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# A versatile and selective chemo-enzymatic synthesis of β-protected aspartic and γ-protected glutamic acid derivatives

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ABSTRACT

Two versatile, high yielding, and efficient chemo-enzymatic methods for the synthesis of β-protected Asp and γ-protected Glu derivatives using Alcalase are described. The first method is based on the α-selective enzymatic hydrolysis of symmetrical aspartyl and glutamyl diesters. The second method involving mixed diesters comprises a three-step protocol using (i) α-selective enzymatic methyl-esterification, (ii) chemical β-esterification, and finally (iii) α-selective enzymatic methyl ester hydrolysis. The yields of the purified β- and γ-esters range from 77% to 91%.



Protecting groups play a pivotal role in organic chemistry and especially in peptide, carbohydrate, or nucleic acid synthesis. In peptide synthesis, amine functionalities are usually protected as carbamates, and carboxylic acid moieties are most often protected as esters. A vast variety of esters have been developed to protect C-terminal and side-chain carboxylic acids for different applications. Orthogonality of these protecting groups and resistance to coupling and deprotection reaction conditions are key factors for high yields and for easy workup. For instance, allyl (All) esters, cleavable with Pd(0),[1] and trimethylsilylethyl (TMSE) esters, cleavable with TBAF,[2] are orthogonal to other commonly used protecting groups such as *tert*-butyl ($^t$Bu) or methyl (Me) esters allowing selective deprotection and modification of certain carboxylic acids during peptide synthesis. Of special interest are selectively protected aspartic acid (Asp) or glutamic acid (Glu) building blocks, which are protected either at their α-carboxylic acid functionality or at their β-(Asp) or γ-(Glu) carboxylic acid moiety. These esters find widespread application in on-resin synthesis of head-to-tail cyclic peptides[3] (using both Fmoc/$^t$Bu- and Boc/Bzl SPPS approaches), side-chain lactam peptides,[4] and branched peptides.[5] Recently, we described the selective α-carboxylic acid esterification of N-protected amino acids, including Asp and Glu residues, using the industrial enzyme Alcalase.[6] Selective synthesis of β-protected Asp and γ-protected Glu derivatives however, remains a challenge.

A number of synthetic strategies have been disclosed for (semi-)selective β- and γ-protection of Asp and Glu derivatives, respectively.[7] The most commonly used methods rely on the intramolecular anhydride formation of N-protected Asp/Glu derivatives using a condensing reagent followed by moderately selective ring opening with a nucleophile.[8] Whereas with Glu residues a relatively good selectivity of 9/1 of γ- over α-ester can be obtained, selective β-protection of Asp residues remains difficult and is often low yielding.

Only a few chemo-enzymatic approaches toward aspartyl β-esters have been reported in the literature. These approaches are based on the α-selective hydrolysis of Asp diesters. However, the reported α-selective hydrolysis with porcine liver esterase is only applicable for N-terminal unprotected aspartic acid derivatives.[9] Most proteases proved to be unselective when N-unprotected aspartyl diesters were used. Hydrolysis of N-protected aspartyl diesters with papain,[10] is limited to Cbz-Asp(OAll)-OAll. The very common N-terminal Fmoc/Boc-protected β-esters of Asp and γ-esters of Glu have not been prepared enzymatically. Herein we demonstrate that the cheap industrial protease Alcalase[11] can catalyze the α-selective hydrolysis of a wide range of N-protected Asp and Glu symmetrical diesters. In addition, we report a versatile and high yielding three-step protocol for the synthesis of N-protected β-aspartyl esters.

Alcalase is often used for the hydrolysis and/or resolution of amino acid esters.[12] Recently,[6] we identified a versatile synthetic method for various N-protected amino acid esters (i.e., methyl, ethyl, benzyl, *tert*-butyl, allyl, and trimethylsilylethyl) using

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Alcalase-cross-linked-enzyme-aggregates (CLEA)[13] in dry organic solvents. The hydrolysis of these esters proved to be easy and quantitative. Encouraged by these results, we decided to focus on the α-selective hydrolysis of N-protected aspartyl and glutamyl diesters (Scheme 1).

N-protected aspartyl ($n = 1$) and glutamyl ($n = 2$) diesters **1–8** were synthesized using EDC and the appropriate alcohol.[14] These symmetrical diesters were hydrolyzed using Alcalase-CLEA in water at pH 7.5 using $^t$BuOH or 1,4-dioxane as a co-solvent for dissolving the starting materials.[15]

Gratifyingly, all the hydrolytic reactions were completely α-selective, that is, no simultaneous β- or γ-ester hydrolysis was observed by HPLC. As shown in Table 1, very high yields of β-aspartyl and γ-glutamyl esters were obtained using a variety of N-protecting groups. To our surprise, even sterically hindered di-(2-phenyl-2-trimethylsilyl)ethyl (PTMSE) esters[16] of Asp were easily and α-selectively hydrolyzed by Alcalase-CLEA. The newly synthesized esters were analyzed by NMR[17] and were found to be identical to those reported in the literature[18] or to commercially available samples,[19] which clearly proved the selective α-hydrolysis of all the diesters used in this study.

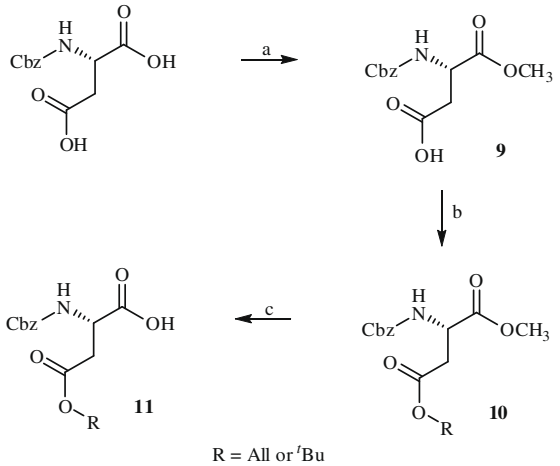
However, as also disclosed by others,[20] the di-$^t$Bu-ester derivatives of Asp and Glu were not easily hydrolyzed enzymatically. We envisioned that, by combining α-selective synthesis of N-protected Asp esters with chemical β-esterification followed by an α-selective hydrolysis of the resulting diesters, we could obtain a feasible approach toward β-protected Asp derivatives. Additionally, this alternative strategy avoids the use of 2 equiv of an expensive (e.g., TMSE-OH or PTMSE-OH) alcohol by preparation of a cheap methyl ester as a temporary protecting group of the α-carboxylic acid followed by chemical esterification of the β- or γ-carboxylic moiety (Scheme 2 and Table 2).

As shown in Table 2, the α-methyl esterification[21] proceeded smoothly and the chemical β-esterification, by activation with either EDC[22] for the All-ester or with $Boc_2O$[23] for the $^t$Bu-ester, furnished the desired esters in high yields. Much to our satisfaction, subsequent Alcalase-CLEA-mediated enzymatic hydrolysis of **10** ($R$ = All or $^t$Bu) proceeded completely α-selectively giving the desired β-protected Asp derivatives **11** in high yields.[24] Although this approach required three-reaction steps, the overall yields (76% for Cbz-Asp(OAll)-OH) were considerably higher compared to the overall yields obtained via hydrolysis of the diesters (57%) or those obtained by chemical means (around 50%). Even more importantly, this method allowed the synthesis of aspartyl β-esters which are not available *via* the diester hydrolysis method, for example, Cbz-Asp($O^t$Bu)-OH.

In conclusion, via two attractive approaches, we have demonstrated that the complete α-selectivity of Alcalase-CLEA in the synthesis and hydrolysis of various esters of N-protected Asp and Glu derivatives, can be utilized to prepare β-esters of N-protected aspartic acid or γ-esters of N-protected glutamic acid in high yields and purity. These derivatives are very useful for the synthesis of peptides and peptide derivatives.

**Table 1**
HPLC and isolated yields of the synthesized β- and γ-esters

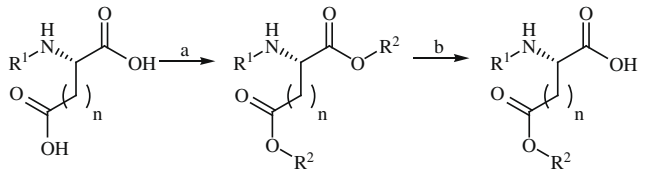
| Compound | Product | HPLC yield (%) | Isolated yield (%) |
|---|---|---|---|
| **1** | Cbz-Asp(OAll)-OH | 96 | 87 |
| **2** | Cbz-Glu(OAll)-OH | 97 | 85 |
| **3** | Boc-Asp(OAll)-OH | 98 | 87 |
| **4** | Boc-Glu(OAll)-OH | 98 | 86 |
| **5** | Fmoc-Asp(OAll)-OH | 95 | 77 |
| **6** | Fmoc-Glu(OAll)-OH | 97 | 78 |
| **7** | Cbz-Asp-(OTMSE)-OH | 95 | 85 |
| **8** | Fmoc-Asp-(OPTMSE)-OH | 96 | n.d. |




**Scheme 2.** Reaction conditions: (a) Alcalase-CLEA, 3 Å MS, MBTE/MeOH (14/1, v/v), 50 °C, 40 h; (b) EDC, DIPEA, AllOH, rt, 24 h or $Boc_2O$, pyridine, DMAP, $CH_3CN$/$^t$BuOH (2/1, v/v), rt, 24 h; (c) Alcalase-CLEA, pH 7.5 phosphate buffer/$^t$BuOH or 1.4-dioxane (1/1, v/v), 37 °C, 16 h.

**Table 2**
Yields of intermediates and Asp β-ester products

| Compound | Product | Isolated yield (%) |
|---|---|---|
| **9** | Cbz-Asp-OMe | 93 |
| **10a** ($R$ = $^t$Bu) | Cbz-Asp($O^t$Bu)-OMe | 94 |
| **10b** ($R$ = All) | Cbz-Asp(OAll)-OMe | 92 |
| **11a** ($R$ = $^t$Bu) | Cbz-Asp($O^t$Bu)-OH | 91 |
| **11b** ($R$ = All) | Cbz-Asp(OAll)-OH | 89 |




| # | $R^1$ | $R^2$ | n |
|---|---|---|---|
| **1** | Cbz | All | 1 |
| **2** | Cbz | All | 2 |
| **3** | Boc | All | 1 |
| **4** | Boc | All | 2 |
| **5** | Fmoc | All | 1 |
| **6** | Fmoc | All | 2 |
| **7** | Cbz | TMSE | 1 |
| **8** | Fmoc | PTMSE | 1 |

**Scheme 1.** Reaction conditions: (a) EDC, HOAt, DIPEA, $CH_2Cl_2$/$R^2$OH (25/1, v/v), rt, 24 h. (b) Alcalase-CLEA, pH 7.5 phosphate buffer/$^t$BuOH or 1,4-dioxane (1/1, v/v), 37 °C, 16 h.

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14. Diallyl ester synthesis of N-protected Asp and Glu; general procedure: 2.0 g of N-protected amino acid, 2.1 equiv of *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide. HCl (EDC·HCl), 2.1 equiv of 7-aza-*N*-hydroxybenzotriazole (HOAt), and 2.1 equiv of diisopropylethylamine (DIPEA) were dissolved in 100 mL of $CH_2Cl_2$ containing 4.0 mL of the appropriate alcohol. The mixture was stirred at ambient temperature for 24 h and concentrated in vacuo. The resulting oil was partitioned between 100 mL of EtOAc and 100 mL of saturated aqueous $NaHCO_3$. The organic phase was washed with 100 mL of saturated aqueous $NaHCO_3$, 100 mL of aqueous HCl (pH 1, 2×), 100 mL of brine and concentrated in vacuo. The resulting oil was purified by preparative HPLC (using 15% of 0.1 mL/L formic acid in $H_2O$ and 85% of 0.1 mL/L formic acid in acetonitrile as eluent).
15. Enzymatic α-hydrolysis of diallyl-protected Asp and Glu; general procedure: 3 g of Alcalase-CLEA (purchased from Codexis (Jülich, Germany) and used as received) was added to 0.5 g of diallyl-protected amino acid, 15.0 mL of $^t$BuOH or 1,4-dioxane, and 15.0 mL of phosphate buffer (pH 7.5, 50 mM). The mixture was shaken at 37 °C at 200 rpm for 16 h. After filtration, the enzyme was re-suspended in 50 mL of saturated aqueous $NaHCO_3$ followed by filtration. This procedure was repeated twice with saturated aqueous $NaHCO_3$ and twice with 50 mL of EtOAc. The combined organic phase was washed with saturated aqueous $NaHCO_3$. The combined aqueous phase was acidified to pH 1 with aqueous HCl (0.1 N) followed by extraction with 100 mL of EtOAc (3×). The combined organic layers were washed with 250 mL of brine, dried over $Na_2SO_4$, concentrated in vacuo, and dried by co-evaporation with 50 mL of toluene (2×) and 50 mL of $CHCl_3$ (2×). Additional column chromatography with EtOAc/*n*-hexane mixtures was performed if necessary. The purity of the β- or γ-ester of the N-protected Asp or Glu derivatives, respectively, was >98% as determined by HPLC.
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21. Alcalase-CLEA (3 g) was added to 0.5 g of Cbz-Asp-OH, 28.0 mL of MTBE, 2.0 mL of MeOH, and 2.0 g of 3 Å molecular sieves. The mixture was shaken at 50 °C at 150 rpm for 16 h. After filtration, the enzyme was washed thoroughly with aqueous HCl (pH 1, 3 × 50 mL) and EtOAc (3 × 50 mL) followed by filtration. The combined organic layers were washed with 100 mL of aqueous HCl (pH 1), dried ($Na_2SO_4$), and concentrated in vacuo. The resulting oil was redissolved in 20 mL of $CH_2Cl_2$/MeOH/AcOH (89.9:10:0.1) followed by a filtration over silica gel. The mixture was concentrated in vacuo and dried by co-evaporation with 50 mL of toluene (2×) and 50 mL of $CHCl_3$ (2×). The NMR data were identical to those of the commercially obtained compound.
22. Cbz-Asp-OMe (281 mg, 1.0 mmol) was dissolved in 50 mL of EtOAc. Subsequently, 209 mg of EDC·HCl (1.1 mmol), 192 μL of DIPEA (1.1 mmol), and 2.0 mL of All-OH were added. The reaction mixture was stirred for 24 h at rt. The mixture was washed with 50 mL of saturated aqueous $NaHCO_3$, 50 mL of aqueous HCl (pH 3, 2×), 50 mL of brine, filtered over basic alumina, dried ($Na_2SO_4$) and concentrated in vacuo. NMR data corresponded to those reported by: Sears, P.; Schuster, M.; Wang, P.; Witte, K.; Wong, C. H. *J. Am. Chem. Soc.* **1994**, *116*, 6521–6530.
23. Cbz-Asp-OMe (281 mg, 1.0 mmol) was dissolved in 60 mL of $CH_3CN$/$^t$BuOH (2/1, v/v). Subsequently, 218 mg of $Boc_2O$ (1.0 mmol), 81 μL of pyridine (1.0 mmol), and 5 mg of (*N,N*)-dimethylaminopyridine were added. The reaction mixture was stirred at rt for 24 h and concentrated in vacuo. The residue was dissolved in 50 mL of EtOAc and washed with 50 mL of saturated aqueous $NaHCO_3$, 50 mL of aqueous HCl (pH 3, 2×), 50 mL of brine, filtered over basic alumina, dried ($Na_2SO_4$) and concentrated in vacuo. NMR data corresponded to those reported by David, C.; Bischoff, L.; Roques, B. P.; Fournie-Zaluski, M. C. *Tetrahedron* **1999**, *56*, 209–215.
24. NMR data corresponded with the commercially available reference compound, Cbz-Asp(O$^t$Bu)-OH: CAS. 5545-52-8.