was extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with H$_2$O (1 × 10 mL), sat. NaHCO$_3$ (2 × 10 mL), and brine (1 × 10 mL). The solvent was dried (Na$_2$SO$_4$) and removed in vacuo. The oily residue (0.48 g) was purified by column chromatography on silica gel using petroleum ether / ethyl acetate (2:1) as eluent. The obtained oil crystallized at room temperature after a few days. The crystals were separated and washed with petroleum ether and dried in a desiccator to obtain 6 (0.22 g, 41 %): mp 77-81 °C; [α]$_D^{20}$ = +15.8 (c = 1.35, MeOH); $^1$H NMR (500 MHz, CDCl$_3$) mixture of cis/trans rotamers (only the data of the major rotamer are disclosed) δ 2.88 (dd, $^2$J = 14.0 Hz, $^3$J = 8.4 Hz, 1H, PhCH/HCH), 3.11 (dd, $^2$J = 14.2 Hz, $^3$J = 5.4 Hz, 1H, PhCH/HCH), 3.19 (s, 3H), 3.23 (s, 3H) (2×CH$_3$N), 4.98 (d, $^2$J = 12.3 Hz, 1H, PhCHHO), 5.04 (d, $^2$J = 12.0 Hz, 1H, PhCHHO), 5.04- 5.10 (m, 1H, NHCHCO), 5.30 (d, $^3$J = 8.2 Hz, 1H, NHCHCO), 7.12-7.40 (m, 10H, H$_{arom}$); $^{13}$C NMR (125 MHz, CDCl$_3$) mixture of cis/trans rotamers, w = weak (refers to minor rotamer), i = intensive (refers to major rotamer) δ 29.99 (w), 30.43 (i), 38.37 (i), 39.63 (w), 40.61 (w), 41.19 (i) (CH$_3$NCN, PhCH$_2$CH, CH$_3$NCO), 51.74 (w), 51.96 (i) (NHCHCO), 67.02 (i), 67.16 (w) (PhCH$_2$O), 113.23 (w), 113.36 (i), (NCN), 127.34 (C-4'), 127.95 (C-2'', C-6''), 128.19 (C-4''), 128.51, 128.78, 129.20 (C-2’, C-6’, C-3’, C-5’, C-3’’, C-5’’), 135.32, 135.96 (C-1’, C-1’’), 155.98 (OCONH), 173.29 (i), 173.35 (w) (CHCONN); MS (ESI) m/z (rel. intensity) (pos.) 389 (47, [M + Na$^+$]), 384 (26, [M + NH$_4^+$]), 367 (100, [M + H$^+$]), 323 (42, [M – CO$_2$ + H$^+$]), (neg.) 365 (26, [M – H$^-$]), 230 (47, [M – BnOH – HCN – H$^-$]), 201 (100, [M – BnOH – CH$_3$NHNCN – H$^-$]); FTIR (KBr, cm$^{-1}$) 3392 (N-H), 2225 (C≡N), 1721 (C=O, OCONH), 1685 (C=O, CON), 1528 (C=O$_{arom}$), 1250 (C-N, OCONH). Anal. C$_{20}$H$_{22}$N$_4$O$_3$ (366.41 g/mol) calcd C 65.56, H 6.05, N 15.29; found C 65.72, H 6.14, N 15.12.
Sodium acetate (0.43 g, 5.21 mmol) and cyanogen bromide (0.22 g, 2.05 mmol) were added to a solution of \(N\)-(benzyloxy carbonyl)-leucine 1,2-dimethylhydrazide (5; 0.57 g, 1.86 mmol) in MeOH (20 mL). The mixture was stirred at room temperature for 2 h, additional cyanogen bromide (0.22 g, 2.05 mmol) was added and stirring was continued for 2 h. The solvent was evaporated and the residue was suspended in H\(_2\)O (10 mL). A pH of 1-2 was adjusted (10 % KHSO\(_4\)), it was extracted with ethyl acetate (1 \(\times\) 40 mL, 3 \(\times\) 30 mL), and the combined organic layers were washed with H\(_2\)O (1 \(\times\) 10 mL), sat. NaHCO\(_3\) (2 \(\times\) 10 mL), and brine (1 \(\times\) 10 mL). The solvent was dried (Na\(_2\)SO\(_4\)) and removed under reduced pressure. The oily crude product (0.70 g) was purified on silica gel with petroleum ether / ethyl acetate (2:1) as eluent to obtain \(7\) (0.46 g, 74 %) as colorless oil: \([\alpha]_D^{20} = +2.96\) (c = 1.01, MeOH); \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 0.95 (d, \(^3\)\(J\) = 6.6 Hz, 3H, CH\(_3\)CHCH\(_3\)), 1.01 (d, \(^3\)\(J\) = 6.3 Hz, 3H, CH\(_3\)CHCH\(_3\)), 1.47-1.53 (m, 2H, CHC\(_2\)H), 1.68-1.80 (m, 1H, CH\(_3\)CHCH\(_3\)), 3.18 (s, 3H), 3.23 (s, 3H) (2 \(\times\) CH\(_3\)N), 4.83 (td, \(^3\)\(J\) = 10.0 Hz, \(^3\)\(J\) = 5.0 Hz, 1H, NHCO), 5.03 (d, \(^2\)\(J\) = 12.0 Hz, 1H, PhCHHO), 5.09 (d, \(^2\)\(J\) = 12.3 Hz, 1H, PhCHHO), 5.20 (d, \(^3\)\(J\) = 8.9 Hz, NHCHCO), 7.27-7.36 (m, 5H, H\(_{arom}\)); \(^1^3\)C NMR (125 MHz, CDCl\(_3\)) \(\delta\) 21.31 (CH\(_3\)CHCH\(_3\)), 23.22 (CH\(_3\)CHCH\(_3\)), 24.79 ((CH\(_3\))\(_2\)CHCH\(_2\)), 30.44 (NCH\(_3\)), 40.99 (NCH\(_3\)), 41.52 (CHCH\(_2\)CH), 49.59 (NHCHCO), 67.04 (PhCH\(_2\)O), 49.59 (NHCHCO), 113.45 (NCN), 127.95 (C-2’, C-6’), 128.20 (C-4’), 128.53 (C-3’, C-4’), 136.02 (C-1’), 156.40 (OCONH), 174.63 (CHCON); MS (ESI) \(m/z\) (rel. intensity) (pos.) 350 (29, [M + NH\(_4\)]\(^+\)), 333 (100, [M + H]\(^+\)), 289 (13, [M – CO\(_2\) + H]\(^+\)), (neg.) 391 (26, [M + CH\(_3\)COO\(^-\)]), 331 (100, [M – H]), 223 (4, [M – BnOH – H]+). Anal. C\(_{17}\)H\(_{24}\)N\(_4\)O\(_3\) (332.40 g/mol) calcd C 61.43, H 7.28, N 16.86; found C 60.38, H 7.46, N 15.77.
N-(Benzyloxycarbonyl)-phenylalanine 1-methyl-2-benzylidenehydrazide

N-(Benzyloxycarbonyl)-phenylalanine 1-methylhydrazide (3; 0.5 g, 1.53 mmol) was dissolved in THF (10 mL), and benzaldehyde (0.15 mL, 1.53 mmol) was added. After stirring at room temperature for 4 h, one more equivalent (0.15 mL, 1.53 mmol) of benzaldehyde was added and stirring was continued for 1 h. The mixture was evaporated to dryness and the crude product was recrystallized from petroleum ether / ethyl acetate to yield a colorless solid (0.34 g, 53%): mp 128-130 °C; $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 3.03 (dd, $^2$J = 13.9 Hz, $^3$J = 6.9 Hz, 1H, PhCH/HCH), 3.17 (dd, $^2$J = 13.7 Hz, $^3$J = 5.5 Hz, 1H, PhCH/HCH), 3.34 (s, 3H, CH$_3$N), 5.04 (d, $^2$J = 12.3 Hz, 1H, PhCHO), 5.09 (d, $^2$J=11.9 Hz, PhCHO), 5.61 (d, $^3$J = 8.6 Hz, 1H, NH/CHO), 5.76-5.83 (m, 1H, NHCHO), 7.07-7.11 (m, 2H, H$_{arom}$), 7.12-7.20 (m, 3H, H$_{arom}$), 7.26-7.36 (m, 5H, H$_{arom}$), 7.38-7.45 (m, 3H, H$_{arom}$), 7.66 (s, 1H, N=CHPh); 7.67-7.71 (m, 2H, H$_{arom}$); $^{13}$C NMR $\delta$ 28.13, 39.22 (CH$_3$N, PhCH$_2$CH), 52.98 (NHCHO), 66.68 (PhCHO), 126.72, 127.34, 127.97, 128.24, 128.45, 128.80, 129.35, 130.07, 134.15, 136.52, 136.56 (C$_{arom}$). 149.21 (N=CHPh), 155.69 (OCONH), 172.73 10 (CHCON). One aromatic carbon signal could not be identified. Anal. C$_{25}$H$_{25}$N$_3$O$_3$ (415.48 g/mol) calcd C 72.27, H 6.06, N 10.11; found C 72.16, H 6.14, N 9.57.
**N-(Benzyloxycarbonyl)-phenylalanine 1-methyl-2-benzylhydrazide**

![Chemical Structure](image)

N-(Benzyloxycarbonyl)-phenylalanine 1-methyl-2-benzylidenehydrazide (0.5 g, 1.2 mmol) was dissolved in CH$_2$Cl$_2$ (5 mL). At 0 °C, a solution of $p$-toluenesulfonic acid (1.37 g, 7.2 mmol) in CH$_2$Cl$_2$ / MeOH (3:1; 10 mL) and dimethylamine borane (DMAB; 0.11 g, 1.92 mmol) were added. The mixture was allowed to react at room temperature for 1.5 h before additional 1.6 equivalents DMAB (0.11 g, 1.92 mol) were added. After 2 h, 1.5 M NaOH (10 mL) was added and stirring was continued for 30 min. The volume was reduced in vacuo, and the aqueous residue was extracted with CH$_2$Cl$_2$ (3 × 15 mL). The combined organic layers were washed with H$_2$O (1 × 15 mL) and brine (1 × 15 mL) and the solvent was dried (Na$_2$SO$_4$). It was evaporated and the residue was purified on silica gel using petroleum ether / ethyl acetate (2:1) as mobile phase to obtain a colorless oil (0.42 g, 84 %).

$^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 2.91 (dd, $^3J = 7.3$ Hz, $^5J = 2.2$ Hz, 2H, PhCH$_2$CH), 3.08 (s, 3H, CH$_3$N), 3.30 (s, 1H, NHCH$_2$), 3.74 (dd, $^2J = 11.8$ Hz, $^3J = 5.5$ Hz, 1H, PhCHNH), 3.87 (dd, $^2J = 11.5$ Hz, $^3J = 6.8$ Hz, 1H, PhCH$_2$NH), 5.02 (d, $^2J = 12.3$ Hz, 1H, PhCHO), 5.06 (d, $^2J = 12.7$ Hz, 1H, PhCHNH), 5.45 ($^3J = 9.2$ Hz, 1H, NHCHCO), 5.49-5.56 (m, 1H, NHCHCO), 7.10-7.38 (m, 15H, H$_{arom}$); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 32.96 (CH$_3$N), 39.97 (PhCH$_2$CH), 51.56 (NHCHCO), 52.95 (PhCH$_2$NH), 66.55 (PhCH$_2$O), 126.67, 127.91, 127.94, 128.21, 128.42, 128.63, 128.86, 129.03, 129.44, 135.91, 136.53, 136.83 (C$_{arom}$), 155.62 (OCONH), 173.92 (CHCON). C$_{25}$H$_{27}$N$_3$O$_3$ (417.21 g/mol).
N-(Benzyloxy carbonyl)-phenylalanyl-methylazaphenylalanine-nitrile (8)

Sodium acetate (0.22 g, 2.69 mmol) and cyanogen bromide (0.15 g, 1.44 mmol) were added to a solution of N-(benzyloxy carbonyl)-phenylalanine 1-methyl-2-benzylhydrazide (0.40 g, 0.96 mmol) in MeOH (10 mL). The mixture was stirred at room temperature for 24 h, three additional equivalents of cyanogen bromide (0.30 g, 2.88 mmol) were added and stirring was continued for 3 h. The solvent was removed under reduce pressure, and the oily residue was suspended in H₂O (5 mL). A pH of 1-2 was adjusted (10 % KHSO₄), it was extracted with ethyl acetate (5 × 15 mL), and the combined organic layers were washed with H₂O (1 × 15 mL), sat. NaHCO₃ (1 × 15 mL) and brine (1 × 15 mL). The solvent was dried (Na₂SO₄) and evaporated. The oily crude product (0.40 g) was purified on silica gel with petroleum ether / ethyl acetate (2:1) as the mobile phase to obtain 8 (0.25 g, 60 %) as a colorless oil: [α]D²⁰ = +47.1 (c = 1.03, MeOH); ¹H NMR (500 MHz, CDCl₃) δ 2.81-3.00 (m, 2H, PhCH₂N), 3.10 (s, 3H, CH₃N), 4.46-4.56 (m, 2H, PhCH₂N), 5.03 (d, ²J = 12.7 Hz, 1H, PhCHO), 5.06 (d, ³J = 12.6 Hz, 1H, PhCH₂O), 5.13-5.17 (m, 1H, NHCHO), 5.30 (d, ³J = 8.5 Hz, 1H, NHCH₂O), 7.11-7.45 (m, 15H, H arom); ¹³C (125 MHz, CDCl₃) δ 32.28 (CH₃C=O), 38.40 (PhCH₂CH), 52.42 (NHCH₂O), 59.06 (PhCH₂NCN), 67.01 (PhCH₂O), 112.24 (NCN), 127.27, 127.99, 128.19, 128.51, 128.73, 129.19, 129.31, 129.83, 130.21, 131.33, 135.37, 136.05 (C arom), 155.97 (OCNH), 173.48 (CHCON); MS (EI) m/z (rel. intensity) 442 (5, M⁺), 307 (3, [M – Bn – CO₂]⁺), 282 (5, [M – N(CH₃)₂N(CH₃Ph)CN]⁺), 91 (100, C₇H₇⁺). Anal. C₂₆H₂₆N₄O₃ (442.52 g/mol) calcd C 70.57, H 5.92, N 12.66; found C 70.81, H 6.49, N 11.69.
**N-(Benzyloxy carbonyl)-phenylalanine 1-methyl-2-phenylethylidenehydrazide**

N-(Benzyloxycarbonyl)-phenylalanine 1-methylhydrazide (3; 0.5 g, 1.53 mmol) was dissolved in THF (10 mL), and phenylacetaldehyde (0.17 mL, 1.53 mmol) was added. After stirring at room temperature for 4 h, one more equivalent (0.17 mL, 1.53 mmol) of phenylacetaldehyde was added and stirring was continued for 1 h. The mixture was evaporated to dryness and the residue was suspended in petroleum ether (5 mL). The product was isolated by suction filtration, washed with petroleum ether and dried in a desiccator to yield a colorless solid (0.55 g, 84%); mp 103-104 °C; $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 2.91 (dd, $^2$J = 13.6 Hz, $^3$J = 7.3 Hz, 1H, PhCH$_2$CH), 3.10 (dd, $^2$J = 13.7 Hz, $^3$J = 5.2 Hz, 1H, PhCH$_2$CH), 3.15 (s, 3H, CH$_3$N), 3.63 (d, $^3$J = 5.4 Hz, 2H, PhCH$_2$CH=N), 5.03 (d, $^2$J = 12.6 Hz, 1H, PhCH$_2$O), 5.07 (d, $^2$J = 12.3 Hz, 1H, PhCHHO), 5.54 (d, $^3$J = 8.8 Hz, 1H, NHCHCO), 5.60-5.67 (m, 1H, NHCHCO), 7.06-7.10 (m, 2H, H$_{arom}$), 7.15-7.36 (m, 14H, H$_{arom}$, CH$_2$CH=N); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 27.86 (NCH$_3$), 39.15 (PhCH$_2$CH), 39.47 (PhCH$_2$C=NC), 52.94 (NHCHCO), 66.62 (PhCH$_2$O), 126.67, 126.96, 127.95, 128.23, 128.43, 128.85, 128.97, 129.37, 136.45, 136.56, 136.73 (C$_{arom}$), 142.09 (CH=N), 155.68 (OCONH), 172.48 (CHCON). One aromatic carbon signal could not be identified. Anal. C$_{26}$H$_{27}$N$_3$O$_3$ (429.21 g/mol) calcd C 72.71, H 6.34, N 9.78; found C 72.83, H 6.42, N 9.26.
**N-(Benzyloxy carbonyl)-phenylalanine 1-methyl-2-phenylethylhydrazide**

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\text{N-(Benzyloxy carbonyl)-phenylalanine 1-methyl-2-phenylethylidenehydrazide (0.59 g, 1.37 mmol) was dissolved in CH}_2\text{Cl}_2 (5 \text{ mL}). \text{ At 0 °C, a solution of p-toluenesulfonic acid (1.56 g, 8.22 mmol) in CH}_2\text{Cl}_2 / \text{MeOH (3:1; 10 mL) and dimethylamine borane (DMAB; 0.13 g, 2.19 mmol) were added. The mixture was allowed to react at room temperature for 1.5 h. 1.5 M NaOH (10 mL) was added and stirring was continued for 30 min. The organic solvents were evaporated and the aqueous residue was extracted with CH}_2\text{Cl}_2 (3 \times 15 \text{ mL}). \text{ The combined organic layers were washed with H}_2\text{O (1 × 15 mL) and brine (1 × 15 mL) and dried (Na}_2\text{SO}_4). The solvent was removed in vacuo to obtain a colorless oil (0.39 g, 66 %):} \]

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1^H \text{ NMR (500 MHz, CDCl}_3) \delta 2.70 (dd, ^2J = 13.7 \text{ Hz, } ^3J = 7.4 \text{ Hz, 1H, PhCHHCH}), 2.80 (br s, 1H, NNHCH}_2), 2.85 (dd, ^2J = 12.9 \text{ Hz, } ^3J = 6.1 \text{ Hz, 1H, PhCHHCH}), 2.89-2.94 \text{ (m, 2H, PhCH}_2\text{CH}_2), 2.94 \text{ (s, 3H, NCH}_3), 2.96-3.07 \text{ (m, 2H, CH}_2\text{CH}_2\text{N}), 5.02 \text{ (d, } ^2J = 12.3 \text{ Hz, 1H, PhCHHO}), 5.07 \text{ (d, } ^2J = 12.0 \text{ Hz, 1H, PhCHHCO}), 5.41-5.52 \text{ (m, 2H, NHCHCO, NHCHCO), 7.00-7.40 \text{ (m, 15H, } H_{\text{arom}});} 1^3C \text{ NMR (125 MHz, CDCl}_3) \delta 32.58 \text{ (NCH}_3), 33.95 \text{ (NCH}_2\text{CH}_2), 39.73 \text{ (PhCH}_2\text{CH}), 49.22 \text{ (CH}_2\text{CH}_2\text{Ph), 51.56 \text{ (NHCHCO), 66.59 (PhCH}_2\text{O), 126.55, 126.67, 127.92, 127.98, 128.21, 128.44, 128.58, 128.65, 129.43, 136.52, 136.81, 138.55 (C}_{\text{arom}), 155.67 (OCONH), 173.85 (CHCONH).} \]

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C_{26}H_{29}N_3O_3 (431.53 \text{ g/mol}).
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N-(Benzyloxy carbonyl)-phenylalanyl-methylazahomophenylalanine-nitrile (9)

Sodium acetate (0.21 g, 2.52 mmol) and cyanogen bromide (0.14 g, 1.35 mmol) were added to a solution of N-(benzyloxy carbonyl)-phenylalanine 1-methyl-2-phenylethylhydrazide (0.39 g, 0.90 mmol) in MeOH (10 mL). The mixture was stirred at room temperature for 4 h, three additional equivalents of cyanogen bromide (0.29 g, 2.70 mmol) were added and stirring was continued for 24 h. The solvent was removed in vacuo, and the oily residue was suspended in H₂O (5 mL). A pH of 1-2 was adjusted (10 % KHSO₄), it was extracted with ethyl acetate (1 × 40; 3 × 10 mL), and the combined organic layers were washed with H₂O (1 × 10 mL), sat. NaHCO₃ (2 × 10 mL), H₂O (1 × 10 mL), and brine (1 × 10 mL). The solvent was dried (Na₂SO₄) and evaporated. The oily residue was purified by column chromatography on silica gel with petroleum ether / ethyl acetate (2:1) as eluent to obtain 8 (0.23 g, 56 %) as a colorless oil: [α]D²⁰ = +23.6 (c = 1.04, MeOH); ¹H NMR (500 MHz, CDCl₃) δ 2.73 (dd, ²J = 14.1 Hz, ³J = 8.4 Hz, 1H, PhCH₂HCH), 2.95 (dd, ²J = 14.2 Hz, ³J = 5.4 Hz, 1H, PhCH/HCH), 2.98-3.13 (m, 2H, PhCH₂CH₂), 3.15 (s, 3H, NCH₃), 3.68 (t, ³J = 7.3 Hz, 2H, NCH₂CH₂), 4.72-4.80 (m, 1H, NHCHCO), 4.99 (d, ²J = 12.3 Hz, 2H, PhCH/HO), 5.06 (d, ²J = 12.3 Hz, 1H, PhCH/HO), 5.14 (d, ³J = 8.5 Hz, 1H, NHCHCO), 6.98 (dd, ³J = 7.3 Hz, 4J = 1.6 Hz, 2H, H arom), 7.20-7.40 (m, 13H, H arom); ¹³C NMR (125 MHz, CDCl₃) δ 31.48 (NCH₃), 32.79 (NCH₂CH₂), 38.02 (PhCH₂CH₂), 51.84 (CH₂CH₂Ph), 54.92 (NHCHCO), 66.96 (PhCH₂O), 112.17 (NCN), 127.25, 127.98, 128.18, 128.50, 128.74, 128.86, 128.96, 129.15, 129.44, 135.13, 136.07, 136.08 (C arom), 155.88 (OCONH), 173.44 (CHCONH); MS (EI) m/z (rel. intensity) 456 (21, M⁺), 321 (14, [M - Bn - CO₂⁺]), 282 (40, [M - N(CH₃)₃N(CH₂CH₂Ph)CN⁺]), 91 (100, C₃H⁺). Anal. C₂₇H₂₆N₄O₃ (456.54 g/mol) calcd C 71.03, H 6.18, N 12.27; found C 71.12, H 6.53, N 11.50.
**N-(Benzyloxy carbonyl)-phenylalanine 1-methyl-2-pentylidenehydrazide**

N-(Benzyloxy carbonyl)-phenylalanine 1-methylhydrazide (3; 0.5 g, 1.53 mmol) was dissolved in THF (10 mL), and valeraldehyde (0.16 mL, 1.53 mmol) was added. After stirring at room temperature for 4 h, one more equivalent (0.16 mL, 1.53 mmol) of valeraldehyde was added and stirring was continued for 2 h. The mixture was evaporated to dryness to obtain a semisolid product (0.59 g, 98 %): $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 0.93 (t, $^3$J = 7.4 Hz, 3H, CH$_3$CH$_2$), 1.33-1.42 (m, 2H, CH$_3$CH$_2$), 1.49-1.58 (m, 2H, CH$_3$CH$_2$CH$_2$), 2.27-2.34 (m, 2H, CH$_2$CH$_2$CH), 2.89 (dd, $^2$J = 13.9 Hz, $^3$J = 7.1 Hz, 1H, PhCHHCH), 3.11 (dd, $^2$J = 13.9 Hz, $^3$J = 5.1 Hz, 1H, PhCHHCH), 3.16 (s, 3H, NCH$_3$), 5.01 (d, $^2$J = 12.6 Hz, 1H, PhCHHO), 5.05 (d, $^2$J = 12.3 Hz, 1H, PhCHHO), 5.55 (d, $^3$J = 9.2 Hz, 1H, NHCHCO), 5.58-5.64 (m, 1H, NHCHCO), 7.02 (t, $^3$J = 5.2 Hz, 1H, CH$_2$CH=N), 7.07-7.12 (m, 2H, H-2’, H-6’), 7.14-7.25 (m, 3H, H-3’, H-4’, H-5’), 7.25-7.37 (m, 5H, H-2”, H-6”’, H-3”, H-5”’, H-4”’); $^{13}$C NMR (125 MHz, CDCl$_3$): $\delta$ 13.86 (CH$_3$CH$_2$), 22.29 (CH$_3$CH$_2$), 27.70 (CH$_3$CH$_2$CH$_2$), 28.65 (CH$_2$CH$_2$CH), 32.54 (CH$_3$N), 39.04 (PhCH$_2$CH), 52.91 (NHCHCO), 66.57 (PhCH$_2$O), 126.61, 127.93, 128.17, 128.37, 128.41, 129.37, 136.59, 136.81 (C$_{arom}$), 143.94 (CH$_2$CH=N), 155.68 (OCONH), 172.35 (CHCON). C$_{23}$H$_{29}$N$_3$O$_3$ (395.49 g/mol).
N-(Benzyloxy carbonyl)-phenylalanine 1-methyl-2-pentylhydrazide

N-(Benzyloxy carbonyl)-phenylalanine 1-methyl-2-pentylidenehydrazide (0.58 g, 1.47 mmol) was dissolved in CH₂Cl₂ (5 mL). At 0 °C, a solution of p-toluenesulfonic acid (1.37 g, 7.2 mmol) in CH₂Cl₂ / MeOH (3:1; 10 mL) and dimethylamine borane (DMAB; 0.14 g, 2.36 mmol) were added. After stirring at room temperature for 1.5 h, one more of dimethylamine borane (0.14 mg, 2.36 mmol) was added. After stirring for 2 h, 1.5 M NaOH (10 mL) was added and stirring was continued for 30 min. The organic solvents were evaporated and the aqueous residue was extracted with CH₂Cl₂ (3 × 15 mL). The combined organic layers were washed with H₂O (1 × 15 mL) and brine (1 × 15 mL) and dried (Na₂SO₄). The solvent was removed in vacuo to obtain a colorless oil (0.55 g, 95 %): ¹H NMR (500 MHz, CDCl₃) δ 0.88 (t, ³J = 6.6 Hz, 3H, CH₃CH₂), 1.22-1.31 (m, 2H, CH₃CH₂), 1.32-1.42 (m, 2H, CH₃CH₂CH₂), 2.50-2.61 (m, 2H, CH₂CH₂NH), 2.68-2.75 (m, 1H, PhCH₂HCH), 2.76-2.81 (m, 1H, PhCH₂HCH), 2.88-3.00 (m, 2H, NHCH₂), 3.00 (s, 3H, NHCH₂), 5.01 (d, ²J = 12.3 Hz, 1H, PhCHHO), 5.06 (d, ²J = 12.3 Hz, 1H, PhCHHO), 5.41-5.54 (m, 2H, NHCH₂CO, NHCH₂CO), 7.09-7.36 (m, 10H, H_arom); ¹³C NMR (125 MHz, CDCl₃) δ 13.93 (CH₂CH₂), 22.52 (CH₂CH₂), 27.24 (CH₂CH₂CH₂), 29.23 (CH₂CH₂NH), 32.54 (CH₃N), 39.76 (PhCH₂CH), 48.35 (CH₂NH), 51.61 (NHCH₂CO), 66.53 (PhCH₂O), 126.64, 127.12, 127.89, 128.17, 128.41, 129.46, 136.55, 136.94 (C_arom), 155.63 (OCONH), 173.81 (CHCON). C₂₃H₃₁N₃O₃ (397.51 g/mol).
Sodium acetate (0.23 g, 2.83 mmol) and cyanogen bromide (0.16 g, 1.51 mmol) were added to a solution of \(N\)-(benzyloxy carbonyl)-phenylalanine 1-methyl-2-pentylhydrazide (0.40 g, 1.01 mmol) in MeOH (10 mL). After stirring at room temperature for 4 h, additional three equivalents of cyanogen bromide (0.29 g, 2.70 mmol) were added and stirring was continued for 20 h. The solvent was removed in vacuo, and the oily residue was suspended in H\(2\)O (5 mL). A pH of 1-2 was adjusted (10 % KHSO\(4\)), it was extracted with ethyl acetate (1\( \times \)40; 3\( \times \)10 mL), and the combined organic layers were washed with H\(2\)O (1\( \times \)10 mL), sat. NaHCO\(3\) (2\( \times \)10 mL), H\(2\)O (1\( \times \)10 mL), and brine (1\( \times \)10 mL). The solvent was dried (Na\(2\)SO\(4\)) and evaporated. The crude product (0.53 g) was purified by column chromatography on silica gel with petroleum ether / ethyl acetate (2:1) as eluent to obtain 10 (0.28 g, 65 %) as a colorless oil: \([\alpha]_D^{20} = +21.2 \) (c = 1.08, MeOH); \(^1\)H NMR (500 MHz, CDCl\(3\)) \(\delta 0.91 \) (t, \(J = 6.9 \) Hz, 3H, \(\text{CH}_3\text{CH}_2\)), 1.33-1.45 (m, 4H, \(\text{CH}_3\text{CCH}_2\text{CCH}_2\)), 1.70-1.79 (m, 2H, \(\text{CH}_2\text{CH}_2\text{N}\)), 2.85 (dd, \(J = 13.9\) Hz, 3\(J = 8.5\) Hz, 1H, PhCHHCH), 3.13 (dd, \(J = 14.4\) Hz, \(J = 4.9\) Hz, 1H, PhCHHCH), 3.20 (s, 3H, NCH\(_3\)), 3.22-3.32 (m, 1H, CH\(_2\)CHHN), 3.40-3.48 (m, 1H, CH\(_2\)CHHN), 4.98 (d, \(J = 12.3\) Hz, 1H, PhCHHO), 5.03 (dd, \(J = 12.0\) Hz, 1H, PhCHHO), 5.01-5.07 (m, 1H, NHCHCO), 5.29-5.34 (d, \(J = 8.9\) Hz, 1H, NHCHCO), 7.15-7.20 (m, 2H, H-2’, H-6’), 7.21-7.35 (m, 8H, H\(_{\text{arom}}\)); \(^{13}\)C NMR (125 MHz, CDCl\(3\)) \(\delta 13.79 \) (C\(_{\text{HCH}}\)), 22.26 (CH\(_3\)CH\(_2\)), 26.26 (CH\(_3\)CH\(_2\)CH\(_2\)), 28.54 (CH\(_2\)CH\(_2\)N), 31.45 (CH\(_3\)N), 38.29 (PhCH\(_2\)CH), 52.22 (NHCHCO), 54.05 (CH\(_2\)CH\(_2\)N), 66.92 (PhCH\(_2\)O), 112.39 (NCN), 127.45, 127.92, 128.13, 128.47, 128.72, 129.19 (C\(_{\text{arom}}\)), 135.49, 136.03 (C-1’, C-1’’), 155.87 (OCONH), 173.39 (CHCON); MS (EI) \(m/z\) (rel. intensity) 422 (21, \(M^+\)), 287 (4, \([M – \text{Bn} – \text{CO}_2]^+\)), 282 (21, \([M – \text{N(CH}_3)_2\text{N(CH}_2\text{CH}_2\text{CH}_2\text{CH}_3\text{CN}]^+\)), 91 (100, C\(_7\)H\(_7^+\)). Anal. C\(_{24}\)H\(_{30}\)N\(_4\)O\(_3\) (422.52 g/mol) calcd C 68.22, H 7.16, N 13.26; found C 67.80, H 7.16, N 13.16.
**N-(tert-Butoxycarbonyl)-alanine-amide**

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\text{O} \quad \text{N} \quad \text{H} \\
\text{O} \quad \text{N} \quad \text{C} \quad \text{NH}_2
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*N-(tert-Butoxycarbonyl)-alanine* (1.0 g, 5.29 mmol) was dissolved in THF (10 mL) and cooled to –25 °C. To the stirred solution, *N*-methylmorpholine (0.59 mL, 5.29 mmol) and isobutylchloroformate (0.69 mL, 5.29 mmol) were added consecutively followed by 25 % NH₃ (1.8 mL, 26.45 mmol). The resulting mixture was stirred at room temperature for 2 h and evaporated. H₂O (10 mL) was added, a pH of 1-2 was adjusted (10 % KHSO₄), and the aqueous mixture was extracted with ethyl acetate (4 × 15 mL). The combined organic layers were washed with H₂O (1 × 15 mL), sat. NaHCO₃ (2 × 15 mL), and brine (1 × 15 mL). The solvent was dried (Na₂SO₄) and evaporated to obtain a colorless solid (0.82 g, 82 %): mp 124-125 °C (lit.¹ mp 124-125 °C); ¹H NMR (500 MHz, CDCl₃) δ 1.35 (d, 3J = 7.3 Hz, 3H, CH₂C₃H₇), 1.42 (s, 9H, C(C₃H₇)₃), 4.18 (br s, 1H, NHC₃H₂CO), 5.04 (br s, 1H, CONHCH), 5.62 (s, 1H, CONH), 6.22 (s, 1H, CONHH); ¹³C NMR (125 MHz, CDCl₃) δ 18.19 (CH₂C₃H₇), 28.30 (C(CH₃)₃), 49.61 (NHCHCO), 80.23 (C(CH₃)₃), 155.54 (OCONH), 175.16 (CHCONH₂). Anal. C₈H₁₆N₂O₃ (188.23 g/mol) calcd C 51.05, H 8.57, N 14.88; found C 50.67, H 8.93, N 14.42.
Alanine-amide hydrochloride

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\text{H}_2\text{N} \overset{\text{O}}{\text{C}} \text{NH}_2 \times \text{HCl}
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Under ice-cooling, acetyl chloride (10.95 ml, 154.13 mmol) was added to dropwise to EtOH (10 ml). To the stirred mixture, a solution of \(N\)-(tert-Butoxycarbonyl)-alanine-amide (0.69 g, 3.66 mmol) in ethyl acetate (10 ml) was added. After 1 h, the formed precipitate was separated by suction filtration, washed with ethyl acetate (5 mL) and dried in a desiccator to obtain a colorless solid (0.40 g, 87%): mp 215-217 °C (lit.\[8\] mp 196-199 °C); \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\) 1.36 (d, \(^3J = 7.3\) Hz, 3H, \(\text{CH}_3\)), 3.75 (q, \(^3J = 7.0\) Hz, 1H, \(\text{CHCO}\)), 7.41 (s, 1H, \(\text{CONH}\)), 7.92 (s, 1H, \(\text{CONHH}\)), 8.25 (s, 3H, \(\text{NH}_3^+\)); \(^{13}\)C NMR (125 MHz, DMSO-\(d_6\)) \(\delta\) 17.18 (\(\text{CH}_3\)), 48.22 (\(\text{CHCO}\)), 171.42 (CO) Anal. \(\text{C}_3\text{H}_8\text{N}_2\text{O} \times \text{HCl}\) (124.57 g/mol) calcd C 28.93, H 7.28, N 22.49; found C 28.65, H 7.57, N 21.87.
N-(Benzyloxy carbonyl)-phenylalanyl-alanine-amide

N-(Benzyloxy carbonyl)-phenylalanine (0.87 g, 2.92 mmol) was dissolved in THF (10 mL) and cooled to –25 °C. To the stirred solution, N-methylmorpholine (0.33 ml, 2.92 mmol) and isobutylchloroformate (0.38 ml, 2.92 mmol) were added consecutively. 1 M NaOH (3.2 mL) was added to alanine-amide hydrochloride (0.36 g, 2.92 mmol) dissolved H₂O (5 mL), and the resulting solution was added to the reaction mixture, which was stirred at room temperature for 2 h. The organic solvent was evaporated and the residue was suspended in H₂O (5 mL), separated by suction filtration, washed with 10 % KHSO₄ (15 mL), sat. NaHCO₃ (15 mL) and H₂O (3 × 15 mL) and dried in a desiccator to obtain a colorless solid (0.83 g, 77 %): mp 223-224 °C (lit.[⁹] mp 212-214 °C); ¹H NMR (500 MHz, DMSO-d₆) δ 1.22 (d, ³J = 7.0 Hz, 3H, CH₃), 2.73 (dd, ²J = 10.9 Hz, ³J = 2.8 Hz, 1H, PhCHCH), 3.03 (dd, ²J = 13.8 Hz, ³J = 3.8 H, 1H, PhCHHCH), 4.24-4.28 (m, 2H, NHCHCONH₂, NHCHCONH), 4.94 (s, 2H, PhCH₂O), 6.98 (s, 1H, NHCHCONH), 7.17-7.48 (m, 11H, NHCHCONH₂, 10H, H_arom), 7.47 (d, 1H, ²J = 8.5 Hz, CON/H), 8.00 (d, 1H, ²J = 7.3 Hz, CON/HH); ¹³C NMR (125 MHz, CDCl₃) δ 18.61 (CH₃), 37.49 (PhCH₂CH), 48.14 (NHCHCONH₂), 56.26 (NHCHCONH), 65.34 (PhCH₂O), 126.35 (C-4’), 127.50 (C-2”, C-6”), 127.77 (C-4”), 128.16, 128.41, 129.32 (C-2’, C-6’, C-3’, C-5’, C-3”, C-5”), 137.16, 138.25 (C-1’, C-1”), 155.98 (OCONH), 171.12 (CHCONH), 174.13 (CHCONH₂). Anal. C₂₀H₂₃N₃O₄ (369.42 g/mol) calcd C 65.03, H 6.28, N 11.37; found C 65.02, H 6.31, N 11.01.
Cyanuric chloride (0.30 g, 1.62 mmol) was added to a solution of N-(benzyloxycarbonyl)-phenylalanyl-alanine-amide (0.60 g, 1.62 mmol) in DMF (10 mL). After stirring for 2 h at room temperature, DMF was evaporated. Ice-cold sat. NaHCO\textsubscript{3} (10 mL) was added and the mixture was extracted with ethyl acetate (4 × 15 mL). The combined organic layers were washed with H\textsubscript{2}O (3 × 15 mL) and brine (1 × 15 mL), dried (Na\textsubscript{2}SO\textsubscript{4}), and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel with petroleum ether / ethyl acetate (1:1) as eluent to obtain 12 (0.45 g, 79 %) as a colorless solid: mp 143-145 °C; [$\alpha$]\textsubscript{D}\textsuperscript{20} = −28.5 (c = 1.00, MeOH); \textsuperscript{1}H NMR (500 MHz, DMSO-\textit{d}_6) δ 1.42 (d, $^3J = 7.3$ Hz, 3H, CH\textsubscript{3}), 2.78 (dd, $^2J = 10.3$ Hz, $^3J = 3.5$ Hz, 1H, PhCHHCH), 2.96 (dd, $^3J = 13.6$ Hz, $^2J = 4.8$ Hz, 1H, PhCHHCH), 4.20-4.25 (m, 1H, NHCCO), 4.75 (quint, $^3J = 7.2$ Hz, 1H, NHCHCN), 4.92 (d, $^2J = 12.9$ Hz, 1H, PhCHHO); 4.96 (d, $^2J = 12.6$ Hz, 1H, PhCHHO), 7.18-7.34 (m, 12H, NHCHCN, NHCHCO, 10H\textsubscript{arom}); \textsuperscript{13}C NMR (125 MHz, CDCl\textsubscript{3}) δ 18.26 (CH\textsubscript{3}), 35.73 (PhCH\textsubscript{2}CH), 37.39 (NHCHCN), 56.01 (NHCHCO), 65.42 (PHCH\textsubscript{2}O), 120.19 (CHCN), 126.49 (C-4’), 127.58 (C-2’’, C-6’’), 127.82 (C-4’’), 128.22, 128.41, 129.31 (C-2’, C-6’, C-3’, C-5’, C-3’’, C-5’’), 137.09, 137.78 (C-1’, C-1’’), 155.96 (OCONH), 171.52 (CHCONH). MS (ESI) \textit{m/z} (rel. intensity) (pos.) 374 (10, [\textit{M} + Na\textsuperscript{+}]), 369 (100, [\textit{M} + NH\textsubscript{4}\textsuperscript{+}]), 352 (93, [\textit{M} + H\textsuperscript{+}]), (neg.) 350 (7, [\textit{M} – H\textsuperscript{−}]), 242 (100, [\textit{M} – BnOH – H\textsuperscript{−}]), 215 (26, \textit{M} – BnOH – HCN – H\textsuperscript{−}). Anal. C\textsubscript{20}H\textsubscript{21}N\textsubscript{3}O\textsubscript{3} (351.40 g/mol) calcd C 68.36, H 6.02, N 11.96; found C 68.25, H 5.91, N 11.66.
**N-(tert-Butoxycarbonyl)-methylalanine-amide**

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\text{O} \quad \text{N} \quad \text{NH}_2
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*N-(tert-Butoxycarbonyl)-methylalanine* (1.0 g, 4.92 mmol) was dissolved in THF (10 mL) and cooled to –25 °C. To the stirred solution, *N*-methyImorpholine (0.54 mL, 4.92 mmol) and isobutylchloroformate (0.64 mL, 4.92 mmol) were added consecutively, followed by 25 % NH₃ (1.68 mL, 24.6 mmol). The resulting mixture was stirred at room temperature for 2 h and the organic solved was removed under reduced pressure. The formed precipitate was suspended in H₂O (10 mL), a pH of 1-2 was adjusted (10 % KHSO₄), and the aqueous mixture was extracted with ethyl acetate (4 × 15 mL). The combined organic layers were washed with H₂O (1 × 15 mL), sat. NaHCO₃ (2 × 15 mL), and brine (1 × 15 mL). The solvent was dried (Na₂SO₄) and evaporated to obtain a colorless solid (0.92 g, 92 %): mp 66-68 °C; ¹H NMR (500 MHz, CDCl₃) δ 1.31 (d, ³J = 7.0 Hz, 3H, CH₃), 1.44 (s, 9H, C(CH₃)₃), 2.77 (s, 3H, NC₃H₃), 4.73 (br s, 1H, CHCO), 5.68 (s, 1H, CONH), 6.08 (s, 1H, CONNH); ¹³C NMR (125 MHz, CDCl₃) δ 13.50 (CH₃), 28.34 (C(CH₃)₃), 29.84 (NCH₃), 53.20 (CHCO), 80.67 (C(CH₃)₃), 156.39 (OCONH), 174.16 (CHCONH₂). Anal. C₉H₁₈N₂O₃ (202.26 g/mol) calcd C 53.45, H 8.97, N 13.85; found C 53.15, H 8.74, N 13.36.
Methylalanine-amide hydrochloride

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\begin{align*}
\text{N} & \text{H} \\
\text{C} & \text{O} \\
\text{N} & \text{H}_2 \\
\text{x HCl}
\end{align*}
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Under ice-cooling, acetyl chloride (10.95 ml, 154.13 mmol) was added to dropwise to EtOH (10 ml). To the stirred mixture, a solution of \(N\)-(tert-Butoxycarbonyl)-methylalanine (0.74 g, 3.66 mmol) in ethyl acetate (10 ml) was added. After 1 h, the precipitate was separated by suction filtration, washed with ethyl acetate (5 mL) and dried in a desiccator to obtain a colorless solid (0.37 g, 73 %): mp 192-194 °C (lit.\[^{[10]}\] mp 158 °C); \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\) 1.39 (d, \(^3 J = 7.3 \text{ Hz}, 3\text{H}, \text{CHCH}_3\)), 2.45 (s, 3H, NCH\(_3\)), 3.71 (q, \(^3 J = 7.0 \text{ Hz}, 1\text{H}, \text{CHCO}\)), 7.55 (s, 1H CONH), 7.55 (s, 1H CONH\(_2\)), 9.18 (s, 2H, NH\(_2^+\)); \(^{13}\)C NMR (125 MHz, DMSO-\(d_6\)) \(\delta\) 15.74 (CH\(_3\)), 30.78 (NCH\(_3\)), 56.09 (CHCO), 170.52 (CONH\(_2\)). Anal. C\(_4\)H\(_{10}\)N\(_2\)O \(\times\) HCl (138.06 g/mol) calcd C 34.66, H 8.00, N 20.21; found C 34.41, H 7.74, N 19.35.
N-(Benzyloxy carbonyl)-phenylalanyl-methylalanine-amide

N-(Benzyloxy carbonyl)-phenylalanine (0.73 g, 2.44 mmol) was dissolved in THF (10 mL) and cooled to –25 °C. To the stirred solution, N-methylmorpholine (0.27 ml, 2.44 mmol) and isobutylchloroformate (0.32 ml, 2.44 mmol) were added consecutively. 1 M NaOH (2.68 mL) was added to methylalanine-amide hydrochloride (0.37 g, 2.68 mmol) dissolved H₂O (5 mL), and the resulting solution was added to the reaction mixture, which was stirred at room temperature for 2 h. The organic solvent was evaporated, the residue was suspended in H₂O (5 mL) and a pH of 1-2 was adjusted (10 % KHSO₄). The aqueous mixture was extracted with ethyl acetate (4 × 15 mL), the combined organic layers were washed with H₂O (1 × 15 mL), sat. NaHCO₃ (2 × 15 mL) and brine (1 × 15 mL), dried (Na₂SO₄) and evaporated to dryness. The crude product was purified by column chromatography on silica gel with CH₂Cl₂ / MeOH (20:1) as eluent to obtain a colorless solid (0.45 g, 51 %): mp 54-58 °C; ¹H NMR (500 MHz, CDCl₃) mixture of cis/trans rotamers δ 0.53, 1.23 (d, ³J = 7.0, 7.0 Hz, Σ 3H, CH₃), 2.679, 2.684 (s, Σ 3H, NCH₃), 2.95-3.08 (m, Σ 2H, PhCH₂CH₂), 4.42 (q, ³J = 6.7 Hz, 1H, CHCONH₂), 4.77-4.81, 4.88-4.93 (m, Σ 1H, NHCHCO), 5.27, 5.45 (s, Σ 2H, PhCH₂O), 5.68 (d, ²J = 8.2 Hz, 1H, NHCHCO), 7.16-7.35 (m, Σ 12H, Hₐrom, CONH₂); ¹³C NMR (125 MHz, CDCl₃) mixture of cis/trans rotamers, w = weak (refers to minor rotamer), i = intensive (refers to major rotamer) δ 13.02 (i), 13.07 (w) (CHCH₃), 28.63 (i), 30.82 (w) (NCH₃), 38.92 (w), 39.54 (i) (PhCH₂CH₂), 51.80 (w), 51.94 (i) (CH₂CONH₂), 52.46 (w), 55.34 (i) (NHCHCO), 66.99 (w), 67.42 (i) (PhCH₂O), 127.31 (w), 127.50 (i) (C-4’), 127.97 (w), 128.00 (i) (C-2’, C-6’), 128.19 (i), 128.31 (w) (C-4”), 128.52 (w), 128.54 (i) (C-3”, C-5’”), 128.74 (w), 128.96 (i) (C-3’, C-5’), 129.28 (w), 129.40 (i) (C-2’, C-6’), 135.53 (i), 135.73 (w), 136.00 (i), 136.16 (w) (C-1’, C-1”’), 155.71 (w), 156.79 (i) (OCONH), 171.80 (w), 171.84 (i), 172.26 (w), 172.38 (i) (CONH₂, CHCONH). Anal. C₂₁H₂₅N₃O₄ (383.45 g/mol) calcd C 65.78, H 6.57, N 10.96; found C 65.72, H 6.71, N 10.54.
Z-Phe-OH (0.72 g, 2.40 mmol) was dissolved in THF (10 mL) and the solution was cooled to −25 °C. N-Methylmorpholine (0.27 mL, 2.40 mmol) and isobutylchloroformate (0.31 mL, 2.44 mol) were added consecutively. 1 M NaOH (2.64 mL) was added to N-methylaminoacetonitrile hydrochloride (0.28 g, 2.64 mmol) dissolved in H₂O (5 mL), and the resulting solution was added to the reaction mixture, which as stirred at room temperature for 2 h. The organic solvent was evaporated, the residue was suspended in H₂O (5 mL) and a pH of 1-2 was adjusted (10 % KHSO₄). The aqueous mixture was extracted with ethyl acetate (3 × 20 mL), the combined organic layers were washed with H₂O (1 × 15 mL), sat. NaHCO₃ (2 × 15 mL) and brine (1 × 15 mL), dried (Na₂SO₄) and evaporated to dryness. The crude product was purified by column chromatography on silica gel with petroleum ether / ethyl acetate (2:1) as eluent to obtain 13 (0.40 g, 34 %) as a colorless solid: mp 82-84 °C; [α]D²⁰ = −3.1 (c = 1.81, MeOH); ¹H NMR (500 MHz, CDCl₃) δ 2.69 (s, 3H, NC₃H₃), 2.97 (dd, 2J = 13.1 Hz, 3J = 9.0 Hz, 1H, PhCHCH), 3.03 (dd, 2J = 12.9 Hz, 3J = 5.7 Hz, 1H, PhCHHCH), 3.94 (d, 2J = 17.1 Hz, 1H, NCH₂CN), 4.46 (d, 2J = 17.1 Hz, NCHHCN), 4.82-4.89 (m, 1H, NHCCH), 5.05 (d, 2J = 12.3 Hz, 1H, PhCHHO), 5.09 (d, 2J = 12.3 Hz, 1H, PhCHH/IO), 5.59 (d, 3J = 8.2 Hz, 1H, NHCHO), 7.16 (d, 3J = 6.9 Hz, 2H, H-2', H-6'), 7.22-7.37 (m, 8H, arom); ¹³C NMR (125 MHz, CDCl₃) δ 35.93 (NCH₂CN), 35.36 (NCH₃), 40.02 (PhCH₂CH), 51.93 (NHCHCO), 67.06 (PhCH₂O), 114.40 (NCH₂CN), 127.41 (C-4'), 128.03 (C-2’’, C-6’’), 128.21 (C-4’’), 128.54, 128.84, 129.31 (C-2’, C-6’, C-3’, C-5’, C-3’’, C-5’’), 135.31, 136.11 (C-1’, C-1’’), 155.58 (OCOCH), 172.10 (CHCON); MS (ESI) m/z (rel. intensity) (pos.) 374 (30, [M + Na]⁺), 369 (40, [M + NH₄]⁺), 352 (100, [M + H]⁺), 308 (60, [M – CO₂ + H]⁺), (neg.) 242 (100, [M – BnOH – H]), 215 (73, [M – BnOH – HCN – H]). Anal. C_{20}H_{21}N_{3}O_{5} (351.40 g/mol) calcd C 68.36, H 6.02, N 11.96; found C 68.64, H 6.11, N 11.73.
N-(Benzyloxycarbonyl)-phenylalanlanyl-methylalanine-nitrile (14)

Cyanuric chloride (0.20 g, 1.09 mmol) was added to a solution of N-(benzyloxycarbonyl)-phenylalanlanyl-methylalanine-amide (0.43 g, 1.09 mmol) in DMF (10 mL). After stirring for 2 h at room temperature, DMF was evaporated. Ice-cold sat. NaHCO$_3$ (10 mL) was added and the mixture was extracted with ethyl acetate (4 \times 15 mL). The combined organic layers were washed with H$_2$O (3 \times 15 mL) and brine (1 \times 15 mL), dried (Na$_2$SO$_4$), and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel with petroleum CH$_2$Cl$_2$ / MeOH (20:1) as eluent to obtain 14 (0.35 g, 83 %) as a colorless solid: mp 65-67 °C; [α]$_D^{20}$ = –19.65 (c = 1.08, MeOH); $^1$H NMR (500 MHz, CDCl$_3$) δ 1.34 (d, $^3$J = 7.0 Hz, 3H, CH$_3$), 2.65 (s, 1H, PhCH$_2$), 2.78 (s, 1H, PhCHHCH), 3.28 (s, 3H, NCH$_3$), 4.60 (q, $^3$J = 7.25, 1H, CHCN), 4.92-5.00 (m, 1H, NHCHCO), 5.43-5.47 (m, 2H, PhCH$_2$O), 7.19-7.35 (m, 10H, H$_{arom}$), 7.78 (d, $^2$J = 7.6 Hz, 1H, NHCHCO); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 14.21 (CH$_3$), 31.16 (NCH$_3$), 37.09 (PhCH$_2$CH), 41.41 (CHCN), 52.63 (NHCHCO), 65.54 (PhCH$_2$O), 118.71 (CHCN), 126.66 (C-4 ''), 127.68 (C-4'), 127.88 (C-2'', C-6''), 128.31, 128.43, 129.43 (C-2', C-6', C-3', C-5', C-3'', C-5''), 137.06, 137.21 (C-1', C-1''), 155.91 (OCNCH), 171.66 (CHCONH); MS (ESI) m/z (rel. intensity) (pos.) 388 (8, [M $+$ Na$^+$]), 383 (67, [M $+$ NH$_4$]$^+$), 366 (100, [M $+$ H]$^+$), (neg.) 364 (100, [M $-$ H]$^-$), 256 (46, [M $-$ BnOH $-$ H]). Anal. C$_{21}$H$_{23}$N$_3$O$_3$ (365.43 g/mol) calcd C 69.02, H 6.34, N 11.50; found C 68.96, H 6.41, N 11.17.
References


