

**Thia-analogues of amino acids
Synthesis of peptide derivatives
containing 3-thia-analogues of amino acids***

P. Hermann, H. Baumann, Ch. Herrnsstadt, and D. Glanz

Institute of Biochemistry, Medical Faculty,
Martin-Luther-University, Halle, Federal Republic of Germany

Accepted November 18, 1991

Summary. N-Protected dipeptides containing L-3-thia-analogues of phenylalanine, p-nitro-phenylalanine, lysine and leucine respectively were prepared applying an enantioselective enzymatic reaction step. Racemic synthetic intermediates of the type $\text{acyl-NH-CH(R}^1\text{)-CO-D,L-NH-CH(S-R}^2\text{)-COOBzl}$ were selectively deprotected at the C-terminus by enzymatic hydrolysis using thermitase or trypsin.

Keywords: Amino acid analogues – Enzymatic hydrolysis – Peptide synthesis – Thermitase – Trypsin

Abbreviations: Ac: acetyl, AcOEt: ethyl acetate, AcOH: acetic acid, Boc: tert.-butoxycarbonyl, Bz: benzoyl, Bzl: benzyl, DMF: dimethyl-formamide, EtOH: ethanol, THF: tetrahydrofuran, Z: benzyloxycarbonyl.

Introduction

Thia-analogues of amino acids are isosteric analogues of natural amino acids in which the positions of carbon-4 or -3 are substituted by the sulphur atom. Due to their chemical nature they can be classified as thioether compounds (e.g. L-4-thia-lysine), as carbonic acid derivatives (e.g. L-2-oxo-thiazolidine-4-carboxylic acid) or N,S-acetals of either formaldehyde (e.g. L-thiazolidine-4-carboxylic acid) or glyoxylic acid (e.g. L-thiazolidine-2-carboxylic acid).

Thia-analogues of amino acids are valuable tools in basic and applied biochemistry. Experimental work with these compounds has contributed among others to our knowledge

* Dedicated to Prof. D. Cavallini at the occasion of his 75th birthday.

- in the fields of amino acid transport, metabolism and its regulation in microorganisms
- the role of enzyme specificity in the different steps in protein biosynthesis
- of structure- activity- relationships in the field of peptide hormones
- of the primary structure of proteins.

The fields of application range from diagnostic methods for determination of enzymatic activities or detection of inborn errors of amino acid catabolism to the selection of analogue-resistant mutants of microorganisms for the production of natural amino acids.

The chemistry and biochemistry of thia-analogues of amino acids has been reviewed by Cavallini (1979) and Hermann (1981).

The open-chain N,S-acetal-type analogues are unstable compounds which are hydrolyzed spontaneously to formaldehyde or glyoxylic acid and ammonia and a thiol or undergo intramolecular aminolysis (see below). In the N-acylated state the open-chain N,S-acetal analogues are stable amino acid derivatives. S-Acetamidomethyl-L-cysteine for instance has found wide application as a S-protected cysteine derivative in peptide synthesis (Veber, 1972; Hermann 1973).

Enzymatic deacylation as described by Greiner (1991) and Willhardt (1991) of S-acylamidomethyl-L-cysteines and deacylation of S-chloroacetamidomethyl-L-cysteine by thiourea derivatives (Hermann, 1976) forms S-aminomethyl-L-cysteine which spontaneously forms thiazolidine-4-carboxylic acid by intramolecular aminolysis.

If, however, the cysteine amino group is protected additionally the selective deacylation of the S-blocking group results in the production of N $^{\alpha}$ -protected-S-aminomethyl-cysteine derivatives which spontaneously decompose to N $^{\alpha}$ -protected cysteine derivatives (Hermann 1976; Greiner, 1991).

Racemic derivatives of open-chain 3-thia-amino acids have been described in the literature (Chemiakine, 1959; Steglich, 1967; Pojer, 1972; Zoller, 1975).

Dipeptide derivatives containing open-chain 3-thia-analogues of phenylalanine or lysine were first synthesized by Gilvarg (1984a and b), Kingsbury (1986) and Hwang (1986) as oligopeptide prodrugs or peptidase substrates via the condensation of N-protected L-amino acid amides (Z-Ala-NH₂ or Z-Phe-NH₂) with glyoxylic acid ester or glyoxylic acid. The racemic N-acylated acetal compounds were O-acetylated and then reacted with a thiol to produce racemic Z-AA₁-D, L-3-thia-AA₂-OBzl (AA₁ : Ala, Phe; AA₂ : Phe, Lys(Boc)), (Scheme 1). Fractional crystallization afforded the L-L and L-D diastereoisomers. Deprotection with HBr/AcOH produced the free dipeptides.

In this paper we describe the synthesis of dipeptide derivatives containing 3-thia-analogues of L-phenylalanine, L-p-nitro-phenylalanine, L-leucine and L-lysine respectively. The key step in our procedure is the enzymatic enantioselective hydrolysis of the C-terminal esters of the type N-acyl-AA₁-D, L-NH-CH(S-R)-COOBzl (AA₁ : Gly, Phe).

Material and methods

pH-stat-method was performed using a Radiometer equipment (TTT 2, ABU 12, SBR 3), Radiometer, Copenhagen.

All compounds were tested for purity by TLC and HPLC using different solvent systems.

TLC: DC-Alufolien (Kieselgel 60 (Merck, Darmstadt)); chloroform : methanol 88 : 12; cyclohexane : AcOEt 1 : 1; butanol : methanol : AcOH : H₂O 4 : 4 : 1 : 2; detection : ninhydrin after N-terminal deprotection; iodoplateat for sulphur compounds, chlorotolidine-reaction for compounds containing amide bonds; Bromcresol Green for compounds containing free carboxyl groups.

HPLC apparatus: Bischoff (Leonberg); columns: 4 × 250 mm, RP18 Tessek (Prague); eluent: acetonitrile: H₂O-mixtures, pH 2 (trifluoroacetic acid), methanol: H₂O-mixtures, pH 2 (trifluoroacetic acid); flow: 1 ml/min; λ = 220 nm.

NMR-spectra were obtained using the Bruker AM 200 and Bruker WM 90 instruments.

Mass spectra were obtained using the Finnigan instrument MAT 312.

Enzymes

Thermitase was prepared by the procedure of Kleine (1981) at the Institute of Biochemistry by Dr. Kettmann. The proteolytic activity, as determined at pH 8.0 and 55°C in 25 mM Tris-HCl buffer using succinyl-(Ala)₃-p-nitroanilide (0.25 mM) as a substrate, was 0.6 μ mol/min · mg.

Trypsina was preparation of SERVA, Heidelberg (from bovine pancreas; DPCC-treated ca. 35 U/mg; lyophil.; salt-free).

Chemicals

A-grade chemicals were used throughout.

Glyoxylic acid monohydrate (Merck, Darmstadt), aminopropylbromide-hydrobromide (Ferrak, Berlin), Na-2-propane-thiolat (Fluka, Buchs) were used without further purification.

Synthesis

The synthesis of dipeptide derivatives containing glycine started from benzyloxycarbonyl-glycine amide (**1a**) or benzoyl-glycine amide (**1b**). The condensation with glyoxylic acid benzyl ester or glyoxylic acid followed the general procedures outlined by Kingsbury (1986). Esterification of the condensation product (**2a**) was done using the cesium salt and benzyl bromide. Acylation of the hydroxy function of the N-acetals (**3a, b**) using acetic anhydride in pyridine affords **4a, b**. Thiolytic of **4a, b** by either thiophenol, p-nitro-thiophenol or N-Boc-l-aminopropane-3-thiol resulted in the racemic dipeptide ester derivatives **5a, 6a, 7a, b** (Scheme 1).

Glyoxylic acid benzyl ester

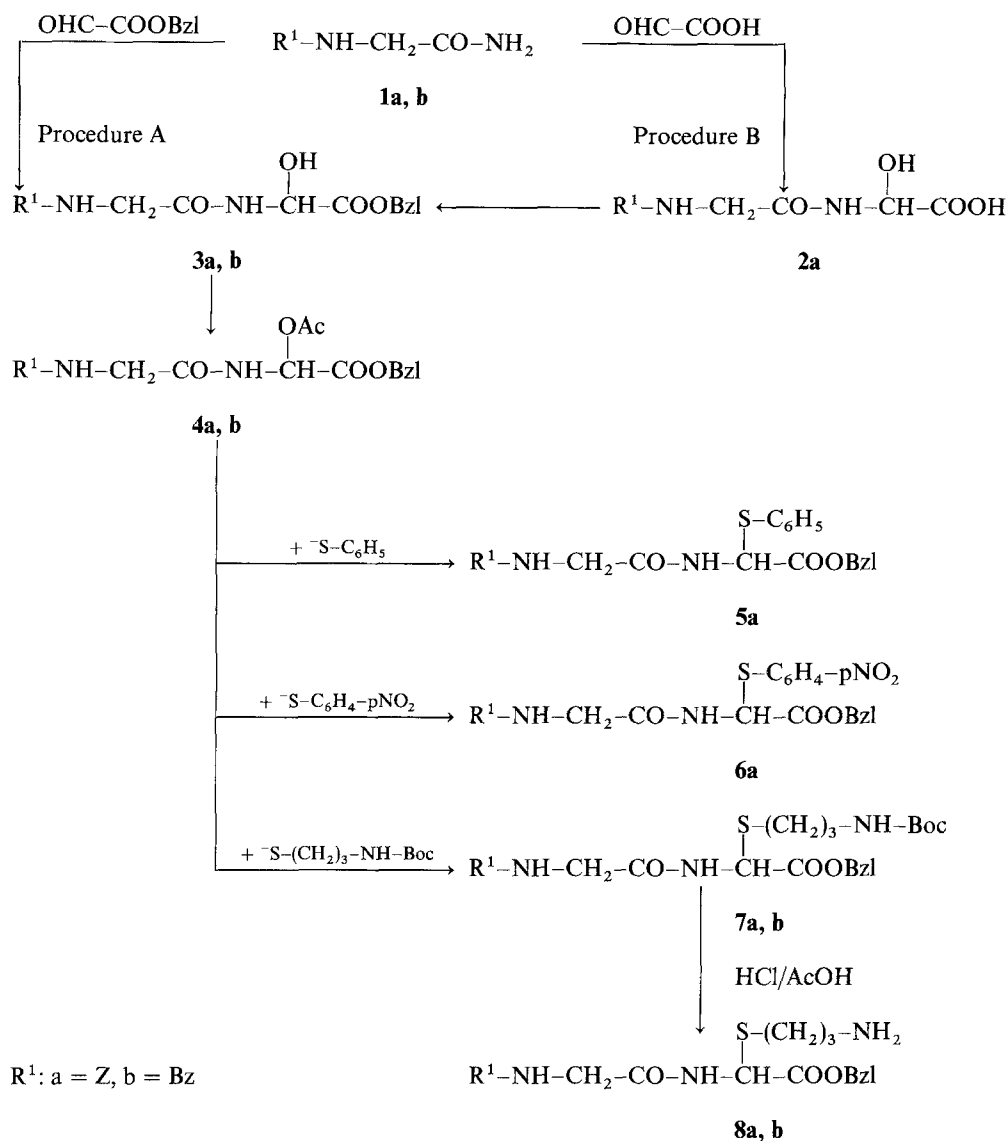
The preparation of glyoxylic acid benzyl ester was done by analogy to the general procedure of Kelly (1972).

yield: 56.8%; b.p.₂: 101–103°C

N-Tert.-butyloxycarbonyl-1-aminopropyl-3-bromide

Boc-NH-(CH₂)₃-Br was obtained from l-amino-propyl-3-bromide by analogy to the general procedure of Bodanszky (1984, p. 20).

yield: 82%, oil



Scheme 1

N-Tert.-butoxycarbonyl-1-amino-propane-3-thiol

18 g (80 mmol) of Boc-NH-(CH₂)₃-Br was dissolved in 100 ml 1 M methanolic NaHS-solution and refluxed under nitrogen 3 hours. The volatiles were taken off with evaporation and the residue was acidified with 10% KHSO₄. The solution was extracted several times with AcOEt. The combined organic phases were dried over Na₂SO₄ and concentrated to dryness.

yield: 10 g (65.4%), oil

Analysis for: C₈H₁₇NO₂S; M_r = 191.295; calc. (%) C: 50.23; H: 8.95; N: 7.32; S: 16.76; found (%) C: 50.64; H: 9.06; N: 7.76; S: 16.85 ¹H-NMR: CDCl₃; ppm; 4.7 (s, 1H), 2.9 (q, 2H), 2.2 (m, 2H), 1.7 (s, 1H), 1.4 (m, 2H); 1.1 (s, 9H)

N-Benzylloxycarbonyl-glycine amide 1a

1a was prepared by analogy to a procedure described by Grüb (1971) and was obtained analytically pure.

yield: 74%

mp: 134–137°C

N-Benzoyl-glycine amide 1b

1b was synthesized by analogy to a procedure of Siemion (1961) and was obtained analytically pure.

yield: 68%

mp: 183–184°C

N-Benzylloxycarbonyl-glycyl-D,L-α-hydroxyglycine benzyl ester 3a

The yields in the preparation of Z-Gly-Gly(OH)-OBzl **3a** were low. Therefore two alternative procedures adopted from the literature were applied.

Procedure A

The α-hydroxydipeptide was prepared by analogy to a procedure described by Kingsbury (1984).

A mixture of 5.7 g (35 mmol) glyoxylic acid benzyl ester and 5.7 g (26 mmol) Z-Gly-NH₂ **1a** in 120 ml methylene chloride was stirred at room temperature for about 5 days.

A white solid, Z-Gly-Gly(OH)-OBzl **3a**, was isolated after flash chromatography (column: 25 × 4 cm, Kieselgel 60 (Merck), eluent: AcOEt/hexane 1 : 1) of the concentrated reaction mixture.

yield: 2.0 g (21%)

mp: 74–75°C

Analysis for: C₁₉H₂₀N₂O₆; M_r = 372.40; calc. (%) C: 61.28 H: 5.41 N: 7.52; found (%) C: 61.12; H: 5.33; N: 7.14 ¹H-NMR: (CD₃)₂O; ppm; 7.3 (m, 10H), 5.75 (d, 1H), 5.15 (m, 2H), 4.62 (s, 2H), 3.9 (d, 2H), 3.33 (s, 2H)

Procedure B

The preparation was done according to the general procedure of Kingsbury (1986).

A mixture of 14.1 g (67.5 mmol) Z-Gly-NH₂ **1a** and 6.9 g (75 mmol) glyoxylic acid monohydrate in 150 ml acetone was heated under reflux for 15 hours. Methylene chloride was added and the solution was stored at 4°C overnight. The resulting solid was filtered to give 6 g of a white solid, Z-Gly-Gly(OH)-OH **2a**, which was not further purified; yield: 6 g (31.5%); mp: 124–127°C.

A solution of 5.7 g (20 mmol) Z-Gly-Gly(OH)-OH **2a** in 20 ml ethanol was mixed with a solution of 3.3 g Cs₂CO₃ in 80 ml of water. The pH of the mixture was brought to about 7.0. The volatiles were taken off with evaporation and successively azeotroping with toluene. The resulting gummy solid was dissolved in 130 ml DMF and 12 ml benzyl bromide were added. The mixture was stirred at room temperature for 3 days. The filtrate was evaporated and triturated with ether to give 6.1 g of white solid Z-Gly-Gly(OH)-OBzl **3a**.

yield: 6.1 g (82.4%)

mp: 76–78°C

N-Benzoyl-glycyl-D,L-α-hydroxyglycine benzyl ester 3b

A mixture of 1 g (5.6 mmol) Bz-Gly-NH₂ **1b** and 1.5 g (9.1 mmol) glyoxylic acid benzyl ester in 20 ml acetonitrile was refluxed for about 15 hours. After cooling Bz-Gly-Gly(OH)-OBzl **3b** separated.

This material was further purified by recrystallization from EtOH/ ether.
 yield: 1.4 g (73.6%)
 mp: 142–147°C

N-Benzylloxycarbonyl-glycyl-D,L-α-acetoxglycine benzyl ester 4a

The acetylation was done according to the general procedure of Hwang (1986).

Acetic anhydride (40 ml) was added to a mixture of 3.7 g (10 mmol) of Z-Gly-Gly(OH)-OBzl **3a** and 10 ml pyridine and cooled to 0°C. After standing in the cold overnight, the reaction mixture was evaporated. The residue was distributed between a H₂O/AcOEt mixture. The organic phase was then extracted with H₂O, 3N HCl, 5% NaHCO₃-solution and dried over Na₂SO₄. The solution was evaporated and the residue triturated with ether.

yield: 3.2 g (78%)

mp: 82–83°C

Analysis for: C₂₁H₂₂N₂O₇; M_r = 414.40; calc. (%) C: 60.87 H: 5.35 N: 6.76; found (%) C: 60.36; H: 5.38, N: 6.36

¹H-NMR: CDCl₃; ppm; 7.26 (s, 10H), 6.48 (d, 1H), 5.2 (s, 2H), 5.11 (s, 2H), 3.93 (d, 2H), 2.15 (s, 1H), 2.06 (s, 3H)

N-Benzoyl-glycyl-D,L-α-acetoxglycine benzyl ester 4b

The preparation was done by analogy to **4a**.

1.15 g (3.3 mmol) Bz-Gly-Gly(OH)-OBzl **3b** was allowed to react with 12 ml pyridine and 15 ml acetic anhydride at 0°C.

yield: 0.8 g (68.2%)

mp: 110–112°C

N-Benzylloxycarbonyl-glycyl-D,L-3-thia-phenylalanine benzyl ester 5a

The dipeptide was synthesized by analogy to the procedure of Hwang (1986).

A mixture of 1.5 g (3.6 mmol) Z-Gly-Gly(OAc)-OBzl **4a**, 0.5 ml triethylamine and 0.8 ml (7.2 mmol) thiophenol in 25 ml dry THF was stirred at room temperature for 48 hours. The mixture was concentrated and the residue was distributed between H₂O/AcOEt. The organic phase was washed with H₂O, dried and evaporated to dryness. For further purification the residue was recrystallized from EtOH/H₂O.

yield: 0.9 g (56%)

mp: 82–83°C

Analysis for: C₂₅H₂₄N₂O₅S; M_r = 464.54; calc. (%) C: 64.63; H: 5.2; N: 6.03; found (%) C: 64.45; H: 5.24; N: 5.96

¹H-NMR: CDCl₃; ppm; 7.31 (m, 15H), 5.74 (d, 1H), 5.16 (d, 2H), 5.12 (s, 2H), 3.86 (d, 2H), 2.16 (s, 1H)

N-Benzylloxycarbonyl-glycyl-D,L-p-nitro-3-thia-phenylalanine benzyl ester 6a

A mixture of 2.1 g (5 mmol) Z-Gly-Gly(OAc)-OBzl **4a**, 0.7 ml triethylamine, 0.78 g (5 mmol) p-nitro-thiophenol in 50 ml DMF was stirred 48 hours at room temperature. The solvent was evaporated and the red residue treated with H₂O. The product was extracted with AcOEt. The combined extracts were washed, dried and concentrated. The red solid was dissolved in chloroform acidified with AcOH. After addition of ether and refrigerating the crude product separated. This material was purified by recrystallization from AcOEt/cyclohexane.

yield: 1.5 g (59.1%)

mp: 138–140°C

Analysis for: C₂₅H₂₃N₃O₇S; M_r = 509.54; calc. (%) C: 58.93; H: 4.55 N: 8.25; S: 6.29; found (%) C: 57.01; H: 4.41; N: 7.96; S: 5.98 ¹H-NMR: d₆ – DMSO + TMS; ppm; 9.2 (d, 1H), 7.3

(m, 14H), 6.1 (d, 1H), 5.1 (s, 2H), 4.9 (s, 2H), 3.7 (d, 2H)
 FAB-MS: M_r found: 510.3

N-Benzoyloxycarbonyl-glycyl-D,L-3-thia-N⁶-tert.-butyloxycarbonyl-lysine benzyl ester 7a

3.3 g (8 mmol) Z-Gly-Gly(OAc)-OBzl **4a** was allowed to react with 2 g (10 mmol) N-Boc-1-aminopropane-3-thiol and 1.5 ml (10 mmol) triethylamine in 50 ml DMF by analogy to Z-Gly-D,L-3-thia-Phe-OBzl **5a**.

yield: 2.8 g (65.1%)

mp: 64–65°C

N-Benzoyloxycarbonyl-glycyl-D,L-3-thia-lysine benzyl ester hydrochloride 8a

1.63 g (3 mmol) Z-Gly-D,L-3-thia-Lys(Boc)-OBzl **7a** was selectively deblocked with HCl/AcOH to yield a hygroscopic solid after evaporation. This material was used without further purification.

yield: 1.2 g (83.3%)

N-Benzoyl-glycyl-D,L-3-thia-N⁶-tert.-butyloxycarbonyl-lysine benzyl ester 7b

3.5 g (10 mmol) of Bz-Gly-Gly(OAc)-OBzl **4b** were reacted with 2.3 g (12 mmol) of N-Boc-1-aminopropane-3-thiol in 70 ml THF and 1.6 ml of triethylamine at 20°C, and stirred for 24 hours. The solvent was evaporated in vacuo to give a yellow oil. This material was diluted with H₂O, then extracted with AcOEt several times. The washed organic extracts were combined and evaporated. The oily residue was further purified using liquid chromatography over silica gel (eluent: cyclohexane/AcOEt 1 : 1) to give 3.2 g of a solid.

yield: 3.2 g (62.2%)

mp: 67–69°C

Analysis for: C₂₆H₃₃N₃O₆S; M_r = 514.6; calc. (%) C: 60.56; H: 6.45; N: 8.14; S: 6.21; found (%) C: 60.17; H: 6.98; N: 8.30; S: 6.51 ¹H-NMR: CDCl₃; ppm; 7.8 (m, 1H), 7.4 (m, 10H), 5.6 (d, 1H), 5.2 (q, 2H), 4.25 (t, 2H), 3.1 (m, 2H), 2.6 (m, 2H), 1.7 (m, 2H), 1.4 (s, 9H)

N-Benzoyl-glycyl-D,L-3-thia-lysine benzyl ester hydrochloride 8b

The tert.-butyloxycarbonyl blocking group of **7b** was removed using HCl/AcOH to give the hydrochloride of Bz-Gly-D,L-3-thia-Lys-OBzl **8b**.

yield: 2.17 g (77%)

mp: 150–152°C

Analysis for: C₂₁H₂₅N₃O₄S · HCl; M_r = 450.96; calc. (%) C: 55.1; H: 5.79; N: 9.29; found (%) C: 54.1; H: 5.69; N: 8.86

¹H-NMR: CDCl₃; ppm; 9.0 (d, 1H), 8.9 (d, 1H), 8.0 (s, 3H), 7.4 (m, 10H), 5.54 (d, 1H), 5.2 (m, 2H), 4.0 (m, 2H), 3.35 (m, 2H), 2.5 (m, 2H), 1.8 (m, 2H)

FD-MS: M_r found 416 (for **8b** minus HCl)

N-Benzoyloxycarbonyl-L-phenylalanyl-D,L-3-thia-leucine benzyl ester 16

Procedure A, by analogy to Zoller (1975)

To a stirred suspension of 231 mg (0.5 mmol) **14**, prepared according to Hwang (1986) in 61% yield, and 152 mg (2 mmol) 2-propanethiol in glacial acetic acid (2 ml) at ice bath temperature conc. H₂SO₄ (50 μ l) was added. After stirring at room temperature for two days the mixture was poured into ice and the organic material was extracted threetimes with AcOEt. The solvent was removed in vacuo and the residue was worked up as described in procedure B, yield: 34%.

Procedure B

2 g (4.3 mmol) **14** were dissolved in freshly distilled thionyl chloride (15 ml) and the mixture stored at room temperature overnight. Thionyl chloride was removed under reduced pressure and the residue **15** (Cl: calc. (%) 7.37, found (%) 7.47) was dried over KOH in vacuo and used without further purification. 481 mg (1 mmol) of **15** was dissolved in acetonitrile (5 ml) and stirred with 201 mg (1.1 mmol) AgSCH(CH₃)₂ (obtained from 2-propanethiol and AgNO₃-solution) for 4 days. Filtration and removal of the solvent under reduced pressure afforded an oily residue which was purified by column chromatography on silica gel (Kieselgel 60, Merck; eluent: cyclohexane/AcOEt 1 : 1 (v/v)) and additional recrystallization from EtOH/H₂O to give **16**.

yield: 0.3 g (57%)

m.p. 91–93°C

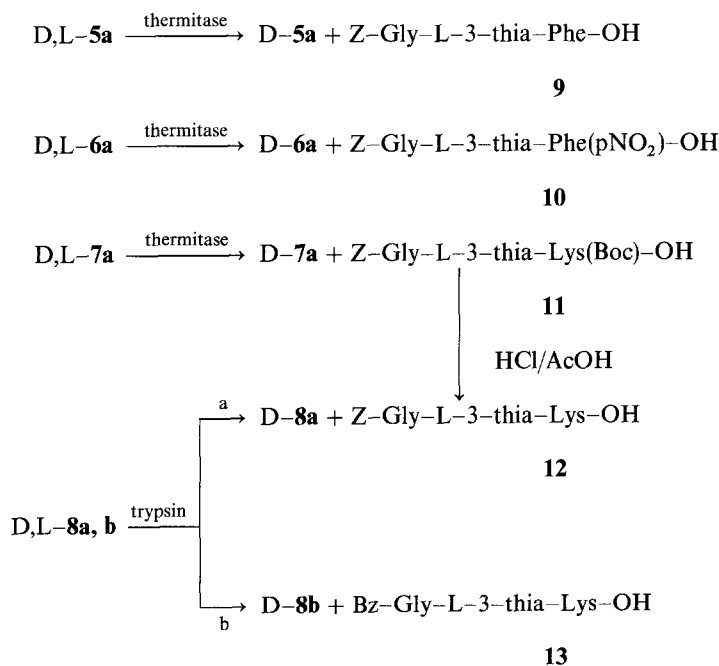
Analysis for: C₂₉H₃₂N₂O₅S; M_r = 520.63; calc. (%) C: 66.89; H: 6.19; N: 5.38; S: 6.16; found (%) C: 65.11; H: 5.93; N: 5.42; S: 6.22

FD-MS: M_r found: 520.20

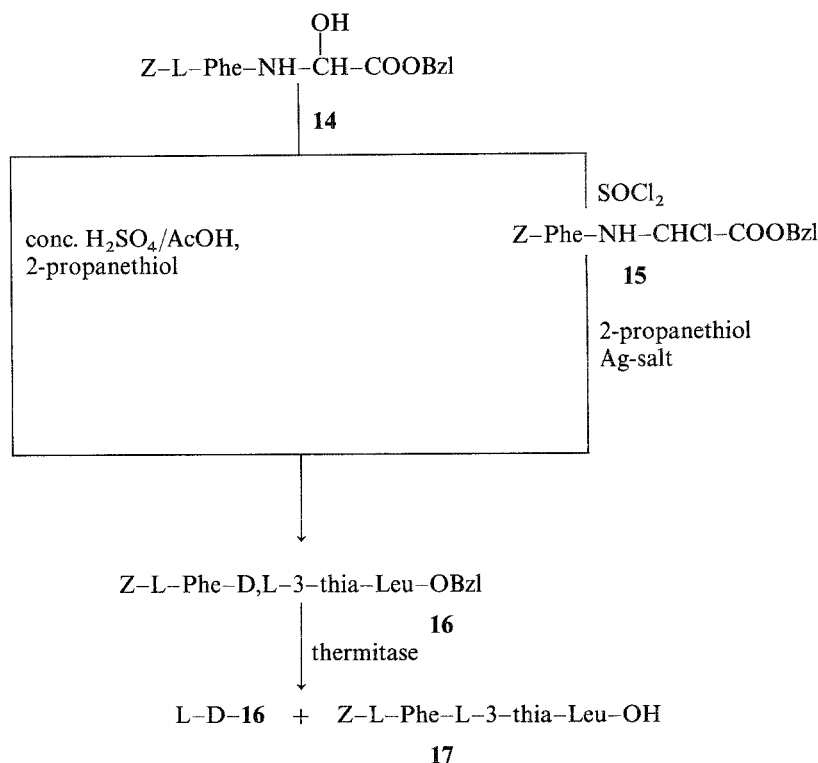
General procedure for the enzymatic hydrolysis of the benzyl esters (Scheme 2)

A: Thermitase

The racemic ester (**5a**, **6a**, **7a**, **16**) was dissolved in a mixture of DMF and water containing CaCl₂ (10⁻⁴ mol/l). A solution of thermitase in 0.01 M ammonium acetate-buffer, pH 6, was added and the pH (Table 1) was kept constant by the pH-stat-method. After completion of the enzymatic hydrolysis the reaction mixture was evaporated to dryness in vacuo and the residue was distributed in a 5% KHCO₃/AcOEt-mixture. The water layer was washed with AcOEt to remove the D-ester. The combined organic layers were dried over anhydrous Na₂SO₄, concentrated and treated with hexane or ether to isolate the D-ester compounds **5a**, **7a**, **16**. **D-6a** was contaminated with a small amount of **L-6a** (see discussion). The compounds were recrystallized from EtOH/H₂O. The yields and some analytical data



Scheme 2



Scheme 3

are documented in Table 1. The water phase was acidified with HCl to pH 2 and extracted with AcOEt. The combined extracts were dried over anhydrous Na₂SO₄ and evaporated in vacuo. Recrystallization from EtOH/H₂O afforded **9**, **10**, **11** (Scheme 2), and **17** (Scheme 3) respectively.

B: Trypsin

The racemic dipeptide ester hydrochloride (**8a**, **8b**) was dissolved in H₂O. A solution of trypsin in 0.01 M ammonium acetate buffer, pH 6, was added and the pH (Table 1) was kept constant by the pH-stat-method. After completion of the enzymatic hydrolysis the L-derivative was isolated by ion exchange chromatography (strong cation exchanger, Wolfen KPS 200; eluent: 1 M pyridine). Compounds **12** and **13** were obtained as crystalline materials after evaporation of the solvent, see Table 1.

N-Benzyloxycarbonyl-glycyl-L-3-thia-phenylalanine **9**

Analysis for **9**: C₁₈H₁₈N₂O₅S; M_r = 374.42; calc. (%) C: 57.7; H: 4.84; N: 7.48; S: 8.56; found (%) C: 57.31; H: 4.86; N: 7.52; S: 8.71

¹H-NMR: CD₃OH; ppm; 7.33 (m, 10H), 5.64 (s, 1H), 5.11 (s, 2H), 4.18 (s, 2H), 3.79 (s, 2H)

N-Benzyloxycarbonyl-glycyl-L-p-nitro-3-thia-phenylalanine **10**

Analysis for **10**: C₁₈H₁₇N₃O₇S; M_r = 419.42; calc. (%) C: 51.54; H: 4.08; N: 10.01; S: 7.64; found (%) C: 51.47; H: 4.08; N: 9.97; S: 7.72

¹H-NMR: CDCl₃; ppm; 13.7 (s, 1H), 9.0 (d, 1H), 7.5 (m, 9H), 5.9 (d, 1H), 5.02 (s, 2H), 3.68 (d, 2H)

FD-MS: M_r found 420

Table 1. Conditions for the enantioselective enzymatic hydrolysis of benzyl ester derivatives of dipeptides containing 3-thia-analogues of amino acids

racem. ester	enzyme/substrate ratio (mol/mol)	% DMF	Temp. (°C)	pH	product	yield %	m.p. (°C)	specific rotation [α] _D ²⁵ (°)	c	solv.
5a	1 : 2 · 10 ⁵	40	45	8.0	9	93.5	140–144	+65.5	0.5	EtOH
6a	1 : 7 · 10 ⁵	30*	50	8.0	D-5a	41.6	84–85	–35.7	2.1	AcOEt
7a	1 : 1 · 10 ⁵	35	50	7.6	10	48.0	148–152	+6.6	0.4	H ₂ O
					11	61.3	103–107	+108.7	1.6	EtOH
16	1 : 1 · 10 ⁵	50	50	7.5	D-7a	74.3	85–88	–67.8	2	EtOH
					17	72.0	136–137	+22.0	0.5	EtOH
8a	7 : 1 · 10 ⁶	—	37	7.2	L-D-16	90.0	96–97	–46.8	0.8	EtOH
8b	2 : 1 · 10 ⁵	—	37	7.2	12	92.0	196–198	+91.8	0.8	1N HCl
					13	87.0	210–211	+127.2	0.8	1N HCl

* Butane-1,4-diol was used instead of DMF

N-Benzyloxycarbonyl-glycyl-L-3-thia-N⁶-tert.-butyloxycarbonyl-lysine **11**

Analysis for **11**: C₂₀H₂₉N₃O₇S; M_r = 455.53; calc. (%) C: 52.73; H: 6.41; N: 9.22; found C: 49.8; H: 5.96; N: 8.44

N-Benzyloxycarbonyl-L-phenylalanyl-L-3-thia-leucine **17**

Analysis for **17**: C₂₂H₂₆N₂O₅S; M_r = 430.526; calc. (%) C: 61.37; H: 6.09; N: 6.50; S: 7.45; found (%) C: 61.62; H: 6.10; N: 6.50; S: 7.52

N-Benzyloxycarbonyl-L-phenylalanyl-D-3-thia-leucine benzyl ester L-D-**16**

Analysis for L-D-**16**: C₂₉H₃₂N₂O₅S; M_r 520.63; calc. (%) C: 66.89; H: 6.19; N: 5.38; S: 6.16 found (%) C: 66.37; H: 6.15; N: 5.31; S: 6.11

N-Benzyloxycarbonyl-glycyl-L-3-thia-lysine **12**

Analysis for **12**: C₁₅H₂₁N₃O₅S; M_r = 355.41; calc. (%) C: 50.69; H: 5.96; N: 11.82; found (%) C: 50.71; H: 5.94; N: 11.85.

12 was also obtained by deprotection of **11** using HCl/AcOH according to Bodanszky (1984, p. 169) followed by desalting by the ion exchange procedure described above.

N-Benzoyl-glycyl-L-3-thia-lysine **13**

Analysis for **13**: C₁₄H₁₅N₃O₄S; M_r = 325.39; calc. (%) C: 51.67; H: 5.88; N: 12.91; found (%) C: 51.61; H: 5.97; N: 12.8

Results

Synthesis of optically active dipeptides containing glycine

Enzymatic resolution of the esters **5a**, **6a**, and **7a** (Scheme 2) was performed by thermitase delivering the N-protected optically active peptides **9**, **10**, **11**.

The enantioselective tryptic hydrolysis of the benzyl ester group of the racemic compounds **8a**, **b**, which were obtained after deprotection of the N⁶-amino groups of **7a**, **b** using HCl/AcOH, afforded the optically active protected dipeptides **12** and **13**.

The dipeptide derivative **12** was obtained also by N⁶-deprotection of the derivative **11** (Scheme 2).

Details of the enantioselective enzymatic hydrolyses are summarized in Table 1. The application of thermitase (M_r: 28 369) allows elevated reaction temperatures resulting in an accelerated turnover. Cosolvents are necessary to achieve appropriate solubility of the ester compounds. The enzyme/substrate ratios were chosen so that enzymatic hydrolyses of the L-compounds were complete within two hours. The yields are calculated for the recrystallized materials.

Synthesis of N-benzyloxycarbonyl-L-phenylalanyl-L-3-thia-leucine

In the synthesis of the N-protected dipeptide derivative Z-L-Phe-L-3-thia-Leu-OH (**17**) the intermediate acetylated N-acetal described by Hwang (1986) re-

sulted in only poor yield in the reaction with 2-propanethiol. Better results were obtained when activation of the hydroxy group and thiolysis were done in a mixture of acetic acid and conc. sulfuric acid according to Zoller (1975).

We describe here an alternative procedure in which activation was achieved with thionylchloride and N,S-acetal **16** formation via the silver salt of 2-propanethiol (Scheme 3). Diastereoselective enzymatic hydrolysis of **16** was performed using thermitase to give the N-protected L-L-dipeptide **17**.

Discussion

Derivatives of 3-thia-analogues of amino acids are usually obtained as racemates. In the case of dipeptide or oligopeptide derivatives containing L-amino acids in addition to the racemic analogues the separation of the diastereoisomers by fractional crystallization or chromatographic procedures are the methods of choice as described by Gilvarg (1984 a and b).

The low yields in the synthesis of Z-Gly-Gly(OH)-OBzl **3a** and Bz-Gly-Gly(OH)-OBzl **3b** are explained mainly by the restricted solubility of Z-Gly-NH₂ and Bz-Gly-NH₂ in the solvents necessary for the condensation with glyoxylic acid benzyl ester. The condensation with e.g. Z-Ala-NH₂ or Z-Phe-NH₂ resulted in yields between 60 and 70% (Hwang, 1986 and own result in the synthesis of compound **14**).

The syntheses of optically active dipeptides containing glycine in addition to the analogue require methods of racemate resolution.

Peptide synthesis makes use of a variety of protecting groups which usually can be split selectively at different steps of the synthesis-scheme. In the last years enzymes had been applied in the manipulation of protecting groups (for review see Hermann, 1986).

For the synthesis of the carboxypeptidase A substrates **9** and **10**, and the carboxypeptidase B substrates **12** and **13** selective C-terminal deprotection of the racemic benzyl ester derivatives **5a**, **6a**, **7a**, **8a** and **8b** can be achieved by an enantioselective enzymatic hydrolysis (Scheme 2).

Thermitase, an alkaline serine protease from *Thermoactinomyces vulgaris* (Kleine, 1981), has a high esterase/peptidase ratio and has therefore been proposed for the selective C-terminal ester hydrolysis (deprotection) in preparative peptide synthesis by Hermann (1983). As compared with chymotrypsin thermitase has a rather broad "side chain specificity" which enables a wide field of applications. Racemate resolutions of amino acid ester derivatives using thermitase were already described by Lankiewicz (1989).

The results of the thermitase-catalyzed ester hydrolyses of racemic dipeptide derivatives containing glycine in addition to the thia-analogue illustrate the high efficiency (enzyme/substrate ratio) of this enzyme for racemate resolutions. The low yields in the synthesis of Z-Gly-L-p-nitro-3-thia-phenylalanine **10** is explained by the low solubility of the ester compound **6a** in butane-1,4-diol; aprotic cosolvents like DMF were inefficient in this case. Enzymatic hydrolysis was stopped after reaching nearly theoretical NaOH consumption in the pH-stat technique. The residual D-ester **6a** could therefore not be isolated in a form free of the L-compound.

In the synthesis of Z-Gly-3-thia-Lys-OH **12** alternative routes were studied (Scheme 2). The enantioselective ester hydrolysis of **7a** using thermitase demonstrates the acceptance of the rather bulky side chain by the enzyme. The application of trypsin for the hydrolysis of the ester compounds **8a** and **8b**, bearing the basic side chains which is in agreement with tryptic specificity, resulted however in better yields. Both procedures produced identical dipeptide material **12**.

The enzymatic approach was also applied in the separation of the diastereoisomeric pair Z-L-Phe-D, L-3-thia-Leu-OBzl **16**. Diastereoselective enzymatic hydrolysis catalyzed by thermitase afforded both the L-L-Z-dipeptide and the L-D-Z-dipeptide ester in high yields.

It is demonstrated here that the enzymatic method can compete successfully with fractional crystallization or chromatographic separation of diastereoisomers.

Acknowledgement

This research was supported by grant WA559/6-1 awarded by the Deutsche Forschungsgemeinschaft.

References

- Bodanszky M, Bodanszky A (1984) The practice of peptide synthesis. Springer, Berlin Heidelberg New York Tokyo
- Cavallini D, Federici G, Dupre S, Canella C, Scandurra R (1979) Pure Appl Chem 52: 147–155
- Chemiakine MM, Tchaman ES, Denisova LI, Ravdel GA, Rodinow WJ (1959) Bull Soc Chim France 1959: 530–536
- Gilvarg C, Kingsbury WD (1984a) US 4 454 065, Int. Cl. C 07 C 103/52, 12.06.1984
- Gilvarg C, Kingsbury WD (1984b) US 4 479 898, Int. Cl. C 07 C 103/52, 30.10.1984
- Greiner G, Hermann P (1991) Application of penicillin amidohydrolase (PAH) for sulphur and amino deprotection in peptide synthesis. In: Giralt E, Andreu D (eds) Peptides 1990, Proceedings 21th European Peptide Symposium, Platja d'Aro 1990. Escom, Leiden, pp 277–278
- Grüb H (1971) Synthese von Gastrinteilsequenzen nach der Festkörpermethode. Thesis, Fachbereich Chemie, Univ. Tübingen, p 55
- Hermann P, Schreier E (1973) Gewinnung von S-Acetamidomethyl-L-cystein. In: Hanson H, Jakubke H-D (eds) Peptides 1972, Proceedings of the 12th European Peptide Symposium, Reinhardtsbrunn Castle 1972. North-Holland, Amsterdam, pp 126–127
- Hermann P, Hoffmann G (1976) Application of S-chloroacetamidomethyl-L-cysteine in peptide synthesis. In: Loffet A (ed) Peptides 1976, Proceedings 14th European Peptide Symposium, Wepion 1976. Editions de l'Université de Bruxelles, Bruxelles, pp 121–122
- Hermann P (1981) Chemistry and biochemistry of thia-analogues of amino acids. In: Freidlina RKh, Skorova AE (eds) Organic sulfur chemistry. Invited lectures, 9th Internat. Symposium on Organic Sulfur Chemistry (IUPAC), Riga 1980. Pergamon Press, Oxford, pp 51–67
- Hermann P, Salewski L (1983) Thermitase-catalyzed hydrolysis of the C-terminal ester group of protected peptide ester derivatives. In: Blaha K, Malon P (eds) Peptides 1982, Proceedings 17th European Peptide Symposium, Prague 1982. de Gruyter, Berlin, pp 399–402
- Hermann P (1986) Enzyme als Reagenzien zur Schutzgruppenabspaltung bei der Peptidsynthese. In: Wilhelmi B (ed) Chemie und Biologie von Peptidwirkstoffen. Jenaer Symposium, Jena, pp 101–112

- Hwang SY, Kingsbury WD, Hall NM, Jakas DR, Dunn GL, Gilvarg C (1986) *Anal Biochem* 154: 552–558
- Kelly TR, Schmidt TE, Haggerty JG (1972) *Synthesis* 1972: 544–545
- Kingsbury WD, Boehm JC (1986) *Int J Peptide Protein Res* 27: 659–665
- Kleine R, Rothe U, Kettmann U, Schelle H (1981) Purification, properties and application of thermitase a microbial serine protease. In: Turk V, Vitale L (eds) *Proteinases and their inhibitors: Structure, function and applied aspects*. Pergamon Press, Oxford, pp 201–212
- Lankiewicz L, Kasprzykowski F, Grzonka Z, Kettmann U, Hermann P (1989) *Bioorg Chem* 17: 275–280
- Pojer PM, Rae ID (1972) *Aust J Chem* 25: 1737–1749
- Siemion IZ, Nowak K (1961) *Rocz Chemii* 35: 153–158
- Steglich W, Tanner H, Hurnaus R (1967) *Chem Ber* 100: 1824–1832
- Veber DF, Milkowski JD, Varga SL, Denkwalter RG, Hirschmann R (1972) *J Am Chem Soc* 94: 5456–5461
- Willhardt I, Pawlikowski K, Hermann P (1991) in preparation
- Zoller U, Ben-Ishai D (1975) *Tetrahedron* 31: 863–866

Authors' address: Prof. Dr. P. Hermann, Institute of Biochemistry, Medical Faculty, Martin-Luther-University, P.O. Box 302, D-O-4010 Halle, Federal Republic of Germany.