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Peptide Synthesis at High Pressure: Activation Volume of a Peptide Coupling, Synthesis of a Glutathione Derivative

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Dedicated to Professor Ekkehard Winterfeldt on the Occasion of his 65th Birthday

Abstract. From the pressure-induced rate enhancement the activation volume of the peptide coupling **1** with the sodium salt of glycine **2** leading to the corresponding dipeptide derivative **3** was determined to be strongly negative ($\Delta V^{\neq}$ = –(19.3 ± 0.5) cm^3mol^{-1} at 51.7 °C, CH_3OH). This finding indicates that an association with the developing of charges proceeds in the rate-determining transition state. The pressure-induced peptide coupling was exploited to synthesize a derivative (**12a, b**) of glutathione (γ-Glu-Cys-Gly), a biologically important tripeptide, starting from either glycine or glutamic acid.

Pressure in the range of 5 to 20 kbar strongly influences the rate and equilibrium position of many chemical reactions. The quantities characteristic for the size of the pressure effect are the volumes of activation and reaction, which can be determined from the pressure dependence of the rate and equilibrium constants, respectively. Processes accompanied by a decrease in volume such as bond formation, charge development (the effect of electrostriction), or cyclization are accelerated by raising the pressure ($\Delta V^{\neq} < 0$) while those accompanied by an increase of volume such as bond dissociation, charge neutralization or diffusion control are retarded ($\Delta V^{\neq} > 0$) [1]. Here, we report on the activation volume of a peptide coupling and its mechanistic and synthetic implication. The utility of high pressure is demonstrated with the synthesis of the derivative **12b** of glutathione, a biologically important tripeptide.

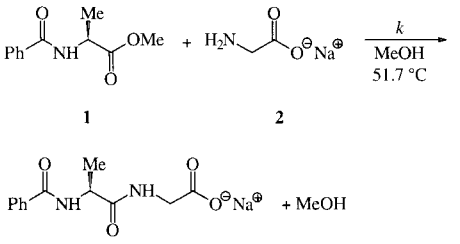
Previously it has been shown, that the aminolysis of methyl or ethyl carboxylates [2] and the peptide coupling with nonactivated amino acid alkylesters [3], which do not react at atmospheric pressure, can be achieved at high pressure. Here, we investigated the pressure-dependence of the rate constants of the reaction of *N*-benzoylalanine methylester **1** with the sodium salt of glycine **2** in methanol at 51.7 °C leading to the dipeptide derivative **3**. At each pressure the rate constant of this bimolecular reaction was determined from the ratio between the starting material **1** and product **3** measured by HPLC for three different ratios of the starting materials **1** and **2** (Table 1). The analysis was carried out under the conditions of pseudo-first-order kinetics where one of the reactants (in this case **1**) was used in a ca. nine-fold excess (over reactant **2**). The details of the kinetic analysis are described in the experimental section. From the pressure dependence of the rate constants (Table 1) the activation volume was calculated to be strongly negative ($\Delta V^{\neq}$ = –(19.3 ± 0.5) cm^3mol^{-1}.

In a more qualitative experiment we found, that the peptide coupling between the highly reactive *N*-ben-

Table 1 Pressure dependence of the rate constants k of the reaction **1** + **2** → **3** in methanol at 51.7 °C and the activation volume ($\Delta V^{\neq}$ in cm^3mol^{-1}) calculated from this pressure dependence by the use of a nonlinear quadratic least-square fit: $\ln k(p) = a + b \times p + c \times p^2$ ($a = \ln k(p=0)$, $\Delta V^{\neq} = -b \times R\,T$).

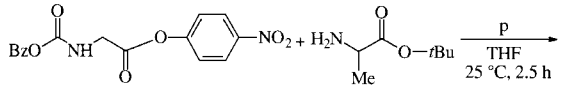
p [bar]	1	743	1800	2043
$10^6 k$ [M^{-1} s^{-1}]	0.684 ± 0.008	0.994 ± 0.002	2.26 ± 0.01	2.61 ± 0.01
p [bar]	2940	3800	5500	6600
$10^6 k$ [M^{-1} s^{-1}]	4.25 ± 0.003	6.88 ±0.02	19.1 ± 0.03	26.9 ± 0.3

$\Delta V^{\neq}$ = – (19.3 ± 0.5) cm^3mol^{-1}

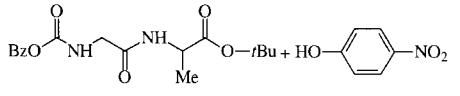


1 2

3



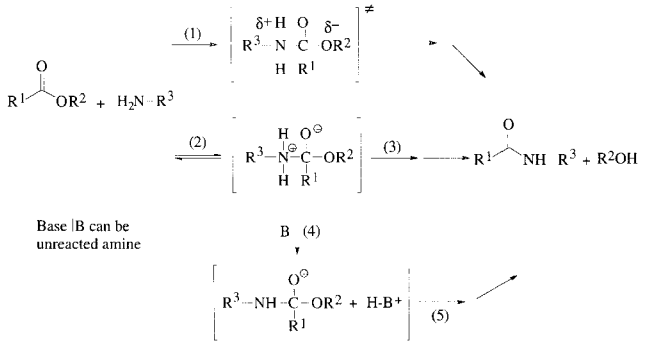
4 5



6

[4] = [5] = 0.4 M : p = 1 bar (10 kbar), yield of **6** : 61 % (> 99 %)

zyloxycarbonyl-glycine *p*-nitrophenylester **4** and alanine *t*-butylester **5** in an aprotic solvent such as tetrahydrofuran (THF) leading to the dipeptide derivative **6** is also accelerated by raising the pressure from 1 bar to 10 kbar. This result is in accord with those reported in the literature [3a].



Scheme 1 Mechanisms postulated for aminolysis of esters: direct S_N2 type substitution (1) or the $B_{AC}2$ type mechanism either with the addition (2) or one of the subsequent steps (proton transfer (4) or elimination (3) and (5), respectively) in the rate-determining step.

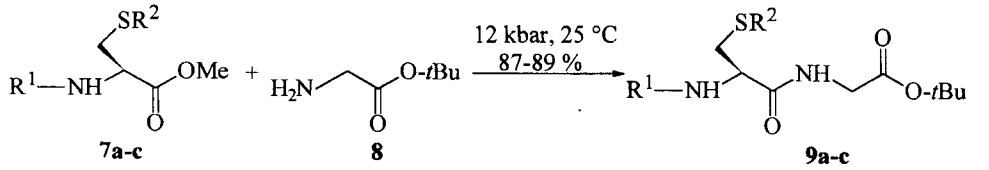
A pressure-induced acceleration can be expected for both types of mechanism postulated for the aminolysis of esters – the direct S_N2 type substitution and the addition-elimination of $B_{AC}2$ type as well-provided that the addition leading to the zwitterionic tetrahedral intermediate occurs in the rate-determing step (Scheme 1, (2)) [4]. It is highly likely that in the peptide coupling of the nonactivated methylester **1** with amino acid salt **2** in the protic solvent methanol the corresponding zwitterionic intermediate is formed in the rate-determining step. The highly negative activation volume provides good evidence for this suggestion. In the aminolysis of activated *p*-substituted phenylesters (*p*-X-C_6H_4-O-C(O)-Ar, X = NO_2, H, CH_3, OCH_3) a positive Hammett-value (ρ = +6.07) indicates that the cleavages of the C-O bond occurs in the rate-determining step which is the case in Scheme 1 for step (1), (3) or (5) [4a]. Only a moderate effect of pressure (or even a pressure-induced retardation) can be expected for the dissociative steps (3) or (5), whereas a pressure-induced acceleration can be predicted for step (1). Therefore, the experimentally observed acceleration of the peptide coupling between the activated *p*-nitrophenylester **4** and the amino component **5** by pressure indicates a direct S_N2 type substitution to be involved in this reaction. Quantitative volume data are, however, required for further elucidation of the intriguing mechanisms of the aminolysis of activated and nonactivated esters in protic or nonprotic solvents.

The synthesis of the glutathione derivatives **12a** and **12b** was accomplished starting from both termini either from glycine or glutamic acid. The *N*- and *S*-protected cystine derivatives **7a** and **7b** or cysteine derivative **7c** react with glycine *t*-butylester **8** at 12 kbar and 20 °C in THF to give the desired peptide derivatives **9a–c** in yields of 87–89%. The amino protecting Boc group could be selectively cleaved leading to **10a** and **10b**, respectively, in yields between 61 and 93% by reaction of **9a** or **9c** with aqueous formic acid. All attempts of the synthesis of glutathione derivatives by coupling **10a** or **10b** with suited derivatives of glutamic acid at high pressure have, hitherto, failed. In order to explain this failure one can speculate that high pressure induces an conformational change in the dipeptides **10a** or **10b** which is unfavorable for the formation of the tripeptide. Since it is already known in the literature that dipeptides of type **10** react with activated glutamic acid derivatives of type **11** leading to the glutathione derivative **12a** [5], the sequence of reactions described here can be regarded as a partial high-pressure synthesis of glutathione.

Finally, we could synthesize the glutathione derivative **12b** by using the biomimetic sequence of coupling reactions starting from the glutamic acid derivative **13**. The peptide coupling between **13** and **14** proceeds at 10 kbar and 40 °C in methanol followed by hydrolysis with phosphoric acid and esterfication with dimethyl sulfate leading to the dipeptidedimethylester **15**. It is noticeable that the coupling between **15** and **8** proceeds almost exclusively at the desired methylester position of the cysteine component and not at the methylester position of the glutamic acid component. A pressure-

Synthesis of Glutathione: γ - Glu - Cys - Gly

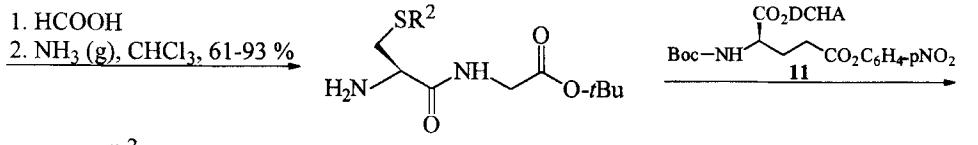
a. Starting from Glycine



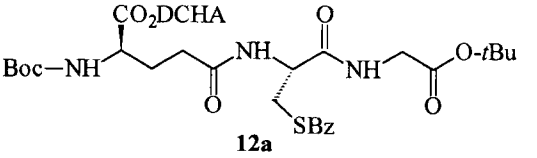
7a-c **8** **9a-c**

a: R^1 = Boc, R^2 = Boc - Cys - OMe
b: R^1 = Z, R^2 = Z - Cys - OMe
c: R^1 = Boc, R^2 = Bz

a: R^1 = Boc, R^2 = Boc - Cys - Gly - O-*t*Bu
b: R^1 = Z, R^2 = Z - Cys - Gly - O-*t*Bu
c: R^1 = Boc, R^2 = Bz

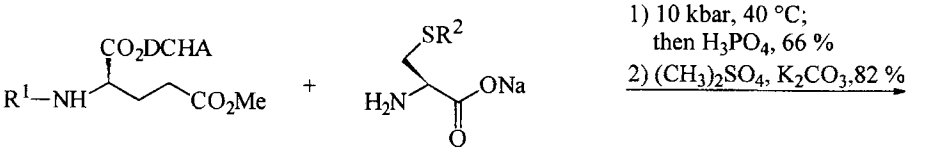


R^2 = H - Cys - Gly - O-*t*Bu: **10a**
R^2 = Bz: **10b**

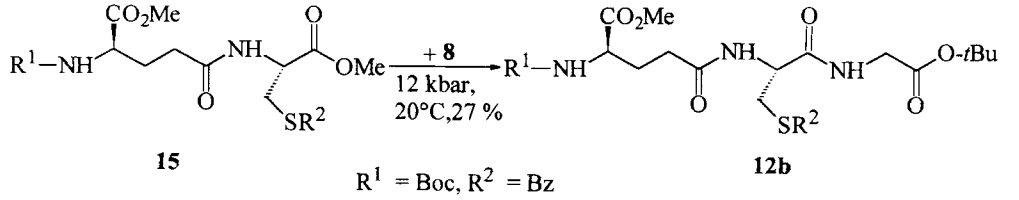


12a

b. Starting from Glutamic acid



13 **14**



15 **12b**

R^1 = Boc, R^2 = Bz

Abbreviations: Cys = Cysteine, Gly = Glycine, Z = Ph-$CH_2O(CO)$, Boc = *t*BuO(CO), DCHA = Dicyclohexylamine, Bz = Benzyl.

induced conformational hindrance in the dipeptide **15** may be responsible for the lower yield of 23% obtained for the formation of the glutathione precursor **12b** compared to the yields observed for the formation of dipeptides which occurs almost quantitatively at high pressure under optimized conditions. We are now planning to investigate the selectivity in α- and γ-coupling of glutamic acid derivatives at high pressure.

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Experimental

IR: Perkin-Elmer FTIR 1600. ^{1}H and ^{13}C NMR: Bruker AMX 300; Varian Gemini XL 200; Bruker WP 80. – MS: Fison Instruments VG ProSpec 3000 (70 eV). – Column Chromatography: Riedel de Haën Silica gel 0.063–0.2 mm and Aldrich Florisil 60–100 mesh. – TLC: Macherey & Nagel Kieselgel Fertigplatten Polygram Sil-G/UV$_{254}$. – HPLC: L-5000 LC-Controller, Merck-Hitachi 655 A-12 Liquid Chromatograph, Shimadzu SPD-6A UV Detector, Hewlett-Packard HP3390A Integrator, Column A; LiChroCart® HPLC-Kartusche 125-4 filled with LiChrospher® 60 RP-select B (5 µm), ColumnB: Hypersil® ODS 125-5 (3 µm). – All melting points are uncorrected. – The preparative pressure experiments were carried out in a 14 kbar apparatus (autoclaves volumes 36 or 100 ml) built by A.W. Birks, Department of Mechanical and Manufacturing Engineering, Queen's University of Belfast, Northern Ireland and Fa. Hofer Hochdrucktechnik, Mülheim/Ruhr, Germany. The kinetic measurements were performed in a 7 kbar apparatus, Fa. Nova-Swiss, Effretikon, Switzerland.

N(*N*-Benzoylalanyl)glycine *N*(3)COH instead of ONa)

A solution of 1.0 mmol each of **1** and **2** in 10 ml of dry methanol was compressed in a sealed polytetrafluoroethylene (PTFE) shrinkage tube at 10 kbar and room temperature for 17 h. The solution was then evaporated *in vacuo* and the residue dissolved in 10 ml of water. The resulting solution was extracted with ethyl acetate. The aqueous layer was carefully acidified to pH ≈ 1 with diluted aqueous HCl, and the product **3** was extracted with ethyl acetate. After drying over Na_2SO_4 the organic extract was evaporated *in vacuo* until the product **3** precipitates. Yield: 64 %, *m.p.* 178–180 °C, $[\alpha]_D^{20} = -6.7°$ (c=1, MeOH). – ^{1}H NMR (200 MHz, DMSO-d_6): δ 1.34 (d, CH-C<u>H</u>$_3$), 3.32 (m, NH-C<u>H</u>$_2$-CO$_2$H), 4.49 (m, C<u>H</u>-CH$_3$), 7.36 (t, N<u>H</u>-CH$_2$-CO$_2$H), 7.40–7.90 (m, C$_6$H$_5$), 8.68 (d, N<u>H</u>-CH(CH$_3$)–CO).

Kinetic Analysis of the Pressure Dependence of the Reaction 1 + 2 → 3

Two portions of a solution containing **1** (466.80 mg, 2.25 mmol), **2** (23.03 mg, 0.237 mmol), and nitrobenzene (12.05 mg, 0.098 mmol, as internal standard) in anhydrous CH_3OH (10 ml, total volume) were diluted to give the concentrations of **1** and **2** listed in Table 2. Portions of 300 µl of each solution were sealed in a PTFE shrinkage tube and thermolyzed at the conditions given in Table 2. The ratio of starting material **1** and product **3** was analyzed by HPLC using column A, UV detection at 254 nm, eluent: H_2O (0.2% trifluoroacetic acid, pH = 2.0)/methanol; 70:30 (ratio of volumes), retention times: **1** (7.8 min), **3** (3.4 min), Bz-Ala-OH (4.5 min, negligible impurities resulting from the hydrolysis of **1** which cannot be avoided even after drying the methanol very carefully), and the internal standard, nitrobenzene (13.5 min). The HPLC response factors $f_i = (F_i\, m_i)/(F_s\, m_i)$ (F_i,F_s-peak areas of **1** or **3** and the internal standard, respectively, m_i/m_s - weights of **1** or **3** and the internal standard, respectively, in mg) were determined by independent measurements to be $f_1 = 3.17$; $f_3 = 3.14$. The pseudo-first order rate constant k_{obs} (s^{-1}) was determined from the different concentrations $[\mathbf{1}]_0$, $[\mathbf{2}]_0$, and $[\mathbf{3}]_t$ at each pressure (Table 2) to be $k_{obs} = (1/t)\times\ln([\mathbf{2}]_0/[\mathbf{2}]_t)$, ($[\mathbf{2}]_t = [\mathbf{2}]_0 - [\mathbf{3}]_t$) and the second-order rate constants in Table 1 to be $k = k_{obs}/[\mathbf{1}]_0$ ($M^{-1}s^{-1}$). The errors given in Table 1 are standard deviations resulting from three measurements at each pressure.

Pressure Dependence of the Formation of *N*-(*N*-Benzyloxy-carbonylglycyl)alanine-*t*-butylester (6)

One 5-ml portion of a solution containing **4** (60 mg, 0.18 mmol), **5** (3 mg, 0.2 mmol), and *p*-nitrophenol (25 mg, 0.18 mmol as internal standard) in anhydrous THF (10 ml) was thermolyzed at 1 bar and 25 °C for 1 h and the other 5 ml portion at 10 kbar and 25 °C for 1 h. Both portions were separately evaporated *in vacuo*, and the residue of ca. 500 µl was filtered over a column (5 cm×0.5 cm) filled with silica gel and washed with pentane/ethyl acetate (2:1, 5 ml). In both samples it was checked by tlc that **5** was quantitatively removed by this work-up procedure. Both samples were evaporated *in vacuo* and the ratios of **4** : **6** : *p*-nitrophenol were determined by integration of the signals at δ 8.1, 6.9 (*p*-NO$_2$-C$_6$<u>H</u>$_4$-OH) 8.3, 4.2 (**4**), and 4.4, 1.4, (**6**) in the ^{1}H NMR spectra of the mixture to be 0.24 : 0.38 : 0.38 at 1 bar and 0 : 0.5 : 0.5 at 10 kbar. From the high pressure sample the product **6** was isolated by evaporating the solvent *in vacuo*, dissolving the residue in a mixture of water (2 ml) and ethyl acetate (2 ml), extracting the organic layer with aqueous Na_2CO_3 (1M, 5 ml) and water, drying the organic layer over Na_2SO_4, and evaporating the solvent *in vacuo*. Yield of **6**: 58 mg (96%) as colorless solid, *m.p.* 88 °C. – ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.34 (m, C$_6$H$_5$), 6.58 (d, -N<u>H</u>-CH(CH$_3$)-), 5.45 (dd, -N<u>H</u>-CH$_2$-), 5.13 (s, C$_6$H$_5$-C<u>H</u>$_2$-O-), 4.40 (quintet, NH-C<u>H</u>(CH$_3$)-, ^3J(NH, CH) ≈ ^3J(CH$_3$, CH) = 7 Hz), 3.96, 3.86 (AB part of an ABX spectrum, -NH-C<u>H</u>$_2$-), 1.4 (s, broad, -C(CH$_3$)$_3$, -CH(C<u>H</u>$_3$)-). – ^{13}C NMR (50.4 MHz, $CDCh_3$): δ 172.0, 168.4, 161.5 (C=O), 136.2 (C, in C$_6$H$_5$-), 128.6, 128.3, 128.2 (CH in C$_6$H$_5$-), 82.3 (C, O-<u>C</u>(CH$_3$)), 67.3 (CH$_2$, C$_6$H$_5$-<u>C</u>H$_2$-O-), 48.7 (CH$_2$, NH-<u>C</u>H$_2$-CO-), 42.0 (CH, NH-<u>C</u>H(CH$_3$)-), 28.0, 18.6 (CH$_3$).

N,N-Bis-t-butyloxycarbonylcystinyldiglycine-t-butylester (9a)

A solution of **7a** (440 mg, 0.86 mmol) and **8** (295 mg, 2.6 mmol) in anhydrous THF (3 ml) sealed in a PTFE shrinkage tube was compressed to 12 kbar at 25 °C for 5 d. The solvent and the excess of **8** were destilled off *in vacuo*. The residue was separated by column chromatography (10 cm × 1 cm, Florisil, eluent: diethylether) into two fractions. The first fraction contained a small amount of starting material **7a** and the second the product **9a** isolated in a yield of 503 mg (88%) as a colorless solid, *m.p.* 131 °C. – ^{1}H NMR (300 MHz, $CDCl_3$) : δ 8.08 (dd(broad), -N<u>H</u>-CH$_2$-), 5.55 (d, -N<u>H</u>-CH(CH$_2$-S-)-, ^3J(NH, CH) = 10 Hz), 4.95 (m, -NH-C<u>H</u>(CH$_2$-S-)-), 4.50, 3.75 (AB part of an ABX spectrum, -NH-C<u>H</u>$_2$-, ^{2}J(CH$_2$) = −18 Hz, ^3J(CH$_2$, NH) = 6.5 Hz and 5.5 Hz, respectively), 3.06 (AB part of an ABX spectrum, -CH(CO-)-C<u>H</u>$_2$-S-, ^2J(CH$_2$) = −15 Hz, J(CH$_2$, CH) = 4 Hz and 11 Hz, respectively), 1.4 (s, C(C<u>H</u>$_3$)$_3$). – ^{13}C NMR (75.5 Hz, $CDCl_3$) : δ 171, 168.5, 156.1 (<u>C</u>=O), 82.1, 80.4 (C, <u>C</u>(CH$_3$)$_3$), 54.7 (CH), 47.2, 41.7, (CH$_2$), 28.5, 28.2 (CH$_3$).

Table 2. Kinetic data obtained for the reaction **1** + **2** → **3** at 51.7 °C and different pressures in methanol.

p [bar]	t (s)	$[\mathbf{1}]_0$ (M^{-1})	$[\mathbf{2}]_0$ (M^{-1})	$[\mathbf{3}]_t \times 10^{-3}$ (M^{-1})	$10^6\, k_{obs} \times$ (s^{-1})
1	236 700	0.1126	0.0119	0.2164	0.077
		0.1802	0.0190	0.5461	0.124
		0.2253	0.0237	0.8503	0.154
743	412 200	0.1126	0.0119	0.535	0.112
		0.1802	0.0190	1.351	0.179
		0.2253	0.0237	2.092	0.224
1800	151 200	0.1126	0.0119	0.4473	0.254
		0.1802	0.0190	1.132	0.407
		0.2253	0.0237	1.755	0.508
2043	72 000	0.1126	0.0119	0.2487	0.294
		0.1802	0.0190	0.6327	0.471
		0.2253	0.0237	0.9843	0.588
2940	65 800	0.1126	0.0119	0.3668	0.476
		0.1802	0.0190	0.9298	0.763
		0.2253	0.0237	1.444	0.955
3800	237 600	0.1126	0.0119	1.996	0.776
		0.1802	0.0190	4.845	1.24
		0.2253	0.0237	7.393	1.55
5500	63 000	0.1126	0.0119	1.506	2.15
		0.1802	0.0190	3.705	3.45
		0.2253	0.0237	5.642	4.31
6600	72 000	0.1126	0.0119	2.322	3.02
		0.1802	0.0190	5.583	4.82
		0.2253	0.0237	8.378	6.07

9b and **9c** were prepared analogously starting either from a solution of **7b** (500 mg, 0.93 mmol) and **8** (282 mg, 2.15 mmol plus 130 mg, 1.0 mmol after a reaction time of 7 d) in THF (3 ml); conditions of reaction: 10 kbar, 25 °C, 7 d plus 11 kbar, 25 °C, 5 d, conditions of column chromatography: Silica gel, 20 cm × 2 cm, eluent: CH_2Cl_2/methanol 5:1, R_f (**9b**) = 0.8 or from a solution of **7c** (515, 1.6 mmol) and **8** (430 mg, 3.2 mmol) in THF (1.5 ml), conditions of reactions: 11.2 kbar, 25 °C, 4 d; conditions of column chromatography: Silica gel, 20 cm × 3 cm, eluent: hexane, methyl-*t*-butylether (MTBE) 3:1 R_f (**9c**) = 0.15. Yield of **9b**: 550 mg (86%) *m.p.* 145 °C. Yield of **9c**: 610 mg (89%), *m.p.* 142 °C. The structures of **9b** and **9c** were accordingly assigned by ^1H and ^{13}C NMR spectroscopy.

Cystinyldiglycine-t-butylester (**10a**)

A solution of **9a** in aqueous formic acid (85%, 2 ml) was stirred at 17 to 19 °C for 4 h. The excess of formic acid and *t*-butanol was evaporated *in vacuo*. Gaseous NH_3 was bubbled into the solution of the residue in $CHCl_3$ until the solution turned cloudy. The precipitate was filtered and the filtrate was evaporated *in vacuo*. The residue was dissolved in ethyl acetate, and dried over Na_2SO_4 for 12 h. After filtration and evaporation of the solvent *in vacuo* the product was obtained as colorless oil. Yield: 70 mg (93%). – ^1H NMR (400 MHz, $CDCl_3$): δ 7.85 (dd(broad),-N<u>H</u>-CH_2-), 3.98, 3.92 (AB part of an ABX-spectrum, -NH-C<u>H</u>$_2$), 3.73 (dd, -C<u>H</u>-CH_2-S-), 3.30, 2.78 (AB part of an ABX-spectrum, -CH-C<u>H</u>$_2$-S-), 1.75 (m, broad, -N<u>H</u>$_2$), 1.48 (s, CH_3). – ^{13}C NMR (100.3 MHz, $CDCl_3$): δ 173, 169 (<u>C</u>=O), 82.4 (C, <u>C</u>$(CH_3)_3$), 54.1 (CH), 43.8, 42.1 (CH_2), 28.3 (CH_3).

S-Benzyl-cysteinylglycine-t-butylester (**10b**)

10b was analogously prepared by treatment of **9c** (210 mg, 0.5 mmol) with aqueous formic acid (85%, 6 ml). Yield of **10b**: 100 mg (61%) as colorless oil. – ^1H NMR (200 MHz, $CDCl_3$) : δ 7.82 (m, broad, N<u>H</u>), 7.25 (m, C_6<u>H</u>$_5$), 3.95, 3.83 (m, -NH-C<u>H</u>$_2$-), 3.69 (s, C_6H_5-C<u>H</u>$_2$-) 3.50 (dd, -C<u>H</u>-CH_2-S-), 2.96, 2.65 (AB part of an ABX spectrum (-CH-C<u>H</u>$_2$-5), 2.68 (s, broad, NH_2) 1.42 (s, CH_3). – ^{13}C NMR (50.4 MHz, $CDCl_3$): δ = 173.3, 168.9 (C=O), 138.1 (C) 128.9, 128.6, 127.1 (CH), 82.1 (C), 53.7 (CH), 41.7, 36.9, 36.4 (CH_2), 28.0 (CH_3).

N-(N-t-Butyloxycarbonyl-γ-glutamyl-α-methylester)-S-benzylcysteine-methylester (**15**)

A solution of **13** (300 mg, 0.7 mmol) and **14** (230 mg, 1 mmol) in anhydrous CH_3OH (2 ml) sealed in a PTFE shrinkage tube was warmed at 10 kbar and 40 °C for 10 d. After evaporation *in vacuo* the residue was dissolved in H_2O (3 ml) and extracted with ethyl acetate until the organic layer remained colorless. The aqueous layer was acidified with aqueous phosphoric acid (8%) to pH ≈ 1 and extracted with ethyl acetate until the cloudy aqueous layer became clear again. The organic layer was dried (Na_2SO_4) and evaporated *in vacuo*. The residue contained a (3:1) mixture of the dipeptide derivative Boc-γ-Glu(Cys(B_Z)-OH and Boc-Glu(OH)-OH according to ^1H NMR analysis. Yield 240 mg of the (3:1) mixture. The suspension of this mixture (237 mg), anhydrous K_2CO_3 (200 mg, 1.5 mmol), and dimethyl sulfate (160 mg = 120 µl, 1.3 mmol) in anhydrous acetone (10 ml) was stirred at 25 °C for 3 h. The solvent was evaporated *in vacuo*. Aqueous K_2CO_3 (5%, 5 ml) and ethyl acetate were added to the residue and the layers were separated. The aqueous layer was extracted with ethyl acetate the solvent and the combined organic layers were dried (Na_2SO_4) and destilled off *in vacuo*. The residue consisting of a mixture of **15** and Boc-Glu(OCH_3)OCH_3 was separated by column chromatography (silica gel, eluent: CH_2Cl_2/MTBE (5:1), UV-

Detection at 254 nm). First fraction: 40 mg of Boc-Glu $(OCH_3)OCH_3$ colorless solid, second fraction: 177 mg of **15** (82%) colorless solid, *m.p.* 122 °C. – ^{1}H NMR (300 MHz, $CDCl_3$) : δ 7.26 (m, C_6H_5), 6.56, 5.28, (d, 2NH-CH(CO_2CH_3)-), 4.76, 4.34 (m, 2NH-CH(CO_2CH_3)-CH_2-), 3.71 (s, CH_3), 3.67 (s, -S-CH_2-C_6H_5), 2.80 (m, CH_2), 2.27 (m, CH_2), 2.16, 1.89 (m, CH_2), 1.41 (s, CH_3). – ^{13}C NMR (75.4 MHz, $CDCl_3$) : δ 172.7, 171.6, 171.2, 155.6 (C=O), 137.6 (C), 128.9, 128.6, 127.2 (CH), 80.1 (C), 52.8 (CH), 52.6, 52.4 (CH_3), 51.4 (CH), 36.5, 33.3, 32.2, 28.6 (CH_2) 28.2 (CH_3).

N-(N-[N-t-Butyloxycarbonyl-γ-glutamyl-α-methylester]-S-benzyl-cysteinyl)-glycine-t-butylester (**12b**)

A solution of **15** (60 mg, 0.13 mmol) and **8** (20 mg, 0.13 mmol) in anhydrous THF (500 µl) sealed in a PTFE shrinkage tube was kept at 12 kbar and 25 °C for 5 d. The solvent was evaporated *in vacuo* and the residue was separated by column chromatography (silica gel, 10 cm × 1 cm, eluent CH_2Cl_2/MTBE (10:1). First fraction: recovered starting material **15**, second fraction: 17 mg of **12b** (27%) MS (70 eV): *m/z* = 567 (M^+), 511 ($M^+-C_4H_{10}$), 476 ($M^+-C_6H_5$-CH_2), 438 (M^+–HGlyO*t*Bu), 420, 394, 91. – ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.30 (m, C_6H_5), 6.85 (dd, NH-CH_2-), 6.66 (d, -NH-CH(CO-)CH_2-S-), 5.33 (d, -NH-CH(CO_2CH_3)-CH_2-), 4.53 (m, NH-CH(CO-)-CH_2-S, ^3J(NH, CH) = 7.0 Hz, ^3J(CH,CH_2) = 6.2 and 6.5 Hz, respectively), 4.36 (m, NH-CH(CO_2CH_3)CH_2-), 3.88 (AB part of an ABX spectrum, NH-CH_2-CO_2 + Bu, ^2J(CH_2) = –17.5 Hz, ^3J(NH, CH_2) = 5.5 and 5.0 Hz, respectively), 3.71 (s, S-CH_2-C_6H_5), 3.65 (s, –COOCH_3), 2.82 (AB part of an ABX spectrum, -CH-CH_2-S-, ^2J(CH_2) = –14.0 Hz, ^3J(CH, CH_2) = 6.2 and 6.5 Hz, respectively), 2.28, 2.15, 1.92 (m, -CO-CH_2-CH_2-CH(CO_2CH_3)-NH), 1.44, 1.41 (s, CH_3). – ^{13}C NMR (70.4 MHz, $CDCl_3$) : δ 172.8, 172.1, 170.2, 168.5, 155.7 (C=O), 138.0 (C), 128.9, 128.6, 127.2 (CH), 82.4, 80.2 (C), 52.7 (CH), 54.5 (CH_3), 52.2 (CH), 41.1, 36.4, 33.1, 32.1, 28.6 (CH_2), 28.3, 28.0 (CH_3). The structural assignment of the signals in the ^{1}H and ^{13}C NMR spectra was confirmed by the use of ^{13}CDEPT and two-dimensional H,H-COSY, H,C-COSY, and H,H-ROESY experiments.

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