Versatile Selective α-Carboxylic Acid Esterification of N-Protected Amino Acids and Peptides by Alcalase

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Abstract: Under continuous removal of water, the industrial protease Alcalase allows selective synthesis of α-carboxylic acid methyl, ethyl, benzyl, allyl, 2-(trimethylsilyl)ethyl, and *tert*-butyl esters of amino acids and peptides under mild conditions in very high yields. The purified yields range from 72% to 92%.

Key words: enzymes, amino acids, esterification, esters, protecting groups

There is a great demand for easily accessible amino acid building blocks for their use in peptide synthesis. For different applications, a large variety of side chain, α-amino and/or α-carboxylic acid protected amino acids is required. Orthogonality of the different protecting groups used is pivotal for conventional peptide synthesis protocols. Whereas the α-amino functions are most often protected as carbamates, the α-carboxylic acid functions are most often protected as esters. Esters can be synthesized by mineral acid catalysis using, for example, hydrochloric or sulfuric acid in the presence of the (unprotected) amino acid and the corresponding alcohol. However, this method is restricted to *n*-alkyl esters such as methyl, ethyl, or benzyl and for a subset of amino acids. More difficult to synthesize, but very commonly used as protecting groups in Fmoc- or Boc-based solid phase peptide synthesis, are the orthogonal esters such as the *tert*-butyl, allyl (All) or 2-(trimethylsilyl)ethyl (TMSE) esters. 2-(Trimethylsilyl)ethyl esters, cleavable with tetrabutylammonium fluoride,[1] and allyl esters, cleavable with palladium(0),[2] are orthogonal to other commonly used carboxylic acid protecting groups thus allowing selective deprotection and modification of certain carboxylic acids during peptide synthesis. Of special interest are α-carboxylic protected aspartic acid (Asp) or glutamic acid (Glu) building blocks, since these also contain a β- or γ-carboxylic acid moiety, respectively. These esters are used for (Fmoc- and Boc-based) on-resin synthesis of head-to-tail cyclic peptides,[3] side-chain lactam peptides,[4] and branched peptides.[5]

In chemoenzymatic peptide synthesis α-carboxylic acid esters are not only used for protection, but also, more importantly, for activation in the case of a kinetically controlled process. Most often, nonsterically hindered esters as methyl, ethyl, or benzyl esters are used as activated substrates.[6]

The chemical synthesis of amino acid esters in which the ester moiety is sterically demanding often requires harsh conditions, which is troublesome for sensitive substrates. For instance, *tert*-butyl esters are often synthesized using a strong mineral acid with isobutene under high pressure,[7] which is laborious in laboratory practice. Furthermore, selective chemical α-carboxylic acid protection of aspartic acid and glutamic acid is difficult due to the similar reactivity of the side chain carboxylic acid functionalities. Although several one step methods have been described for (semi)selective side chain esterification,[8] α-carboxylic acid protection of aspartic acid and glutamic acid usually needs multistep and/or low yielding protocols. For instance, the internal anhydride method[9] and the chloroformate method[10] both give approximately 3:2 mixtures of α-carboxy vs β/γ-carboxy esterification.

To overcome the selectivity issue and to avoid racemization, as is often encountered in chemical esterification, N-protected amino acids have been esterified using enzymes.[11] However, the water produced during the reaction and the water required for enzyme activity prohibits a favorable position for the esterification equilibrium resulting in low to moderate yields.[12] High yields were reported using esterases or lipases in dry organic solvents, but these enzymes are usually not selective for the α-carboxylic moiety. Proteases such as papain,[13] chymotrypsin,[14] and α-chymotrypsin[15] are α-selective, but only demonstrated esterification with sterically undemanding alcohols such as methanol, ethanol, or benzyl alcohol. Esterification of amino acids to aliphatic esters with Alcalase has, to our knowledge, only been disclosed for Boc-Phe-OEt (65% in 48 h using subtilisin). Herein we demonstrate that the inexpensive industrial protease Alcalase[16] (from Novozymes, 10 wt% Alcalase solution, 6.75 €/kg) can catalyze the esterification of a wide range of N-protected proteinogenic and nonproteinogenic amino acids in organic solvents at low water content with high α-carboxylic selectivity.

During an investigation of the Alcalase-catalyzed peptidic bond formation between Cbz-Phe-OMe and H-Leu-O*t*-Bu in *tert*-butyl alcohol at low water content,[17] we discovered

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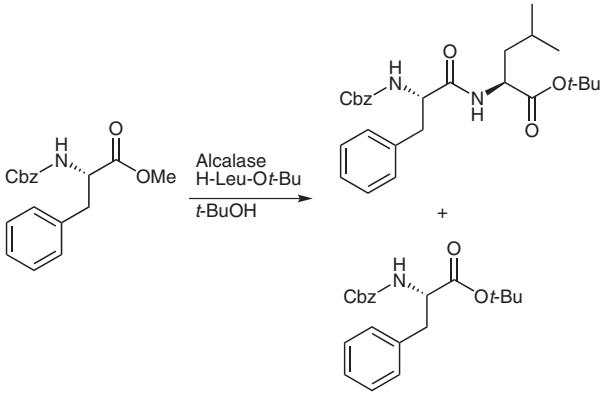
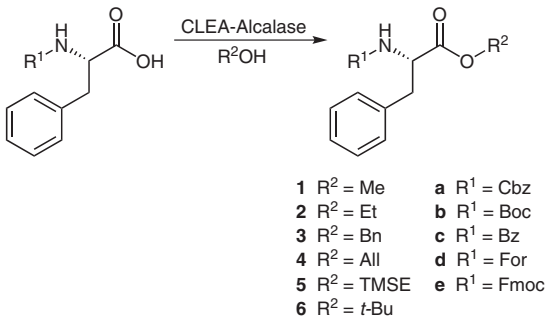


Scheme 1



Scheme 2

a small percentage of byproduct (Scheme 1). HPLC/MS and NMR analysis proved this byproduct to be Cbz-Phe-O*t*-Bu.

To our knowledge, this transesterification to give a *tert*-butyl ester had not been previously reported. This is much to our surprise, because *tert*-butyl alcohol is generally the solvent of choice for protease-catalyzed peptide synthesis, and enzymes tend to have very good stability in this solvent.[18]

Further investigation of the *tert*-butyl ester formation from Cbz-Phe-OMe showed that, in the absence of the amino acid nucleophile H-Leu-O*t*-Bu, in neat *tert*-butyl alcohol the *tert*-butyl ester could be obtained in 75% yield. Upon addition of 4 Å molecular sieves Cbz-Phe-OMe was converted into Cbz-Phe-O*t*-Bu in 96% yield (50 °C, 48 h), indicating that the amounts of water and methanol were critical for the reaction outcome. Most strikingly, equally high yields could be obtained starting from the free carboxylic acid Cbz-Phe-OH (50 °C, 16 h, 96% yield) using 3 Å molecular sieves whereas in the absence of molecular sieves only low yields (48 h, 37% yield) of Cbz-Phe-O*t*-Bu were obtained. Apparently, continuous removal of water shifts the equilibrium towards product, with the enzyme remaining active. We decided to investigate the scope of the methodology using N-terminal protected phenylalanine as the model substrate (Scheme 2). Cross-linked enzyme aggregate (CLEA)[19] Alcalase was used since it could be easily dried and recovered after the reaction by filtration. Due to the well-known inactivation of Alcalase by methanol and polar aprotic solvents (such as DMSO and DMF), apolar co-solvents were used (i.e., *n*-heptane, toluene, or MTBE) in the esterification reactions. All reported esterification reactions with primary alcohols were performed using a mixture of the appropriate alcohol with methyl *tert*-butyl ether (1:14, v/v, respectively).

As is shown in Table 1, Cbz-protected phenylalanine was smoothly converted into a variety of esters **1a–6e** in very high yields. The choice of N-terminal protection (**6a–e**) did not influence the esterification reaction. As is known from the literature, Alcalase accepts a wide variety of proteinogenic (**7a–e**) and nonproteinogenic (**7f**) amino acids, which was also demonstrated for *tert*-butyl ester synthesis

Table 1 Yields of Esterification Reactions of **1a–6e**

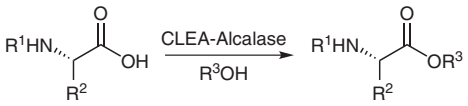
Product		HPLC yield (%)	Isolated yield (%)
1a	Cbz-Phe-OMe[a]	92	83
2a	Cbz-Phe-OEt[a]	95	85
3a	Cbz-Phe-OBn[a]	98	90
4a	Cbz-Phe-OAll[a]	95	86
5a	Cbz-Phe-OTMSE[a]	97	87
6a	Cbz-Phe-O*t*-Bu[b]	96	85
6b	Boc-Phe-O*t*-Bu[b]	98	85
6c	Bz-Phe-O*t*-Bu[b]	94	90
6d	For-Phe-O*t*-Bu[b]	99	92
6e	Fmoc-Phe-O*t*-Bu[b]	93	88

[a] CLEA-Alcalase (300 mg), Cbz-Phe-OH (50 mg), R^2OH (200 μL), MTBE (2.8 mL), 3 Å MS (200 mg), shaken at 150 rpm, 50 °C.
[b] CLEA-Alcalase (300 mg), protected-Phe-OH (50 mg), *t*-BuOH (3.0 mL), 3 Å MS (200 mg), shaken at 150 rpm, 50 °C.

(Table 2). Only amino acids with a tertiary β-carbon, e.g., threonine, isoleucine, and valine were not efficiently accepted by Alcalase. The dipeptide Cbz-Phe-Leu-OH proved to be a good substrate for esterification reactions giving **8–10**. Under the dry conditions used, neither hydrolysis nor alcoholysis of the peptidic bond was observed.

Since the use of molecular sieves is inconvenient on large scale, azeotropic water removal was investigated. Gratifyingly, the *tert*-butyl esters were formed in high yields (>95%) when *tert*-butyl alcohol, which forms an azeotrope with water, was continuously evaporated and dry *tert*-butyl alcohol was simultaneously added to keep the reaction volume constant. Alcohols, which do not form an azeotrope with water, such as allylic alcohol and 2-(trimethylsilyl)ethanol, could be combined with the azeotropic solvent toluene (1:14, v/v, respectively), under reduced pressure, to obtain ester yields of up to 98%. The CLEA-Alcalase could be recycled with minimal activity loss; after filtration, new starting materials were added and only 1% activity loss occurred after five esterification cycles.

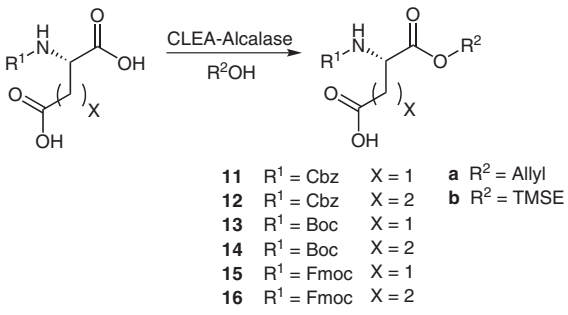
Table 2 Esterification Yields for Various Amino Acids and Peptides **7–10**



7a–e,8–10

Product		HPLC yield (%)	Isolated yield (%)
7a	Cbz-Ala-O*t*-Bu	90	86
7b	Cbz-Leu-O*t*-Bu	92	88
7c	Cbz-Ser-O*t*-Bu	83	72
7d	Cbz-Met-O*t*-Bu	89	82
7e	Cbz-Lys(Cbz)-O*t*-Bu	93	90
7f	Cbz-DOPA-O*t*-Bu	95	90
8	Cbz-Phe-Leu-OMe	98	88
9	Cbz-Phe-Leu-OBn	98	80
10	Cbz-Phe-Leu-O*t*-Bu	96	90

To investigate the α-carboxylic acid selectivity of Alcalase we decided to apply the conditions as for phenylalanine to aspartic acid and glutamic acid derivatives with various N-protecting groups (Scheme 3). Gratifyingly, all aspartic acid and glutamic acid derivatives were converted into their corresponding monoesters **11a–16b** in very high yields and with complete α-selectivity (Table 3).



11 R^1 = Cbz X = 1
12 R^1 = Cbz X = 2
13 R^1 = Boc X = 1
14 R^1 = Boc X = 2
15 R^1 = Fmoc X = 1
16 R^1 = Fmoc X = 2

a R^2 = Allyl
b R^2 = TMSE

Scheme 3

In conclusion, we have developed a general versatile method for enzymatic α-carboxylic acid selective esterification of N-protected amino acids and peptides towards a variety of esters by Alcalase in very high to excellent yields. Even esters that are difficult to synthesize chemically, e.g. *tert*-butyl esters, were obtained in high yields. Furthermore, amino acids with an additional carboxylic acid functionality in the side chain, e.g. aspartic acid and glutamic acid, were esterified with complete selectivity for the α-carboxylic moiety. The processes appeared amenable to scale-up since the water could also efficiently be removed by azeotropic distillation and the CLEA-Alcalase could be recycled with minimal loss of activity.

Table 3 Esterification Yields of Aspartic Acid and Glutamic Acid Derivatives **11a–16b**

Product		HPLC yield (%)	Isolated yield (%)
11a	Cbz-Asp-OAll	98	84
11b	Cbz-Asp-OTMSE	95	86
12a	Cbz-Glu-OAll	97	88
12b	Cbz-Glu-OTMSE	96	88
13a	Boc-Asp-OAll	97	89
13b	Boc-Asp-OTMSE	94	88
14a	Boc-Glu-OAll	96	87
14b	Boc-Glu-OTMSE	92	85
15a	Fmoc-Asp-OAll	94	88
15b	Fmoc-Asp-OTMSE	93	85
16a	Fmoc-Glu-OAll	96	89
16b	Fmoc-Glu-OTMSE	93	86

Unless stated otherwise, chemicals were obtained from commercial sources and used without further purification. Before use CLEA-Alcalase was dried as follows: CLEA-Alcalase (3 g, Codexis, 650 AGEU/g, 3.5 wt% H_2O), was suspended in *t*-BuOH (100 mL) and crushed with a spatula. After filtration, the enzyme was resuspended in MTBE (50 mL) followed by filtration. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance 300 MHz NMR spectrometer; chemical shifts are given relative to TMS. TLC was performed on pre-coated silica gel 60 F_{254} plates (Merck); spots were visualized using UV light, ninhydrin, or permanganate soln. 3 Å molecular sieves (8–12 mesh, Acros) were activated under reduced pressure at 200 °C and *t*-BuOH was stored on these activated molecular sieves. *t*-BuOH was pre-heated to liquid (45 °C) before use. Column chromatography was carried out using silica gel, Merck grade 9385 60 Å. Analytical HPLC was performed on an HP1090 Liquid Chromatograph, using a reversed-phase column (Inertsil ODS-3, C18, 5 μm particle size, 150 × 4.6 mm internal diameter) at 40 °C. UV detection was performed at 220 nm using a UV-Vis 204 Linear spectrophotometer (Varian). The gradient program was: 0–25 min linear gradient from 5% to 98% eluent B and from 25.1–30 min 5% eluent B (eluent A: 0.5 mL/L MsOH in H_2O; eluent B: 0.5 mL/L MsOH in MeCN). The flow was 1 mL/min from 0–25.1 min and 2 mL/min from 25.2–29.8 min, then back to 1 mL/min until stop at 30 min. Injection volumes were 20 μL.

The flow-injection analysis (FIA) experiments to determine the exact mass were performed on an Agilent 1100 LC-MS system (Agilent, Waldbronn, Germany), which consists of a binary pump, degasser, autosampler, column oven, diode-array detector, and a TOF-MS. The ESI-MS was run in positive mode, with the following conditions: *m/z* 50–3200, 175 V fragmentor, 0.94 cycl/sec, 350 °C drying gas temperature, 10 L N_2/min drying gas, 45 psig nebulizer pressure and 4 kV capillary voltage. The exact mass was determined using an internal reference to recalibrate the *m/z* axis for each measurement. The samples were directly introduced into the ESI by

injection of 5 μL into the eluent flow of 0.5 mL/min (MeOH–H_2O, 1:1).

Preparative HPLC was performed on a Varian PrepStar system using a stationary-phase column (Pursuit XRs, C18, 10 μm particle size, 500 × 41.4 mm internal diameter) at room temperature. UV detection was performed at 220 nm and 254 nm using a UV-Vis Varian ProStar spectrometer. The isocratic program was 85% eluent B and 15% eluent A (eluent A: 0.1 mL/L HCO_2H in H_2O; eluent B: 0.1 mL/L HCO_2H in MeCN) with a flow rate of 80 mL/min, injection volume of 10 mL and stop time after 30 min. Pure fractions were pooled and concentrated in vacuo and co-evaporated with toluene (2 × 25 mL) and $CHCl_3$ (2 × 25 mL).

Spectral data of Cbz-Phe-OEt (**2a**),[20] Cbz-Phe-OAll (**4a**),[21] Boc-Phe-O*t*-Bu (**6b**),[22] Bz-Phe-O*t*-Bu (**6c**),[23] For-Phe-O*t*-Bu (**6d**),[24] Fmoc-Phe-O*t*-Bu (**6e**),[25] Cbz-Ala-O*t*-Bu (**7a**),[26] Cbz-Ser-O*t*-Bu (**7c**),[27] Cbz-Phe-Leu-OMe (**8**),[28] Cbz-Asp-OAll (**11a**),[29] Boc-Asp-OTMSE (**13b**),[30] and Boc-Glu-OTMSE (**14b**)[31] were consistent with those reported in the literature. The identity of Cbz-Phe-OMe (**1a**), Cbz-Phe-OBn (**3a**), Cbz-Phe-O*t*-Bu (**6a**), Fmoc-Asp-OAll (**15a**), and Fmoc-Glu-OAll (**16a**) was ascertained by NMR spectroscopy and by comparison with commercially available samples (Bachem or Senn, Switzerland).

Enzymatic Esterification with Primary Alkyl Alcohols; General Procedure

CLEA-Alcalase (300 mg) and 3 Å molecular sieves (200 mg) were added to N-protected amino acid (0.167 mmol) dissolved in MTBE (2.8 mL) and the appropriate alcohol (200 μL). The mixture was shaken at 50 °C with 150 rpm for 16 h. After filtration, the enzyme was washed three times by resuspension in EtOAc (20 mL) followed by filtration. The combined organic layers were washed with sat. aq $NaHCO_3$ (2 × 40 mL), aq HCl (pH 1, 2 × 40 mL), and sat. aq NaCl (40 mL) and subsequently dried (Na_2SO_4), concentrated in vacuo, and co-evaporated with toluene (2 × 20 mL) and $CHCl_3$ (2 × 20 mL). Additional preparative HPLC was required for Cbz-Phe-OBn (**3a**) and Cbz-Phe-Leu-OBn (**9**).

N-(Benzyloxycarbonyl)phenylalanine 2-(Trimethylsilyl)ethyl Ester (5a)

^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.00 (s, 9 H, $SiCH_3$), 0.94–0.89 (m, 2 H, CH_2Si), 3.09–3.05 (dd, *J* = 6.0 Hz, 2 H, $C_\beta H_2$), 4.07–4.20 (m, 2 H, $COOCH_2$), 4.57–4.60 (dd, *J* = 3.3, 9.0 Hz, 1 H, $C_\alpha H$), 5.06 (s, 2 H, CH_2OCO), 5.23 (d, *J* = 9.0 Hz, 1 H, NH), 7.06 (dd, *J* = 3.3, 5.7 Hz, 2 H, $C_{Ar}H$), 7.19–7.34 (m, 8 H, $C_{Ar}H$).

^{13}C NMR (75 MHz, $CDCl_3$): δ = –1.2, 17.2, 29.1, 55.1, 64.0, 67.1, 127.2, 128.2, 128.6, 129.6, 136.0, 136.5, 155.8, 171.8.

MS [FIA-ESI(+)-TOF]: *m*/*z* [M + Na]$^+$ calcd for $C_{22}H_{29}NNaO_4Si$: 422.1758; found: 422.1757.

N-(Benzyloxycarbonyl)phenylalanylleucine Benzyl Ester (9)

^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.78 (d, *J* = 6.0 Hz, 6 H, $C_{\delta Leu}H_3$), 1.37–1.57 (m, 3 H, $C_{\gamma Leu}H$, $C_{\beta Leu}H_2$), 2.96–3.01 (dd, *J* = 6.3 Hz, 2 H, $C_{\beta Phe}H_2$), 4.41–4.49 (dd, *J* = 12.0, 3.3 Hz, 1 H, $C_{\alpha Phe}H$), 4.49–4.55 (m, 1 H, $C_{\alpha Leu}H$), 5.00 (s, 2 H, CH_2OCO), 5.05 (s, 2 H, $COOCH_2$), 5.25 (d, *J* = 7.8 Hz, 1 H, NH), 6.12 (d, *J* = 8.1 Hz, 1 H, NH), 7.07–7.29 (m, 15 H, $C_{Ar}H$).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 21.9, 22.6, 24.7, 38.2, 41.7, 51.2, 56.3, 67.2, 127.2, 128.1, 128.2, 128.5, 128.7, 128.9, 129.4, 135.5, 136.4, 156.1, 170.7, 172.3.

MS [FIA-ESI(+)-TOF]: *m*/*z* [M + Na]$^+$ calcd for $C_{30}H_{34}N_2NaO_5$: 525.2359; found: 525.2324.

Enzymatic Esterification with *tert*-Butyl Alcohol; General Procedure

CLEA-Alcalase (300 mg) was added to N-protected amino acid (0.167 mmol), *t*-BuOH (3.0 mL), and 3 Å molecular sieves (200 mg). The mixture was shaken at 50 °C with 150 rpm for 16 h. After filtration, the enzyme was washed three times by resuspension in EtOAc (20 mL) followed by filtration. The combined organic layers were concentrated in vacuo and the resulting oil redissolved in EtOAc (50 mL). This soln was washed with sat. aq $NaHCO_3$ (2 × 40 mL), aq HCl (pH 1, 2 × 40 mL), and brine (40 mL) and subsequently dried (Na_2SO_4), concentrated in vacuo, and co-evaporated with toluene (2 × 20 mL) and $CHCl_3$ (2 × 20 mL). For Cbz-Ser-O*t*-Bu (**7c**) additional column chromatography (*n*-heptane–EtOAc, 1:1) was required.

N-(Benzyloxycarbonyl)leucine *tert*-Butyl Ester (7b)

^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.86 (d, *J* = 6.3 Hz, 6 H, $C_\delta H_3$), 1.38 (s, 9 H, CCH_3), 1.39–1.70 (m, 3 H, $C_\gamma H$, $C_\beta H_2$), 4.19–4.22 (m, 1 H, $C_\alpha H$), 5.03 (s, 2 H, CH_2OCO), 5.12 (d, *J* = 7.2 Hz, 1 H, NH), 7.24–7.28 (m, 5 H, $C_{Ar}H$).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 22.6, 25.4, 28.6, 42.5, 53.7, 67.4, 82.4, 128.2, 128.7, 137.0, 156.5, 172.9.

MS [FIA-ESI(+)-TOF]: *m*/*z* [M + Na]$^+$ calcd for $C_{18}H_{27}NNaO_4$: 344.1838; found: 344.1828.

N-(Benzyloxycarbonyl)methionine *tert*-Butyl Ester (7d)

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.39 (s, 9 H, CCH_3), 1.80–1.89 (m, 2 H, $C_\beta H_2$), 2.01 (s, 3 H, SCH_3), 2.40–2.48 (m, 2 H, $C_\gamma H_2$), 4.28–4.30 (m, 1 H, $C_\alpha H$), 5.03 (s, 2 H, CH_2OCO), 5.33 (d, *J* = 7.5 Hz, 1 H, NH), 7.26–7.29 (m, 5 H, $C_{Ar}H$).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 22.2, 23.0, 28.2, 42.1, 53.3, 67.0, 82.0, 128.2, 129.1, 136.6, 156.1, 171.4.

MS [FIA-ESI(+)-TOF]: *m*/*z* [M + Na]$^+$ calcd for $C_{17}H_{25}NNaO_4S$: 362.1396; found: 362.1334.

N^2,N^6-Bis(benzyloxycarbonyl)lysine *tert*-Butyl Ester (7e)

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.25–1.45 (m, 13 H, CCH_3, $C_\gamma H_2$, $C_\delta H_2$), 1.46–1.71 (m, 2 H, $C_\beta H_2$), 3.01–3.12 (m, 2 H, $C_\varepsilon H_2$), 4.13–4.19 (m, 1 H, $C_\alpha H$), 4.81–4.85 (m, 1 H, NH), 5.00 (s, 2 H, CH_2OCO), 5.01 (s, 2 H, CH_2OCO), 5.42 (d, *J* = 7.2 Hz, 1 H, NH), 7.18–7.26 (m, 10 H, $C_{Ar}H$).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 22.4, 28.2, 29.9, 32.6, 40.9, 54.3, 66.8, 67.1, 82.3, 128.2, 128.7, 136.6, 136.8, 156.2, 156.7, 171.7.

MS [FIA-ESI(+)-TOF]: *m*/*z* [M + Na]$^+$ calcd for $C_{26}H_{34}N_2NaO_6$: 493.2309; found: 493.2216.

N-(Benzyloxycarbonyl)-3,4-dihydroxyphenylalanine *tert*-Butyl Ester (7f)

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.34 (s, 9 H, CCH_3), 2.83–2.88 (dd, *J* = 9.3, 4.2 Hz, 2 H, $C_\beta H_2$), 4.37–4.40 (dd, *J* = 13.0, 4.5 Hz, 1 H, $C_\alpha H$), 5.00 (s, 2 H, CH_2OCO), 5.25 (d, *J* = 8.1 Hz, 1 H, NH) 5.90–6.05 (m, 2 H, OH), 6.45 (d, *J* = 7.2 Hz, 1 H, $C_{Ar}H$), 6.58–6.65 (m, 2 H, $C_{Ar}H$), 7.19–7.30 (m, 5 H, $C_{Ar}H$).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 28.2, 38.0, 55.7, 67.3, 82.8, 115.4, 116.5, 121.9, 127.2, 128.2, 128.4, 128.7, 136.3, 143.2, 144.0, 156.2, 171.2.

MS [FIA-ESI(+)-TOF]: *m*/*z* [M + Na]$^+$ calcd for $C_{21}H_{25}NNaO_6$: 410.1574; found: 410.1530.

N-(Benzyloxycarbonyl)phenylalanylleucine *tert*-Butyl Ester (10)

^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.80 (d, *J* = 6.0 Hz, 6 H, $C_{\delta Leu}H_3$), 1.36–1.50 (m, 12 H, $C_{\gamma Leu}H$, $C_{\beta Leu}H_2$, CCH_3), 3.00 (d, *J* = 6.3 Hz, 2 H, $C_{\beta Phe}H_2$), 4.32–4.39 (m, 2 H, $C_{\alpha Leu}H$, $C_{\alpha Phe}H$), 5.00 (s, 2 H,

CH_2OCO), 5.29 (d, J = 5.4 Hz, 1 H, NH), 6.23 (d, J = 6.9 Hz, 1 H, NH), 7.09–7.25 (m, 10 H, $C_{Ar}H$).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 22.1, 22.6, 24.8, 28.0, 38.4, 41.8, 51.4, 56.0, 67.0, 81.9, 127.0, 128.0, 128.1, 128.7, 128.8, 129.5, 136.3, 155.8, 170.3, 171.5.

MS [FIA-ESI(+)-TOF]: m/z [M + Na]$^+$ calcd for $C_{27}H_{36}N_2NaO_5$: 491.2516; found: 491.2525.

Enzymatic α-Selective Esterification of N-Protected Aspartic Acid and Glutamic Acid; General Procedure

CLEA-Alcalase (3 g) was added to N-protected amino acid (0.5 g), MTBE (28.0 mL), the appropriate alcohol (2.0 mL), and 3 Å molecular sieves (2.0 g). The mixture was shaken at 50 °C with 150 rpm for 16 h. After filtration, the enzyme was washed three times by resuspension in aq HCl (pH 1, 50 mL) and three times by resuspension in EtOAc (50 mL) followed by filtration. The combined EtOAc and HCl phases were separated and the organic phase was washed with aq HCl (pH 1, 100 mL), dried (Na_2SO_4), and concentrated in vacuo. The resulting oil was redissolved in CH_2Cl_2–MeOH–AcOH (89.9:10:0.1, 20 mL) followed by silica gel filtration. The mixture was concentrated in vacuo and co-evaporated with toluene (2 × 50 mL) and $CHCl_3$ (2 × 50 mL).

***N*-(Benzyloxycarbonyl)aspartic Acid α-[2-(Trimethylsilyl)ethyl] Ester (11b)**

^1H NMR (300 MHz, $CDCl_3$): δ = 0.00 (s, 9 H, $SiCH_3$), 0.92–1.01 (m, 2 H, CH_2Si), 2.85 (dd, J = 4.5, 17.4 Hz, 1 H, $C_\beta H_2$), 2.90 (dd, J = 4.2, 16.8 Hz, 1 H, $C_\beta H_2$), 4.18–4.25 (m, 2 H, $COOCH_2$), 4.58–4.64 (m, 1 H, $C_\alpha H$), 5.10 (s, 2 H, CH_2OCO), 5.84 (d, J = 8.1 Hz, 1 H, NH), 7.30–7.39 (m, 5 H, $C_{Ar}H$).

^{13}C NMR (75 MHz, $CDCl_3$): δ = –1.5, 17.3, 36.7, 50.4, 53.5, 64.5, 128.0, 128.7, 136.2, 159.9, 170.9, 175.4.

MS [FIA-ESI(+)-TOF]: m/z [M + Na]$^+$calcd for $C_{17}H_{25}NNaO_6$: 390.1343; found: 390.1336.

***N*-(Benzyloxycarbonyl)glutamic Acid α-Allyl Ester (12a)**

^1H NMR (300 MHz, $CDCl_3$): δ = 1.91–2.20 (m, 2 H, $C_\gamma H_2$), 2.38–2.45 (m, 2 H, $C_\beta H_2$), 4.37–4.45 (m, 1 H, $C_\alpha H$), 4.60 (d, J = 5.7 Hz, 2 H, $COOCH_2$), 5.07 (s, 2 H, CH_2OCO), 5.20–5.26 (m, 2 H, $=CH_2$), 5.54 (d, J = 7.5 Hz, 1 H, NH), 5.80–5.91 (m, 1 H, CH=), 7.23–7.30 (m, 5 H, $C_{Ar}H$), 8.95 (s, 1 H, COOH).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 27.8, 30.2, 53.2, 66.6, 67.6, 119.6, 128.5, 128.6, 128.9, 131.7, 136.5, 171.9, 178.1.

MS [FIA-ESI(+)-TOF]: m/z [M + Na]$^+$calcd for $C_{16}H_{19}NNaO_6$: 344.1104; found: 344.1134.

***N*-(Benzyloxycarbonyl)glutamic Acid α-[2-(Trimethylsilyl)ethyl] Ester (12b)**

^1H NMR (300 MHz, $CDCl_3$): δ = 0.00 (s, 9 H, $SiCH_3$), 0.91–1.00 (m, 2 H, CH_2Si), 1.93–2.00 (m, 1 H, $C_\gamma H_2$), 2.10–2.18 (m, 1 H, $C_\gamma H_2$), 2.31–2.41 (m, 2 H, $C_\beta H_2$), 4.10–4.22 (m, 2 H, $COOCH_2$), 4.31–4.37 (m, 1 H, $C_\alpha H$), 5.07 (s, 2 H, CH_2OCO), 5.42 (d, J = 8.1 Hz, 1 H, NH), 7.23–7.31 (m, 5 H, $C_{Ar}H$).

^{13}C NMR (75 MHz, $CDCl_3$): δ = –1.4, 17.5, 27.9, 30.5, 53.5, 63.0, 64.2, 128.2, 128.3, 128.6, 136.4, 156.0, 172.9, 176.9.

MS [FIA-ESI(+)-TOF]: m/z [M + Na]$^+$ calcd for $C_{18}H_{27}NNaO_6Si$: 404.1505; found: 404.1507.

***N*-(*tert*-Butoxycarbonyl)aspartic Acid α-Allyl Ester (13a)**

^1H NMR (300 MHz, $CDCl_3$): δ = 1.43 (s, 9 H, CCH_3), 2.88 (dd, J = 4.5, 17.1 Hz, 1 H, $C_\beta H_2$), 3.00 (dd, J = 3.9, 16.8 Hz, 1 H, $C_\beta H_2$), 4.45–4.64 (m, 3 H, $COOCH_2$, $C_\alpha H$), 5.24–5.34 (m, 2 H, $=CH_2$), 5.60 (d, J = 8.4 Hz, 1 H, NH), 5.82–5.92 (m, 1 H, CH=), 8.89 (s, 1 H, COOH).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 28.4, 36.8, 50.1, 66.0, 80.7, 119.0, 131.7, 155.9, 171.1, 175.9.

MS [FIA-ESI(+)-TOF]: m/z [M + Na]$^+$ calcd for $C_{12}H_{19}NNaO_6$: 296.1134; found: 296.1117.

***N*-(*tert*-Butyloxycarbonyl)glutamic Acid α-Allyl Ester (14a)**

^1H NMR (300 MHz, $CDCl_3$): δ = 1.42 (s, 9 H, CCH_3), 1.94–2.03 (m, 1 H, $C_\gamma H_2$), 2.15–2.21 (m, 1 H, $C_\gamma H_2$), 2.41–2.47 (m, 2 H, $C_\beta H_2$), 4.34–4.36 (m, 1 H, $C_\alpha H$), 4.61 (d, J = 5.7 Hz, 2 H, $COOCH_2$), 5.26–5.35 (m, 3 H, NH, $=CH_2$), 5.83–5.94 (m, 1 H, CH=), 9.44 (s, 1 H, COOH).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 27.1, 28.2, 30.1, 52.9, 66.0, 80.1, 118.9, 131.5, 155.5, 172.0, 177.5.

MS [FIA-ESI(+)-TOF]: m/z [M + Na]$^+$ calcd for $C_{13}H_{21}NNaO_6$: 310.1261; found: 310.1266.

***N*-(9*H*-Fluoren-9-ylmethoxycarbonyl)aspartic Acid α-[2-(Trimethylsilyl)ethyl] Ester (15b)**

^1H NMR (300 MHz, $CDCl_3$): δ = 0.00 (s, 9 H, $SiCH_3$), 0.80–0.96 (m, 2 H, CH_2Si), 2.91 (dd, J = 3.9, 16.8 Hz, 1 H, $C_\beta H_2$), 3.06 (dd, J = 3.6, 16.8 Hz, 1 H, $C_\beta H_2$), 4.19–4.57 (m, 6 H, CH_2OCO, $COOCH_2$, $C_\alpha H$, fluorenyl CH), 5.79 (d, J = 7.8 Hz, 1 H, NH), 7.22–7.73 (m, 8 H, $C_{Ar}H$).

^{13}C NMR (75 MHz, $CDCl_3$): δ = –1.3, 17.5, 30.0, 36.6, 47.4, 64.9, 67.7, 120.3, 125.4, 127.3, 128.1, 141.6, 144.0, 156.1, 170.3, 175.5.

MS [FIA-ESI(+)-TOF]: m/z [M + Na]$^+$ calcd for $C_{24}H_{29}NNaO_6Si$: 478.1656; found: 478.1651.

***N*-(9*H*-Fluoren-9-ylmethoxycarbonyl)glutamic Acid α-2-(Trimethylsilyl)ethyl Ester (16b)**

^1H NMR (300 MHz, $CDCl_3$): δ = 0.00 (s, 9 H, $SiCH_3$), 0.86–1.02 (m, 2 H, CH_2Si), 1.91–2.06 (m, 1 H, $C_\gamma H_2$), 2.17–2.28 (m, 1 H, $C_\gamma H_2$), 2.39–2.45 (m, 2 H, $C_\beta H_2$), 4.18–4.21 (m, 3 H, CH_2OCO, fluorenyl CH), 4.35–4.42 (m, 3 H, $COOCH_2$, $C_\alpha H$), 5.42 (d, J = 6.6 Hz, 1 H, NH) 7.23–7.74 (m, 8 H, $C_{Ar}H$).

^{13}C NMR (75 MHz, $CDCl_3$): δ = –1.3, 17.4, 22.3, 27.9, 47.5, 53.8, 63.4, 64.4, 67.5, 120.0, 125.2, 127.2, 128.3, 141.4, 144.2, 156.4, 171.5, 177.4.

MS [FIA-ESI(+)-TOF]: m/z [M + Na]$^+$ calcd for $C_{25}H_{31}NNaO_6Si$: 492.1812; found: 492.1824.

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