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Are PhAcOZ and Nsc suitable N^{α}-protecting groups for protease-catalysed peptide synthesis?

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Protecting groups are necessary chemical tools in peptide synthesis. In protease-catalysed peptide synthesis they exert influence both on enzyme-substrate binding and on solubility. In this study, we have investigated the usability of various PhAcOZ- and Nsc-protected amino acids for protease-catalysed peptide synthesis. PhAcOZ-protected peptides were obtained in high yields using papain and thermolysin. In contrast to this, Nsc, as a base-labile α-amino protecting group, is not suitable for biocatalytic synthesis under the conditions employed.

1. Introduction

Protein conjugates in which the amino acid backbone is covalently linked to carbohydrates, lipids or phosphates play important roles in many biological processes. Lipid-modified model peptides were for instance used in the study of membrane binding and membrane targeting of Ras-proteins. The synthesis of lipopeptides requires various orthogonally stable protecting groups, which subsequently have to be removed under the mildest conditions due to the presence of acid- and base-sensitive structures [1].

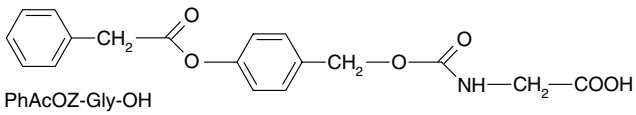
Enzymatic methods offer advantageous alternatives to classical chemical techniques for the solution of these problems. Biocatalysts often operate at neutral, weakly acid or weakly basic pH values. In many cases, they combine a high selectivity for the catalysed reactions and the recognized structure with a broad substrate tolerance.

It is well known that Bz-Phe as well as Z-Arg and Ac-Arg can be used as enzyme-labile N^{α}-protecting groups and split off by chymotrypsin and trypsin [2]. Unfortunately, these proteases are able to cleave behind these amino acids also in other positions of the peptide backbone. The urethane hydrolases I–III and IV, for example, are suitable for removing the N^{α}-protecting groups Z-, Boc- and Ac at least from a part of the amino acids [3, 4].

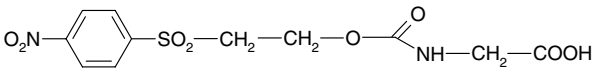
The enzyme-labile urethane protecting group PhAcOZ, well established by Pohl and Waldmann [5], contains a phenylacetic acid ester moiety which is recognized by penicillin G acylase. This biocatalyst operates under mild conditions. It is highly specific for the phenylacetyl group and does not attack any peptide, phosphate and glycosyl bonds [6]. Another urethane protecting group, AcOZ, can be specifically attacked by acetyl esterase and lipases [1].

PhAcOZ-protected amino acids were successfully used for the chemical synthesis of several peptide fragments [5].

Enzymes have proved to be an attractive alternative to chemical methods in peptide synthesis, since several proteases effect peptide bond formation under mild conditions, with minimum side-chain protection, and avoiding racemization. Two strategies for enzyme-catalysed peptide synthesis are possible: equilibrium-controlled and kinetically-controlled synthesis [7, 8]. The equilibrium-controlled approach is based on direct reversal of proteolysis, and can be performed with proteases like thermolysin [9, 10]. In the kinetic approach, a serine- or cysteine protease reacts with a suitable ester substrate to form the acylenzyme intermediate that can be deacylated by the nucleophilic amino component or water [11, 12]. In the following, we want to report on the papain- and thermolysin-catalysed peptide synthesis using PhAcOZ-protected amino acids. Additionally, we tested the suitability of the base-labile N^{α}-protecting group Nsc for enzymatic peptide synthesis. Nsc-amino acids have, in comparison to Fmoc-protected ones, good solubility properties [13], and this is an important aspect for peptide synthesis.



PhAcOZ-Gly-OH



Nsc-Gly-OH

2. Investigations, results and discussion

At first, PhAcOZ-protected Gly, Ala, Phe, Met and the ester PhAcOZ-Gly-OMe were synthesized, and on the example of PhAcOZ-Gly-OH (Scheme) the substrate stability in buffered aqueous methanolic solutions at different pH was studied (Fig. 1). Under the conditions used, PhAcOZ-Gly-OH is very stable at pH 6 and 7. In the alkaline pH region the protecting group undergoes decomposition due to its ester structure, forming phenyl acetic acid and fragmentation products. At pH 9, PhAcOZ-Gly-OH sharply decreased to 19% within a period of 6 h. In the following the prepared PhAcOZ-protected amino acids

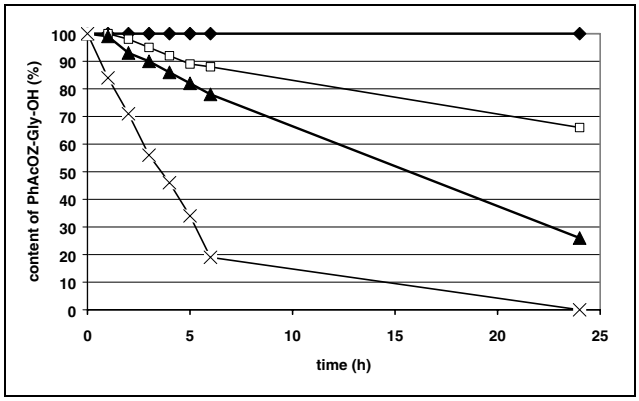


Fig. 1: Stability of PhAcOZ-Gly-OH at pH 6–9
Conditions: 5 mM PhAcOZ-Gly-OH; buffer (0.5 M); 40% (v/v) MeOH; 40 °C; ◆ pH 6; □ pH 7; ▲ pH 8; × pH 9

Table 1: Thermolysin-catalysed synthesis of PhAcOZ-Xaa$_1$-Xaa$_2$-NH$_2$

Substrate	Nucleophile				
	Peptide yield (%); (reaction time, h)				
	Leu-NH$_2$	Ile-NH$_2$	Phe-NH$_2$	Met-NH$_2$	Val-NH$_2$
PhAcOZ-Gly-OH	40 (24)	85 (24)	99 (24)	38 (24)	84 (120)
PhAcOZ-Ala-OH	52 (24)	94 (96)	75 (48)	99 (72)	85 (72)
PhAcOZ-Phe-OH	86 (72)	99 (48)	99 (48)	86 (48)	78 (24)
PhAcOZ-Met-OH	94 (48)	98 (24)	76 (48)	58 (24)	84 (24)

Experimental conditions: 100 mM acyl donor; 200 mM nucleophile; 0.27 mM thermolysin; 50% (v/v) ethyl acetate; Hepes-buffer (pH 6.5, 0.5 M, 5 mM $CaCl_2$); total volume: 500 µl; 40 °C

Table 2: Papain-catalysed peptide synthesis using PhAcOZ-Gly-OMe as acyl donor

Nucleophile	Peptide yield (%)	Reaction time (h)
Leu-NH$_2$	80	5.5
Ile-NH$_2$	90	24
Met-NH$_2$	18	24

Experimental conditions: 100 mM acyl donor; 400 mM nucleophile; papain 1 mM; 50% (v/v) ethyl acetate; Sörensen-buffer (pH 7, 0.5 M, EDTA, DTT); total volume: 500 µl; 40 °C

were used for the thermolysin-catalysed equilibrium-controlled reaction with amino acid amides under biphasic conditions at pH 6.5 (Table 1). This model system was chosen according to established enzymatic peptide synthesis reactions [14]. The protected glycine methyl ester was the substrate for kinetically-controlled peptide syntheses in the presence of papain at pH = 7 (Table 2). The results clearly demonstrate that PhAcOZ-protected peptides can be obtained in high yields with the help of thermolysin and papain, respectively.

The N$^\alpha$-protecting group Nsc, recently proposed as an alternative to Fmoc in solid phase peptide synthesis [13, 15, 16], was used for the synthesis of Nsc-Gly-OH (Scheme) and Nsc-Gly-OMe. The stability of Nsc-Gly-OH at pH 5–7 is shown in Fig. 2. This compound is fairly stable at lower pH and at pH 6 at least for 5 h. In slightly basic media, which are the preferred ones for kinetically-controlled enzymatic peptide synthesis [7], the Nsc group is to a certain extent subject to β-elimination resulting in the 4-nitrophenyl vinyl sulfone. This on its part alkylates the liberated amino acid at the α-amino group [17]. Therefore, the enzyme-catalysed reactions of Nsc-Gly-OH and Nsc-Gly-OMe with Leu-NH$_2$ and Ile-NH$_2$ were carried out at

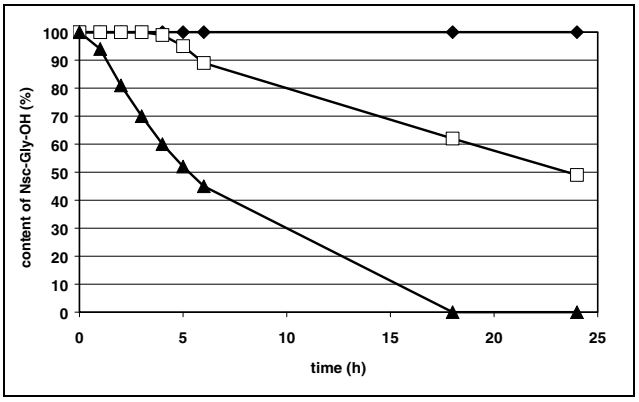


Fig. 2: Stability of Nsc-Gly-OH at pH 5–7
Conditions: 5 mM Nsc-Gly-OH; buffer (0.5 M); 40% (v/v) MeOH; 40 °C; ◆ pH 5; □ pH 6; ▲ pH 7

Table 3: Protease-catalysed synthesis of Nsc-Gly-Xaa-NH$_2$

Substrate	Nucleophile		
	Peptide yield (%); (reaction time, h)		
	Leu-NH$_2$	Ile-NH$_2$	Enzyme
Nsc-Gly-OH	20 (24)	40 (120)	Thermolysin[a]
Nsc-Gly-OMe	21 (24)	40 (16)	Papain[b]

Experimental conditions: [a] 100 mM acyl donor; 200 mM nucleophile; 0.27 mM thermolysin; 50% (v/v) ethyl acetate; Hepes-buffer (pH 6, 0.5 M, 5 mM $CaCl_2$); total volume: 500 µl; 40 °C;
[b] 100 mM acyl donor; 200 mM nucleophile; papain 1 mM; 50% (v/v) ethyl acetate; Sörensen-buffer (pH 6, 0.5 M, EDTA, DTT); total volume: 500 µl; 40 °C

pH 6 (Table 3). Under these conditions rather low peptide yields were achieved. Because of increasing side reactions, reasonable syntheses with these two nucleophiles at pH 7 could not be performed.

Our preliminary results suggest that PhAcOZ is a recommendable N$^\alpha$-protecting group also for protease-catalysed peptide synthesis. Thus, it can be well used for chemoenzymatic synthesis strategies. We could prepare PhAcOZ-protected peptides in high yields using papain and thermolysin. In contrast to this, Nsc as a base-labile α-amino protecting group is less suitable for biocatalytic syntheses.

3. Experimental

3.1. Materials

Papain (Merck) and thermolysin (Sigma) were used without further purification. Amino acids were purchased from Degussa. Organic solvents and other reagents were used without further purification from Aldrich, Lancaster, Merck and Sigma, solvents for HPLC-analyses were HPLC-grade. Nsc-Cl was a gift from Chemtec Leuna AG.

3.2. Preparation of N$^\alpha$-protected amino acid compounds

3.2.1. PhAcOZ-Xaa-OR

3.2.1.1. 4-(Phenylacetoxy)benzylalcohol (PBA)

Firstly, 1-methyl-3-(phenylacetyl)imidazolium chloride was produced as an intermediate compound for the synthesis of PBA starting from 1-methylimidazole and phenylacetyl chloride according to Guibé-Jampel et al. [18]. 6.2 mmol of the intermediate compound and 4.8 mmol (0.591 g) 4-hydroxybenzyl alcohol (dissolved in 10 ml 0.62 N KOH solution), were stirred at room temperature for 20 min according to Le Corre et al. [19]. The solution was twice extracted with ether. The combined organic phases were dried overnight with Na_2SO_4 and the organic phase was evaporated in vacuo. The pure substance resulted from purification by column chromatography on silica gel; chloroform/methanol; 5 : 1 (v/v). The alcohol was isolated as a white or a slightly yellow solid (yield 43%).

3.2.1.2. 4-(Phenylacetoxy)benzyl chloroformate (PhAcOZ-Cl)

PhAcOZ-Cl was synthesized at the Chemtec Leuna AG using PBA and phosgene according to Pohl and Waldmann [5].

3.2.1.3. PhAcOZ-Xaa-OH

The PhAcOZ-protected amino acids were prepared according to Pohl and Waldmann [5] using amino acid, trimethylchlorosilane and PhAcOZ-Cl. PhAcOZ-Gly-OH, PhAcOZ-Ala-OH and PhAcOZ-Phe-OH were obtained as white crystals and PhAcOZ-Met-OH was isolated as a slightly yellow oil (yields: 40%–70%).

3.2.1.4. PhAcOZ-Gly-OMe

4.3 mmol (0.542 g) Gly-OMe hydrochloride were dissolved in 15 ml methylene chloride and 8.6 mmol (0.867 g) trimethylamine and 4.3 mmol (0.467 g) trimethylchlorosilane were added. Then the mixture was heated under reflux for 90 min. After cooling down to 0 °C, 4.3 mmol (1.131 g) PhAcOZ-Cl were added and the mixture was stirred for 30 min at 0 °C. Then, it was stirred for 12 h at room temperature and finally evaporated in vacuo. The residue was dissolved in 50 ml ether and washed with 2.5% $NaHCO_3$ solution and thrice with water. The organic phase was dried with Na_2SO_4 and after evaporating the solvent in vacuo, the product was isolated as a white crystalline compound (yield: 12%).

3.2.2. Nsc-Gly-OR

Nsc-Gly-OH was prepared according to Samukov et al. [17] using glycine, trimethylchlorosilane and Nsc-Cl (yield: 89%). The synthesis of Nsc-Gly-OMe was carried out by esterification of Nsc-Gly-OH with methanol in the presence of thionyl chloride under the usual Brenner and Huber conditions (yield: 63%) [20]. Both compounds were obtained as a white powder.

3.2.3. Characterization of the compounds

The purity of all compounds was checked by TLC and HPLC. The newly synthesized substrates were characterized by ^{1}H spectroscopy. NMR spectra were recorded on a DRX 500 Bruker spectrometer at working frequencies of 500 MHz for ^{1}H.

PhAcOZ-Gly-OH: m.p.: 91–93 °C; (^{1}H NMR, DMSO-d_6); 3.66 (d, J = 6.2 Hz, 2 H, $C_\alpha H_2$), 3.96 (s, 2 H, CH_2-CO), 5.03 (s, 2 H, CH_2-O), 7.11 (d, J = 8.4 Hz, 2 H, 2 × OCCH arom.), 7.27–7.40 (m, 7 H, 2 × $CH_2-C\underline{CH}$ arom., $\underline{C_6H_5}-CH_2$), 7.57 (t, $J_1 = J_2 = 6.1$ Hz, 1 H, NH), 12.57 (br, 1 H, COOH).

PhAcOZ-Phe−OH: m.p.: 47–50 °C; (^{1}H NMR, DMSO-d_6); 2.87–2.89 (m, 1 H, $C_\beta H_a$), 3.17–3.19 (m, 1 H, $C_\beta H_b$), 3.98 (s, 2 H, CH_2-CO), 4.40–4.44 (m, 1 H, $C_\alpha H$), 5.01 (s, 2 H, CH_2-O), 7.10 (d, J = 8.4 Hz, 2 H, 2 × OCCH arom.), 7.22–7.37 (m, 12 H, 2 × $CH_2-C\underline{CH}$ arom., 2 × C_6H_5), 7.55 (t, $J_1 = J_2 = 6.0$ Hz, 1 H, NH), 12.57 (br, 1 H, COOH).

PhAcOZ-Met-OH: ^{1}H NMR, DMSO-d_6); 1.15 (m, 2 H, CH_2-S), 1.81–1.85 (m, 1 H, $C_\beta H_a$), 1.90–1.94 (m, 1 H, $C_\beta H_b$), 2.01 (s, 3 H, CH_3), 3.96 (s, 2 H, CH_2-CO), 4.03 (t, $J_1 = J_2 = 7.1$ Hz, 1 H, $C_\alpha H$), 5.0 (s, 2 H, CH_2-O), 7.12 (d, J = 8.5 Hz, 2 H, 2 × OCCH arom.), 7.28–7.40 (m, 7 H, 2 × $CH_2-C\underline{CH}$ arom., C_6H_5), 7.57 (t, $J_1 = J_2 = 6.0$ Hz, 1 H, NH), 12.5 (br, 1 H, COOH).

PhAcOZ-Gly-OMe: m.p.: 45–47 °C; (^{1}H NMR, DMSO-d_6); 3.62 (s, 3 H, CH_3), 3.76 (d, J = 6.1 Hz, 2 H, $C_\alpha H_2$), 3.96 (s, 2 H, CH_2-CO), 5.03 (s, 2 H, CH_2-O), 7.12 (d, J = 8.5 Hz, 2 H, 2 × OCCH arom.), 7.27–7.39 (m, 7 H, 2 × $CH_2-C\underline{CH}$ arom., $\underline{C_6H_5}-CH_2$), 7.71 (t, $J_1 = J_2 = 6.1$ Hz, 1 H, NH).

Nsc-Gly-OH: m.p.: 151–152 °C; (^{1}H NMR, DMSO-d_6); 3.52 (d, J = 6.1 Hz, 2 H, $C_\alpha H_2$), 3.84 (t, $J_1 = J_2 = 5.6$ Hz, 2 H, $S-CH_2$), 4.26 (t, $J_1 = J_2 = 5.6$ Hz, 2 H, CH_2-O), 7.06 (t, $J_1 = J_2 = 6.1$ Hz, 1 H, NH), 8.16 (AA′BB′, J = 8.8 Hz, 2 H, 2 × SO_2CCH arom.), 8.44 (AA′BB′, J = 8.9 Hz, 2 H, 2 × NO_2CCH arom.), 12.58 (br, 1 H, COOH).

Nsc-Gly-OMe: m.p.: 86–88 °C; (^{1}H NMR, DMSO-d_6); 3.61 (s, 3 H, CH_3), 3.64 (d, J = 6.1 Hz, 2 H, $C_\alpha H_2$), 3.84 (t, $J_1 = J_2 = 5.6$ Hz, 2 H, $S-CH_2$), 4.27 (t, $J_1 = J_2 = 5.6$ Hz, 2 H, CH_2-O), 7.22 (t, $J_1 = J_2 = 6.1$ Hz, 1 H, NH), 8.16 (AA′BB′, J = 8.8 Hz, 2 H, 2 × SO_2CCH arom.), 8.44 (AA′BB′, J = 8.8 Hz, 2 H, 2 × NO_2CCH arom.).

3.3. Stability of PhAcOZ-Gly-OH and Nsc-Gly-OH

N^α-protected amino acids (5 mM) were incubated in mixture of buffer (Sörensen or Kalthoff, 0.5 M, pH 6–9) and MeOH (40% v/v) in a total volume of 1 ml and agitated in an Eppendorf Thermomixer in 1.5 ml-vials at 40 °C. After a defined period, 20 µl of the mixture were taken and the content of the protected amino acid determined by HPLC.

3.4. Enzymatic peptide synthesis

3.4.1. Papain-catalysed peptide synthesis

In a typical procedure, papain (1 mM) was incubated in 50 µl Sörensen-buffer (pH 6.0 or 7.0, 0.5 M, EDTA, DTT) at 40 °C in 1.5 ml-vials. After 15 min, the reaction was started by adding 250 µl acyldonor (100 mM in ethyl acetate) and 200 µl nucleophile (200 mM or 400 mM). The nucleophile was dissolved in the same buffer and the pH was corrected. The vials were then agitated at 40 °C on an Eppendorf Thermomixer. After a given reaction time, the solvent was evaporated to dryness. The residue was dissolved in a mixture of acetonitrile and water (1 ml), filtered and analysed by HPLC. The optimal reaction time was determined by running parallel experiments.

3.4.2. Thermolysin-catalysed peptide synthesis

The reactions were performed in 1.5 ml-vials in a total volume of 500 µl containing 100 mM substrate, dissolved in 250 µl ethyl acetate, and 200 mM nucleophile, dissolved in 200 µl Hepes-buffer (0.5 M, 5 mM $CaCl_2$), followed by correction of the pH (6 and 6.5, resp.). The reaction was started by adding a solution of 50 µl thermolysin (0.27 mM in Hepes-buffer). The reactions were monitored and analysed as described in *3.4.1.*

3.4.3. Analyses

The composition of the reaction mixtures was determined by HPLC. The system was equipped with a Spectra Series P 100 pump and a Spectra Series UV 100 UVIS detector using a Merck LiChrospher 100 RP18 (5 µm, 4 × 4 mm) precolumn and a LiChrospher 100 RP18 (5 µm, 250 × 4 mm) column. Analyses were performed under isocratic conditions using various mixtures of acetonitrile in water containing 0.1% trifluoroacetic acid at a flow of 0.8 ml/min. Compounds were detected at a wavelength of 217 nm and at room temperature. The peptide products were identified by LC-MS. The results are the means of two reactions. The reproducibility of the results amounts to ± 2%.

Abbreviations:

AcOZ, 4-acetoxybenzyloxycarbonyl; Fmoc, 9-fluorenylmethoxycarbonyl; Nsc, 2-(4-nitrophenylsulfonyl)ethoxycarbonyl; PhAcOZ, 4-(phenylacetoxy)-benzyloxy-carbonyl; PBA, 4-(phenylacetoxy)benzylalcohol; PhAcOZ-Cl, 4-(phenylacetoxy)-benzyl chloroformate

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