# Preparation and Application of *O*-Amino-Serine, Ams, a New Building Block in Chemoselective Ligation Chemistry

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Abstract: The non-codable amino acid *O*-amino-serine, Ams, has been prepared in both L- and D-forms as the orthogonally protected derivative, Fmoc-Ams(Boc)-OH (**1** and **2**). This new amino acid derivative is useful for chemoselective ligations. Under acidic conditions and in the presence of all other common amino acid functionalities, the oxyamine function selectively forms oxime linkages with aldehydes. The Ams residue has been incorporated into both ends of the peptide sequence Asp-Leu-Trp-Gln-Lys using standard SPPS. The deprotected peptide has been used for chemical ligation to afford a peptide dimer as well as a glycopeptide. Ams racemization was found to be negligible, as monitored by HPLC separation of Ams dipeptide diastereomers. Copyright © 1999 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: Ams; *O*-amino-serine; cycloserine; ligation; oxime; peptide dimer; glycopeptide; solid-phase peptide synthesis

## INTRODUCTION

The solid-phase peptide synthesis (SPPS) technique introduced by Merrifield [1] has made it possible to routinely synthesize linear peptides of good quality using stepwise build-up. However, preparation of very long peptides or peptides with unusual architecture, such as branched or cyclic peptides [2,3], is still often problematic. The conventional protected segment condensation is an option for complicated peptides, but the solubilities of protected peptide segments are often unpredictable and coupling rates are often low [4], leading to problems such as epimerization. Recently, chemoselective ligation approaches have been developed for the synthesis of long, branched and cyclic peptides [5–10]. These approaches allow ligation of unprotected peptides through the formation of peptide bonds or surrogate peptide bonds between two complementary reactive functions. Amino acid functionalities like amines, thiols or carboxylic acids must be unreactive or reversibly reactive under the conditions used. Several strategies are available for chemoselective ligation, e.g. those based on carbonyl or thiol chemistry. Carbonyl ligation chemistry involves reaction under acidic conditions between an aldehyde and a weak base such as 1,2-aminothiols, hydrazines or oxyamines to afford thiazolidines, hydrazones or oximes [11,12]. The higher basicity of side-chain and $\alpha$-amines of amino acids render these groups relatively unreactive below neutral pH. For peptide-to-peptide ligations, the weakly basic groups and aldehydes may be incorporated during SPPS, usually at the termini or through acylation of selectively protected side-chain amines [13,14]. For the assembly of peptide dimers and dendrimers,

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templates containing multiple aldehydes may be used. Examples of thiol ligation chemistries involve the formation of pseudo-peptide bond thioethers [15,16] or thioesters [17]. Elegantly, native peptide bonds have been formed by peptide-to-peptide ligations between a *C*-terminal thioester and an *N*-terminal cysteine or *N*-oxyethanethiol glycine [18–20]. Common to the different thioester methods is their limitation for choice of protecting group strategy. Because thioesters are not stable to the basic reagents used for Fmoc-removal, peptide thioesters usually cannot be synthesized by Fmoc-based chemistry. An alternative approach involves reaction of *C*-terminal *S*-cyano cysteine peptides with peptide nucleophiles. This chemistry likewise yields native peptide bonds, although several byproducts may co-form, e.g. dehydroalanine sequences [21,22].

For the purpose of incorporation of branch points at any position in a peptide sequence, we wanted access to amino acids with oxyamine functions in the side-chain. Acylation of a selectively deprotected lysine with protected 2-oxyamino acetic acid has been used [5,11,13,14,23], but the long flexible side-chain of lysine is not always desirable. Short-chain lysine analogues may be used, but we preferred an amino acid with the oxyamine directly on the amino acid alkyl side-chain. This would keep the side-chain length minimal and eliminate the need for specialized amino group protections. In nature, the oxyamino side-chain functionality is found in the canaline amino acid (L-2-amino-4-aminooxy-butyric acid) [24,25]. An oxyamine analogue of lysine, L-homocanaline, was recently prepared as the Fmoc/Boc-protected derivative through a seven-step synthesis [26]. We preferred the shortest possible side-chain in order to limit structural flexibility. This made us target *O*-amino-serine (Ams) [27], preferably as the Fmoc/Boc-derivative. *N*(α)-Z-derivatives of Ams have previously been synthesized as racemates from the alpha-bromo acid [28,29].

The present paper describes a three-step synthetic route to the D- and L-forms of orthogonally-protected *O*-amino-serine, Fmoc-Ams(Boc)-OH (**1** and **2**, respectively). We propose Ams as an abbreviation for *O*-amino-serine. The starting point for the synthesis is the appropriate enantiomer of the chiral natural product cycloserine **3** (4-amino-isoxazolidin-3-one) [30]. The new building block has been used in stepwise peptide synthesis to afford peptides that contain the oxyamino side-chain. Two applications of the unprotected peptides for

chemoselective ligation have been demonstrated and evaluated. In addition, Ams racemization has been shown to be very low, via preparation of two dipeptide diastereomers and their separation using HPLC.

## MATERIALS AND METHODS

Suitably protected *N*(α)-Fmoc-amino acids were purchased from Novabiochem or Senn, Switzerland. 1-Hydroxy-7-azabenzotriazole (HOAt) was purchased from Perseptive Biosystems. Other chemicals were purchased from Aldrich, Sigma or Fluka, and used as received, except where noted. Dried THF was collected by distillation from Na/benzophenone. Analytical thin layer chromatography (TLC) was performed on silica gel 60 $F_{254}$ aluminium sheets with detection by UV light, ninhydrin or 0.04% sodium bromocresol green in EtOH, which identifies β-lactones as yellow spots on a blue background. Melting points are reported uncorrected. ^{1}H- and ^{13}C-NMR spectra were recorded on a 400 MHz Varian instrument. Chemical shifts are reported relative to $CHCl_3$ at 7.27 ppm, DMSO-$d_6$ at 2.50 ppm or acetone-$d_6$ at 2.05 ppm. Hz-values denote coupling constants ($J$). Molecular weights of the peptides were determined using matrix-assisted laser desorption time of flight mass spectroscopy (MALDI-MS), recorded on a Voyager-DE (Perseptive Biosystems). A matrix of sinapinic acid was used. Column chromatography was carried out on silica gel 60 (Merck). Analytical and semi-preparative high-performance liquid chromatography (HPLC) were performed using a Waters RCM $8 \times 10$ module with C-18 columns ($19 \times 300$ mm or $25 \times 300$ mm) at 40°C. The solvent system for both analytical and semi-preparative HPLC was buffer A (0.1% TFA in water) and buffer B (0.07% TFA in MeCN). UV detection was at 215 nm or 200–400 nm (photo-diode array). The gradient for analytical HPLC was 5–60% buffer B over 25 min at 1.5 ml/min and the gradient for semi-preparative HPLC was 5–60% buffer B over 40 min at 4 ml/min, unless otherwise indicated.

### Fmoc-L-Serine β-Lactone, 4

Triphenylphosphine (8.1 g, 31 mmol) in dried THF (125 ml) was cooled with $CO_2$/acetone under an argon atmosphere. Diethyl azodicarboxylate (4.8 ml, 31 mmol) was added dropwise over 10 min with stirring. After standing for 10 min, Fmoc-serine (10.1 g, 31 mmol) in dried THF (125 ml) was added

dropwise over 15 min. The mixture was left with stirring for 20 min under cooling and 3.5 h at room temperature. The reaction was monitored using TLC (AcOEt/hexane, 1:1). The solvent was removed *in vacuo* and the lactone was isolated by column chromatography (AcOEt/hexane, 1:1). The later fractions were contaminated with diethyl hydrazinedicarboxylate, which was removed by washing the solid material with $CHCl_3$. The total yield was 4.5 g (47%), m.p. 201–202°C.

$^1$H-NMR (acetone-$d_6$) $\delta$: 7.87 (d, 2H, 7.7 Hz, ArH), 7.70 (d, 2H, 7.6 Hz, ArH), 7.42 (t, 2H, 7.5 Hz, ArH), 7.33 (t, 2H, 7.5 Hz, ArH), 5.31 (q, 1H, 6.3 Hz, $\alpha$H), 4.46 (m, 4H, FlCH$_2$ and CH$_2$ON), 4.26 (d, 1H, $J$ = 6.7, ArCH).

$^{13}$C-NMR (acetone-$d_6$) $\delta$: 169.5, 155.8, 144.1, 141.4, 127.9, 127.3, 125.3, 120.1, 67.0, 65.9, 60.0, 47.4.

### Fmoc-L-Ser-ONH-Boc, 5

The $\beta$-lactone **4** (50 mg, 0.16 mmol) was dissolved in dried THF, DCM or $CH_3CN$ (1–2 ml), either with or without DIEA (27 μl, 0.16 mmol), and was treated with Boc-NH-OH (22 mg, 0.16 mmol). The mixtures were stirred at room temperature or at 50°C for several days. TLC showed formation of the ester primarily and no Fmoc-Ams(Boc)-OH could be detected. The mixtures were worked up by addition of AcOEt and washing with 0.1 M HCl and water. The solutions were dried (MgSO$_4$) and evaporated *in vacuo*. An analytical sample was purified by chromatography on silica with AcOEt/hexane/AcOH, 20:20:1 as eluent, yielding Fmoc-Ser-ONH-Boc, 40 mg (56%).

$^1$H-NMR (CDCl$_3$) $\delta$: 8.13 (bs, 1H, NH), 7.76 (d, 2H, 7.4 Hz, ArH), 7.60 (t, 2H, 6.2 Hz, ArH), 7.40 (t, 2H, 7.5 Hz, ArH), 7.31 (t, 2H, 7.4 Hz, ArH), 5.88 (bd, 1H, 8.7 Hz, NH), 4.62 (d, 1H, 9.0 Hz, CHO), 4.40 (t, 2H, 8.1 Hz, FlCH$_2$), 4.23 (m, 2H, ArCH and $\alpha$H), 3.87 (d, 1H, 12 Hz, CH'O), 1.49 (s, 9H, CH$_3$).

$^{13}$C-NMR (CDCl$_3$) $\delta$: 171.3, 158.8, 156.4, 144.0, 141.5, 127.9, 127.3, 125.3, 120.2, 82.6, 67.7, 64.0, 55.8, 47.4, 28.3.

### Fmoc-D-cycloserine, 6[2]

D-cycloserine **3** (1.0 g, 9.79 mmol) in water (12 ml) was cooled with an ice-bath and treated with DIEA (1.12 ml, 6.53 mmol), followed by FmocOSu (2.2 g,

6.53 mmol) in DMF (20 ml). The pH of the solution was adjusted to a value of 8–9 by the addition of extra DIEA after 2 and 3 h. The mixture was stirred overnight at room temperature and then poured into 0.1 M HCl (100 ml). After 2 h at 5°C, the precipitate was collected by filtration and recrystallized from isopropanol/water, 1.73 g (82%), m.p. 122–124°C.

$^1$H-NMR (CDCl$_3$) $\delta$: 8.03 (bs, 1H, NH), 7.78 (d, 2H, 7.8 Hz, ArH), 7.59 (d, 2H, 7.3 Hz, ArH), 7.42 (t, 2H, 7.5 Hz, ArH), 7.33 (t, 2H, 7.2 Hz, ArH), 5.44 (bs, 1H, NH), 4.82 (dd, 1H, 7.5 Hz, $\alpha$H), 4.65 (m, 1H, CHON), 4.45 (d, 2H, 4 Hz, 6.3 Hz, FlCH$_2$), 4.23 (t, 1H, ArCH), 4.12 (dd, 1H, 6.9 Hz, CH'ON, 8.8 Hz).

$^{13}$C-NMR (CDCl$_3$) $\delta$: 170.8, 156.4, 143.7, 141.5, 128.0, 127.3, 125.2, 120.2, 75.1, 67.7, 54.0, 53.1.

### Fmoc-D-Ams, HCl, 7

Fmoc-D-cycloserine **6** (2.5 g, 6.53 mmol) in 2 M HCl/THF (80 ml, 1:1) was heated to 60°C for 2–3 days. Alternatively, the mixture was stirred at room temperature for 2–3 weeks. The reaction progress was monitored by TLC (DCM:AcOEt:AcOH, 5:5:1). The cooled solution was evaporated to remove THF, and was then filtered and evaporated to dryness and dried *in vacuo*, 1.38 g (56%).

$^1$H-NMR (DMSO-$d_6$) $\delta$: 7.87 (d, 2H, 7.4 Hz, ArH), 7.83 (d, 1H, 8.4 Hz, NH), 7.70 (d, 2H, 7.7 Hz, ArH), 7.40 (t, 2H, 7.5 Hz, ArH), 7.31 (t, 2H, 7.2 Hz, ArH), 4.4–4.2 (m, 6H).

MALDI-MS, $m/z$ 342.6 (M + H)$^+$, $C_{18}H_{18}N_2O_5$ requires $m/z$ 342.4.

### Fmoc-D-Ams(Boc)-OH, 1

Fmoc-D-Ams hydrochloride **7** (1.26 g, 3.3 mmol) in DMF (40 ml) was treated with DIEA (1.13 ml, 6.6 mmol) and di-*tert*-butyl dicarbonate (0.77 g, 3.6 mmol). The mixture was stirred for 2–3 h, and the pH was adjusted to a value of 8–9 every hour, by addition of DIEA. TLC was used for monitoring (AcOEt:hexane:AcOH, 20:20:1). At reaction endpoint, ether and 0.1 M HCl were added. The organic phase was washed with 0.1 M HCl and water ( $\times$ 2). The solution was then extracted with 2% NaHCO$_3$ ( $\times$ 3). The resulting aqueous solution was washed with ether, acidified with 1 M HCl to pH 2, and the precipitated material was dissolved in ether and dried (MgSO$_4$). The solvent was removed and the crude product was crystallized from acetone/hexane, 0.90 g (62%), m.p. 74–78°C.

$^1$H-NMR (CDCl$_3$) $\delta$: 7.76 (d, 2H, 7.6 Hz, ArH), 7.61 (d, 2H, 7.3 Hz, ArH), 7.40 (t, 2H, 7.5 Hz, ArH), 7.31

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[2] An alternative route to Fmoc-cycloserine was reported very recently. The synthesis was performed via bis-silyated cycloserine [56].

(t, 2H, 7.5 Hz, ArH), 6.20 (bd, 1H, NH), 4.57 (m, 1H, CHON), 4.40 (t, 2H, 7.7 Hz, FlCH$_2$), 4.24 (m, 2H, ArCH and $\alpha$H), 4.00 (m, 1H, CH'ON), 1.51 (s, 9H, CH$_3$).

$^{13}$C-NMR (CDCl$_3$) $\delta$: 163.4, 158.2, 156.5, 144.0, 141.4, 128.1, 127.2, 125.4, 120.2, 83.7, 76.2, 67.7, 53.1, 47.4, 37.1, 28.5.

### Fmoc-L-Ams(Boc)-OH, 2

Prepared in analogy with the D-amino acid, **1**, and identical by NMR.

### SPPS and Analysis, General Protocol

All of the peptides were synthesized manually in plastic syringes using either a preloaded Rink amide linker RAM-TentaGel (0.23–0.28 mmol/g) or a RAM-MBHA-resin (0.55 mmol/g). Fully protected $N(\alpha)$-Fmoc amino acids (3 equiv.) were used and the temporary Fmoc protecting group was removed after each cycle by 30% piperidine in $N$-methylpyrrolidone (NMP). Amino acids were coupled in NMP using DIC (3 equiv.) and HOAt (3 equiv.). The completion of the acylation reaction was monitored visually by the use of bromophenol blue. Between the Fmoc-deprotection and the acylation reaction, the resin was washed with NMP ( $\times$ 6). Following synthesis, the peptides were washed with DCM ( $\times$ 3). The peptides were cleaved from the resin and the side-chain protecting group removed by treatment with 95% aq. TFA containing triisopropylsilane (TIS) (4 molar equiv.) for 1.5 h. The resin was rinsed with 95% aq. acetic acid ( $\times$ 4). Both TFA and acetic acid were evaporated and the peptides were precipitated from diethyl ether and lyophilized overnight. The peptides were analysed by HPLC and MALDI-MS.

### Protocol Used for Racemization Studies

The diastereomers D-Ams-Phe-NH$_2$ and L-Ams-Phe-NH$_2$ were synthesized as described above by coupling Fmoc-Ams (3 equiv.) to Phe-Rink resin (50 mg, 0.55 mmol/g). After cleavage from the resin, the dipeptides were analysed by analytical HPLC using an isocratic gradient of 10% buffer B over 5 min, followed by a linear gradient 10–25% buffer B over 25 min, $t_R$ 17.7 min for LL and $t_R$ 20.6 min for DL. The diastereomers were also mixed and analysed by analytical HPLC, which provided two peaks. Analysis by MALDI-MS, $m/z$ 266.6 (M + H)$^+$ (LL) and 266.6 (M + H)$^+$ (DL), C$_{12}$H$_{18}$N$_4$O$_3$ requires $m/z$, 266.2, confirmed the products.

### Ams-Asp-Leu-Trp-Gln-Lys-NH$_2$, 8

The precursor Fmoc-Asp(OtBu)-Leu-Trp(Boc)-Gln-(Trt)-Lys(Boc)-RAM-TentaGel (0.28 mmol/g) was synthesized as described above. After removal of Fmoc with 30% piperidine in NMP, Fmoc-Ams(Boc)-OH (91.2 mg, 208 μmol) was coupled to the peptide resin (52 μmol) using DIC (32 μl, 208 μmol) and HOAt (27.1 mg, 208 μmol). The completion of the coupling was monitored by the use of bromophenol blue. The $N(\alpha)$-Fmoc protecting group was removed by treatment with 30% piperidine in NMP for 20 min. The side-chain deprotection and cleavage from the resin was mediated by 95% aq. TFA containing TIS (4 molar equiv.). The crude peptide was analysed by analytical HPLC ($t_R$ 12.3 min) and purified by semi-preparative HPLC. Analysis by MALDI-MS, $m/z$ 789.5 (M + H)$^+$, C$_{35}$H$_{55}$N$_{11}$O$_{10}$ requires $m/z$ 789.4, confirmed the expected product. Peptide **8** was obtained as a solid (26 mg, 63%).

### Asp-Leu-Trp-Gln-Lys-Ams-NH$_2$, 9

Fmoc-Ams(Boc)-OH (69.0 mg, 156 μmol) was coupled to the RAM TentaGel (100 mg, 0.26 mmol/g) using DIC (24 μl, 156 μmol) and HOAt (20.3 mg, 156 μmol). The Fmoc group was deprotected with 30% piperidine in NMP for 20 min. Then the synthesis of **9** was performed as described above. The crude peptide was analysed by analytical HPLC ($t_R$ 12.5 min) and purified by semi-preparative HPLC. Analysis by MALDI-MS, $m/z$ 789.5 (M + H)$^+$, C$_{35}$H$_{55}$N$_{11}$O$_{10}$ requires $m/z$ 789.4, confirmed the expected product. Peptide **9** was obtained as a solid (7.9 mg, 39%).

### D-Ams-Asp-Leu-Trp-Gln-Lys-NH$_2$, 10

The synthesis of **10** was performed as described for the preparation of **8** on the RAM TentaGel (0.28 mmol/g). Fmoc-D-Ams(Boc)-OH (91.2 mg, 208 μmol) was coupled to the protected peptide resin (52 μmol) using DIC (32 μl, 208 μmol) and HOAt (27.1 mg, 208 μmol). The crude peptide was analysed by analytical HPLC ($t_R$ 12.6 min) and purified by semi-preparative HPLC. Analysis by MALDI-MS, $m/z$ 790.2 (M + H)$^+$, C$_{35}$H$_{55}$N$_{11}$O$_{10}$ requires $m/z$ 789.4, confirmed the expected product. Peptide **10** was obtained as a solid (24 mg, 58%).

### Asp-Leu-Trp-Gln-Lys-D-Ams-NH$_2$, 11

The synthesis of **11** was performed as described for the preparation of **9** on the RAM TentaGel (0.26 mmol/g). Fmoc-Ams(Boc)-OH (69.0 mg, 156 μmol)

was coupled to the resin (100 mg, 26 μmol) using DIC (24 μl, 156 μmol) and HOAt (21.0 mg, 156 μmol). The crude peptide was analysed by analytical HPLC ($t_R$ 12.02 min) and purified by semi-preparative HPLC. Analysis by MALDI-MS, $m/z$ 789.6 (M + H)$^+$, $C_{35}H_{55}N_{11}O_{10}$ requires $m/z$ 789.4, confirmed the expected product. Peptide **11** was obtained as a solid (8.4 mg, 41%).

### Glyoxal-linked Dimers 12 and 13

To peptide **9** or **11** (1 mg, 1.27 μmol) was added glyoxal (0.13 μg, 0.63 μmol) dissolved in 0.02 M NaOAc buffer, pH 5.1/DMSO (7:3, v/v) (15 μl). Water (5 μl) was then added to the solution. The pH was then adjusted to 5 with solid sodium acetate. The solution was stirred for 1 h at room temperature and progress of the reaction was monitored by reverse phase (RP)-HPLC. The formed dimer was purified by semi-preparative HPLC. Yields: dimer of **9**, 0.82 mg (81%), and dimer of **11**, 0.79 mg (78%). The expected product gave a single peak in analytical HPLC, $t_R$ 12.3 min dimer **12** (Figure 3(a)), and $t_R$ 14.7 min dimer **13**. Analysis by MALDI-MS, $m/z$ 1601.6 (M + H)$^+$, dimer **12**, and 1601.3 (M + H)$^+$, dimer **13**, $C_{72}H_{108}N_{22}O_{20}$ requires $m/z$ 1600.8.

### Galactose Derivatives 14 and 15

To peptide **9** or **11** (2 mg, 2.5 μmol) was added D-(+)-galactose (0.450 mg, 2.5 μmol) dissolved in 0.02 M NaOAc buffer, pH 5.1 (10 μl) and DMSO (2 μl) and the pH was adjusted to 5 with solid sodium acetate. The solution was then stirred overnight at room temperature. The two glycopeptides were purified by RP-HPLC. Yields: glycopeptide **14**, 1.4 mg (59%) and glycopeptide **15**, 1.2 mg (68%). The expected product gave a single main product in analytical HPLC, $t_R$ 12.3 min glycopeptide **14** (Figure 3(b)), and $t_R$ 12.5 min, glycopeptide **15**. Analysis by MALDI-MS, $m/z$ 951.2 (M + H)$^+$, glycopeptide **14**, and 951.2 (M + H)$^+$, glycopeptide **15**, $C_{41}H_{65}N_{11}O_{15}$ requires $m/z$ 951.5.

## RESULTS AND DISCUSSION

### Synthesis of Building Blocks Fmoc-D-Ams(Boc)-OH, 1, and Fmoc-L-Ams(Boc)-OH, 2

Several routes are open for the introduction of functionality at the $\beta$-position of $\alpha$-amino acids. Among others, a few $\beta$-halo amino acids are commercially available. In the Fmoc-family, $\beta$-chloro-alanine is
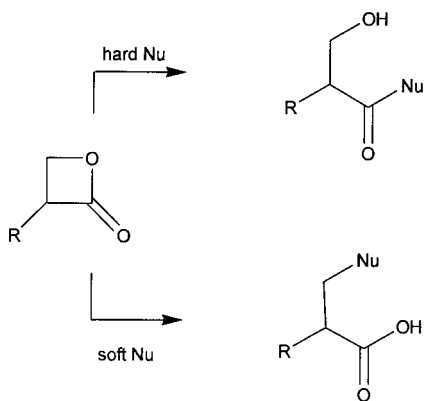
the only commercially available halide. However, a general problem with $\beta$-activated amino acids is their tendency to undergo elimination to yield dehydroalanine (Scheme 1) [31]. Accordingly, the failure of our attempts to substitute the chloro atom of Fmoc-$\beta$-chloro-alanine **16** with N-Boc-hydroxylamine was not surprising. The reaction was relatively sluggish, and under basic conditions (THF with tertiary amines or heterogenous NaHCO$_3$) slow formation of Fmoc-dehydroalanine **17** was the major event. Under more forcing conditions (heating to 40–70°C), deprotection of the Fmoc-group began to interfere. Attempts to perform the reaction without external base by heating the two reaction partners to high temperatures (50–150°C in DMSO) likewise resulted primarily in the formation of dehydroalanine. The Mitsunobu reaction was considered, but this reportedly yields dehydroalanine from N-urethane protected Ser and Thr esters [32]. Although it may be possible to add N-Boc-hydroxylamine to dehydroalanine, this would of course only give the racemic product. It should be noted that N-acylated hydroxylamines may be ambident nucleophiles. The protective effect for the nitrogen atom is in some cases only partial [33]. Thus, the non-eliminative reaction of Boc-hydroxylamine with a suitably $\beta$-activated alanine could lead to the formation of a mixture of O- and N-alkylated products.
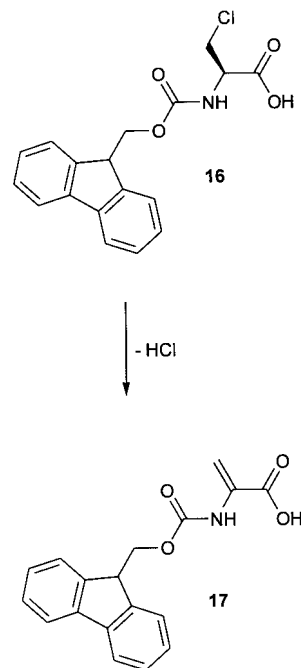


Scheme 1 Formation of Fmoc-dehydroalanine from $\beta$-activated Fmoc-alanine.

However, *N*-alkoxycarbonyl hydroxylamines (such as Boc-NH-OH) are often efficiently protected, so that reaction will only take place in oxygen [32]. Furthermore, the O–N bond is reductively labile, so catalytic hydrogenation for the removal of Z-protecting groups is problematic with oxyamines such as Ams [34,35].

An alternative route to the desired compounds is the ring opening of a serine $\beta$-lactone [36]. $\beta$-lactones may react with nucleophiles by C–O bond cleavage to form $\beta$-functionalized carboxylic acids [37]. This has been useful in the synthesis of several unusual amino acids, amongst other $\beta$-alkyl branched Z-amino acids [38] and $\beta$-phosphono Fmoc-amino acids [39,40]. The opening of $\beta$-lactones is, however, dependent on the nature of the nucleophile [37]. Soft nucleophiles primarily cleave the C–O bond, while hard nucleophiles tend to attack the carbonyl group, yielding $\beta$-hydroxy carboxyls (Scheme 2). Some dependence on the type of solvent is also in effect [41]. Although the $\beta$-lactone system has been described in numerous reports, we could not find any information on ring opening with hydroxylamines. Alcohols and hydrazines are close analogues of hydroxylamine, but these relatively hard nucleophiles react with $\beta$-lactones to usually give the $\beta$-hydroxy product [37,42,43]. This is the 'wrong' product from our point of view. Nevertheless, we wanted to explore this route and prepared the Fmoc-serine $\beta$-lactone **4** by the Mitsunobu reaction, using diethylazodicarboxylate (DEAD) and Ph$_3$P. For preparation of *N*-protected amino acid $\beta$-lactones, the methyl variant of DEAD has previously been described as preferable (dimethylazodicarboxylate, DMAD), because it is easier to separate from amino acid $\beta$-lactones during column chromatography [36]. However,

DMAD was not easily available to us. We found that **4** and the byproduct of DEAD, diethyldicarboxyhydrazine, was indeed difficult to separate on a column, but we also found that the solubility of **4** in CHCl$_3$ is rather low. Thus, isolation of Fmoc-serine $\beta$-lactone **4** in acceptable yield could be accomplished by column chromatography (AcOEt:hexane, 1:1) followed by washing with CHCl$_3$, to remove diethyl hydrazinodicarboxylate impurities. Unfortunately, but not surprisingly, the reaction of **4** with *N*-Boc-hydroxylamine proceeded by the undesired route. In THF, CH$_3$CN and DCM at 20–50°C under both neutral and basic conditions (DIEA) the major product was the *N*-Boc-hydroxylamine ester of Fmoc-serine (**5**, Scheme 3) [44]. There was no trace of the desired product, Fmoc-Ams(Boc)-OH.

Cycloserine **3** (4-amino-3-isoxazolidinone) is a natural product of microbial origin [30]. D-enantiomer cycloserine is the most abundant and thus the cheapest ($30/g). Hydrolysis of cycloserine under acidic conditions is known to produce Ams [27,45,46], Exploiting this chemistry, we succeeded in making Fmoc-D-Ams(Boc)-OH from cycloserine in three steps (Scheme 4):

1. Cycloserine was *N(α)*-protected using Fmoc-OSu in DMF/water. The ring nitrogen holds some



Scheme 2  Ambident $\beta$-lactone opening by nucleophiles.
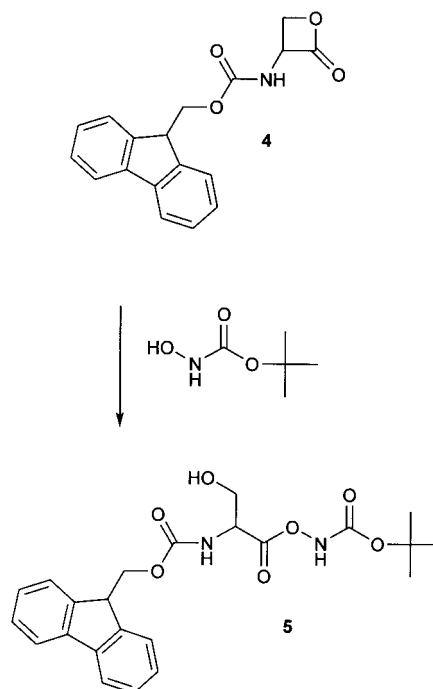


Scheme 3  Formation of Fmoc-Ser-ONH-Boc from the $\beta$-lactone.
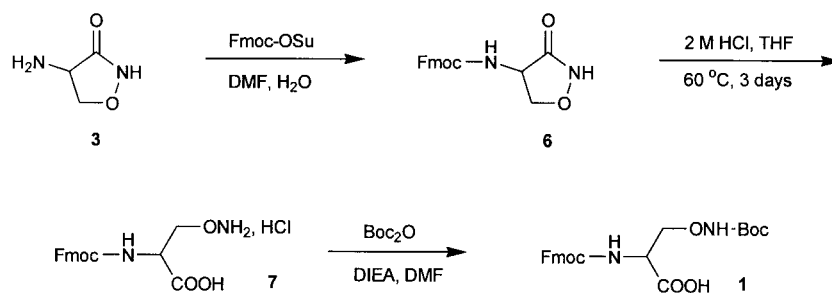
Scheme 4   Synthesis of Fmoc-D-Ams(Boc)-OH from D-cycloserine.

nucleophilicity, analogous to acylated hydroxyl-amines as discussed above [47], and in some batches the *N,N'*-bis-Fmoc compound was formed as a byproduct [56]. Recrystallization from iso-propanol yielded pure Fmoc-cycloser-ine **6**.

2. The hydroxamic function of **6** was hydrolyzed with 2 M aqueous HCl/THF. Reaction at 50–60°C for several days was required. As an alternative, reaction at room temperature for 2 weeks was used. Heating to higher temperatures must be avoided, as byproducts will appear. Among others, O–N bond cleavage has been described previously [46]. The crude hydrochloride **7** was used directly in the next step.

3. Hydrochloride **7** was neutralized and the oxyamine was protected using Boc-anhydride in DMF. A few minor side-products appeared as monitored by TLC, but upon extractive work-up and crystallization from acetone/hexane, the pure compounds (**1** or **2**) were isolated without using chromatography.

Cycloserine and some other small ring α-amino acids, such as pyroglutamic acid, appear to be more susceptible to racemization than α-amino acids in general. The reason for this phenomenon is not well understood, but it is nevertheless described in a few instances [48,49]. In order to investigate any racemization during the synthesis of Fmoc-Ams(Boc)-OH (the prolonged acidic environment during the ring hydrolysis may be a danger) we made Ams from both D- and L-cycloserine. The D-form was made in two batches using acidic hydrolysis at either 20°C or 60°C. The L- and D-Ams residues were coupled to L-Phe-NH$_2$ in solid-phase reactions using DIC with HOAt and the peptides were cleaved from resin with 95% TFA. RP-HPLC analysis separated the diastereomeric peptides D-Ams-L-Phe-NH$_2$ and L-Ams-L-Phe-NH$_2$, and showed no sign of Ams racemization (Figure 1).

## Incorporation of the Ams Building Blocks in Peptide Synthesis

The two building blocks **1** and **2** were incorporated into the *C*- or *N*-terminal of the peptide sequence Asp-Leu-Trp-Gln-Lys. The synthesized peptide sequences are given below:

| | |
|---|---|
| **8** | L-Ams-Asp-Leu-Trp-Gln-Lys-NH$_2$ |
| **9** | Asp-Leu-Trp-Gln-Lys-L-Ams-NH$_2$ |
| **10** | D-Ams-Asp-Leu-Trp-Gln-Lys-NH$_2$ |
| **11** | Asp-Leu-Trp-Gln-Lys-D-Ams-NH$_2$ |
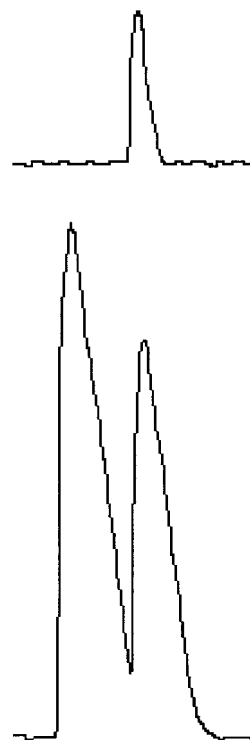


Figure 1   HPLC profiles of D-Ams-Phe-NH$_2$ (top) and mixture of diastereomers (bottom).

The parent penta-peptide has been isolated from the ultra-filtrate of a uremic patient and has shown immunomodulating properties [50]. The sequence was chosen because it contains a side-chain carboxylic acid (Asp) as well as an amine (Lys), thus exemplifying chemoselective ligation. The peptides **8**–**11** were synthesized on a Tentagel resin by standard SPPS using Fmoc-chemistry [51,52]. The Rink amide linker *p*-[(*α*-Fmoc-amino)-2,4-dimethoxybenzyl]phenoxyacetic acid was employed to provide an *α*-carboxamide [53]. All couplings, including those for the building blocks **1** and **2**, were mediated by DIC/HOAt [54]. Progress of the acylation reactions was monitored visually by the presence of bromophenol blue [55]. The protected Ams residues **1** and **2** were used in four equivalent excess in the synthesis of peptides **8** and **10** and the couplings were finished within 1 h. However, anchoring of **1** and **2** to the Rink linker, for the preparation of peptides **9** and **11**, required 3 h with six equivalents of amino acid. Cleavage of the *N(α)*-Fmoc protecting group was carried out with 30% piperidine in *N*-methylpyrrolidone (NMP) for 20 min. Side-chain deprotection and cleavage of the peptides from the resin was carried out by treatment with 95% TFA containing 4 equivalent of triisopropylsilane (TIS). Cleavage of the Ams peptides from resin by 95% aq. TFA without additional scavenger gave peptides with an additional mass of 243, corresponding to the trityl group. Cleavage of the native sequence Asp-Leu-Trp-Gln-Lys by 95% aq. TFA afforded only the expected product. When building Ams-containing peptides, the presence of TIS or other scavengers during TFA-cleavage appears, therefore, to be required for catching the released trityl group. Fortunately, TIS does not seem to affect the otherwise somewhat reductively sensitive Ams O–N bond.

The deprotected peptides were analysed by MALDI-MS and their purity was evaluated by RP-HPLC. Finally, the peptides were purified by semi-preparative RP-HPLC. The pure peptides available for ligation chemistry were obtained in yields of 39–63%. The previously mentioned partially nucleophilic character of acyl-protected oxyamines was apparently not a serious problem for Ams peptide build-up (-ONH-Boc nucleophilicity). This is in accordance with observations from other oxyamine amino acids used in peptide synthesis (e.g. homocanaline) [26].

## Application of Ams Peptides in Chemoselective Ligation

The Ams peptides were used for the synthesis of a peptide dimer and a glycopeptide using chemoselective ligation chemistry. The two ligation reactions in Scheme 5 were performed in water and DMSO. The presence of DMSO is expected to accelerate the oxime formation and to prevent peptide aggregation [11]. The dimers **12** and **13** were prepared by assembling two units of peptide **9** or **11** onto a template containing two aldehyde groups (glyoxal, Scheme 5). The unprotected peptides **9** and **11** were used in one equivalent per aldehyde moiety (peptide:glyoxal 2:1), and the ligation was completed within 1 h as monitored by RP-HPLC (Figure 2). The dimers **12** and **13**, analysed by MALDI-MS, afforded the correct mass and were formed in 81% and 78% yields, respectively. The glycopeptides **14** and **15** were synthesized by the reaction of peptide **9** and **11** with D-galactose (Scheme 5) forming an oxime bond. The cyclic saccharide structure is preserved in this kind of construct. The ligation reaction was monitored by analytical HPLC (Figure 3). The
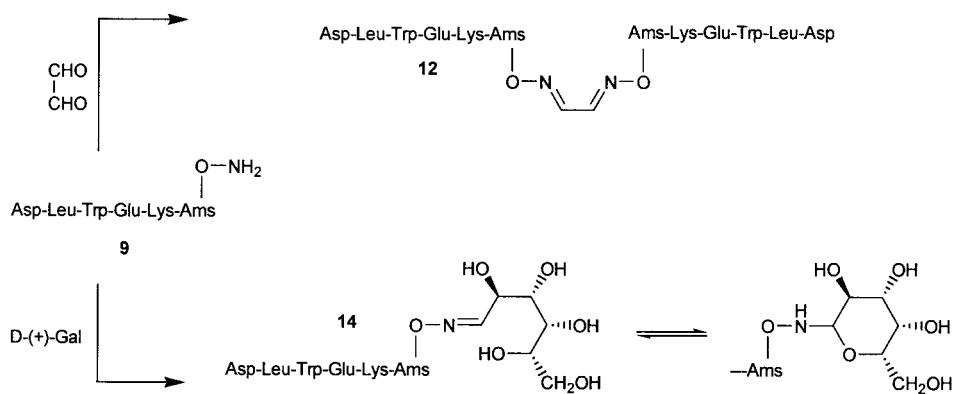


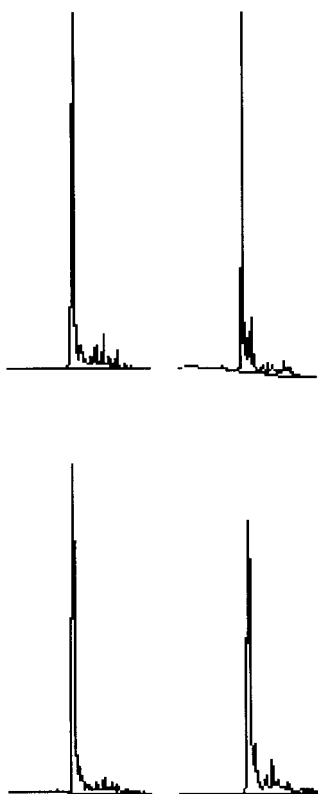Scheme 5   Chemical ligation to give peptide dimer and glycopeptide.

Figure 2 HPLC profiles of crude peptides **8–11** (top left to bottom right).

corresponding glycopeptides **14** and **15** were obtained in 59% and 68% yields. Analysis by MALDI-MS identified the expected mass. Because this approach does not require any protecting groups, a broad spectra of glycopeptides can be prepared directly [13,14,23].

In conclusion, we have prepared D- and L-forms of Fmoc-Ams(Boc)-OH (**1** and **2**), a new orthogonally-
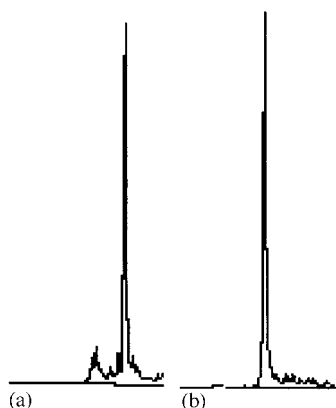


Figure 3 HPLC profiles of crude dimer **12** (left) and crude glycopeptide **14** (right).

protected amino acid with a short oxyamine-containing side-chain, in three steps from the readily available natural product cycloserine. The utility of the new amino acid in chemoselective ligation has been demonstrated, and it has been found that scavengers (TIS) are important when Ams are used in conjunction with the trityl protecting group. Side-reactions of the partially nucleophilic Boc-protected oxyamine appear negligible, as does Ams racemization under both the amino acid synthesis and under standard peptide synthesis conditions.

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## REFERENCES

1. Merrifield RB. Solid phase peptide synthesis I. The synthesis of a tetrapeptide. *J. Am. Chem. Soc.* 1963; **85**: 2149–2154.
2. Tam JP. Synthetic peptide vaccine design: synthesis and properties of a high-density multiple antigenic peptide system. *Proc. Natl. Acad. Sci. USA* 1988; **85**: 5409–5413.
3. Mutter M, Tuchscherer GC, Miller C, Altmann KH, Carey RI, Wyss DF, Labhardt, AM, Rivier JE. Template-assembled synthetic proteins with four-helix-bundle topology. Total chemical synthesis and conformational studies. *J. Am. Chem. Soc.* 1992; **114**: 1463–1470.
4. Sasaki T, Finderis MA, Kaiser ET. Evaluation of the oxime resin based segment synthesis-condensation approach using RNAse T1 as a model synthetic target. *J. Org. Chem.* 1991; **56**: 3159–3168.
5. Rose K. Formation of homogeneous artificial proteins. *J. Am. Chem. Soc.* 1994; **116**: 30–33.
6. Tam JP, Rao C. Synthesis of peptide dendrimers. *J. Am. Chem. Soc.* 1994; **116**: 6975–6976.
7. Tam JP, Spetzler JC. Multiple antigen peptide system. *Methods Enzymol.* 1997; **289**: 612–637.
8. Zhang LS, Tam JP. Lactone and lactam library synthesis by silver ion-assisted orthogonal cyclization of unprotected peptides. *J. Am. Chem. Soc.* 1999; **121**: 3311–3320.
9. Veprek P, Jezek J. Peptide and glycopeptide dendrimers. Part I. *J. Pep. Sci.* 1999; **5**: 5–23.
10. Veprek P, Jezek J. Peptide and glycopeptide dendrimers. Part II. *J. Pep. Sci.* 1999; **5**: 203–220.
11. Shao J, Tam JP. Unprotected peptides as building blocks for the synthesis of peptide dendrimers with oxime, hydrazone and thiazolidine linkages. *J. Am. Chem. Soc.* 1995; **117**: 3894–3899.

12. Pallin TD, Tam JP. Cyclization of totally unprotected peptides in aqueous solution by oxime formation. *J. Chem. Soc.*, *Chem. Commun.* 1995; 2021–2022.

13. Zhao Y, Kent SBH, Chait BT. Rapid, sensitive structure analysis of oligosaccharides. *Proc. Natl. Acad. Sci. USA* 1997; **94**: 1629–1633.

14. Cervigni SE, Dumy P, Mutter M. Synthesis of glycopeptides and lipopeptides by chemoselective ligation. *Angev. Chem., Int. Ed. Engl.* 1996; **35**: 1230–1232.

15. Schnolzer M, Kent SB. Constructing proteins by dovetailing unprotected synthetic peptides: backbone-engineered HIV protease. *Science* 1992; **256**: 221–225.

16. Muir TW, Williams MJ, Ginsberg MH, Kent SB. Design and chemical synthesis of a neoprotein structural model for the cytoplasmic domain of a multisubunit cell-surface receptor-integrin $\alpha$IIb-$\beta$3 (platelet GPIIb–IIIa). *Biochemistry* 1994; **33**: 7701–7708.

17. Dawson PE, Kent SB. Convenient total synthesis of a 4-helix tasp molecule by chemoselective ligation. *J. Am. Chem. Soc.* 1993; **115**: 7263–7266.

18. Dawson PE, Muir TW, Clark-Lewis I, Kent SB. Synthesis of proteins by native chemical ligation. *Science* 1994; **266**: 776–779.

19. Shao Y, Lu WY, Kent SB. A novel method to synthesize cyclic peptides. *Tetrahedron Lett.* 1998; **39**: 3911–3914.

20. Tam JP, Lu Y-A, Yu Q. Thia zip reaction for synthesis of large cyclic peptides: mechanisms and applications. *J. Am. Chem. Soc.* 1999; **121**: 4316–4324.

21. Takenawa T, Oda Y, Ishihama Y, Iwakura M. Cyanocysteine-mediated molecular dissection of dihydrofolate reductase – occurrence of intramolecular and intermolecular reactions forming a peptide bond. *J. Biochem.* 1998; **123**: 1137–1144.

22. Ishihama Y, Ito O, Oda Y, Takenawa T, Iwakura M. A novel method for peptide block synthesis using unprotected peptides. *Tetrahedron Lett.* 1999; **40**: 3415–3418.

23. Peri F, Dumy P, Mutter M. Chemoselective and stereoselective glycosylation of hydroxylamino derivatives – a versatile approach to glycoconjugates. *Tetrahedron* 1998; **54**: 12269–12278.

24. Knobler Y, Frankel M. Synthesis of DL-Canaline and some of its derivatives. *J. Chem. Soc.* 1958; 1632–1635.

25. Oziskas AJ, Rosenthal GA. Synthesis of L-canaline and $\gamma$-functional 2-aminobutyric acid derivatives. *J. Org. Chem.* 1986; **51**: 5047–5050.

26. Lang I, Donze N, Garrouste P, Dumy P, Mutter M. Chemoselectively addressable HCan building blocks in peptide synthesis: L-homocanaline derivatives. *J. Pep. Sci.* 1998; **4**: 72–80.

27. Stammer CH. $\beta$-Aminoxy-D-Alanine. *J. Org. Chem.* 1962; **27**: 2957–2958.

28. Khomutov RM, Karpeiskii MY, Severin ES. Hydroxylamines derivatives, communication 6. Synthesis and some reactions of 3-(aminooxy)alanine. *Bull. Acad. Sci. USSR, Div. Chem. Sci.* 1964; **6**: 631–634.

29. Severin ES, Gnuchev NV, Khomutov RM. Synthesis of conformational inhibitors of pyridoxal enzymes. *Bull. Acad. Sci. USSR, Div. Chem. Sci.* 1967; **3**: 1345–1347.

30. Stammer CH, Wilson AN, Spencer CF, Bachelor FW, Holly FW, Folkers K. Synthesis of cycloserine and a methyl analog. *J. Am. Chem. Soc.* 1957; **79**: 3236–3240.

31. Cherney RJ, Wang L. Efficient Mitsunobu reactions with *N*-phenylfluorenyl or *N*-trityl serine esters. *J. Org. Chem.* 1996; **61**: 2544–2546.

32. Wisniewski K, Koldziejczyk AS, Falkiewicz B. Applications of the Mitsunobu reaction in peptide chemistry. *J. Pep. Sci.* 1998; **4**: 1–14.

33. Katritzky AR, Rao MSC. A novel synthesis of acyclic *N*-($\alpha$-hydroxybenzyl)benzamides. *Synthesis* 1990; 663–666.

34. Kamiya T. Synthesis of tricholomic acid. VI. Synthesis of DL-tricholomic acid and its threo-isomer from diastereoisomers of $\beta$-hydroxyglutamic acid $\alpha$-benzyl ester. *Chem. Pharm. Bull.* 1969; **17**: 886–889.

35. Pajpanova T, Stoev S, Golovinsky E, Krauss GJ, Miersch J. Canavanine derivatives useful in peptide synthesis. *Amino Acids* 1997; **12**: 191–204.

36. Arnold LD, Kalantar TH, Vederas JC. Conversion of serine to stereochemically pure $\beta$-substituted $\alpha$-amino acids via $\beta$-lactones. *J. Am. Chem. Soc.* 1985; **107**: 7109–7115.

37. Gregory JT, Gresham TL, Jansen JE, Shaver FW. $\beta$-propiolactones I–V. *J. Am. Chem. Soc.* 1948; **70**: 998–1006.

38. Arnold LD, Drover JCG, Vederas JC. Conversion of serine $\beta$-lactones to chiral $\alpha$-amino acids by copper-containing organolithium and organomagnesium reagents. *J. Am. Chem. Soc.* 1987; **109**: 4659.

39. Hutchinson JPE, Parkes KEB. A short synthesis of (S)-3-(dimethylphosphono)-2-((9-fluorenyl)methoxycarbamoyl)propionic acid, a protected phosphonic acid analogue of aspartic acid. *Tetrahedron Lett.* 1992; **33**: 7065–7066.

40. Lohse PA, Felber R. Incorporation of a phosphonic acid isostere of aspartic acid into peptides using Fmoc-solid phase synthesis. *Tetrahedron Lett.* 1998; **39**: 2067–2070.

41. Ratemi ES, Vederas JC. Reaction of trimethylsilylamines with *N*-Cbz-L-serine-$\beta$-lactone – a convenient route to optically pure $\beta$-amino-L-alanine derivatives. *Tetrahedron Lett.* 1994; **35**: 7605–7608.

42. Gresham TL, Jansen JE, Shaver FW, Bankert RA, Feidorek FT. $\beta$-Propiolactone XI. *J. Am. Chem. Soc.* 1951; **73**: 3168–3171.

43. Kim KS, Ryan PC. Synthesis of an aminopyrazolidine substituted quinolone acid. *Heterocycles* 1990; **31**: 79–86.

44. Baldwin JE, Adlington RM, Mellor LC. Synthesis of chiral isoxazolidin-5-ones and their applications to the

synthesis of $\beta$-amino-alanines and $\beta$-($N$-hydroxy-amino)-alanines. *Tetrahedron* 1994; **50**: 5049–5066.

45. Weaver N, Busch NF, Stammer CH. Serine carbamates. *J. Med. Chem.* 1974; **17**: 1033–1035.

46. Korpela T, Makela M. Preparation and properties of *O*-amino-D-serine; acid hydrolysis of D-4-amino-3-isoxazolidinone (D-cycloserine). *Synthesis* 1980; 997–998.

47. Kim KS. Synthesis of an aminoisoxazolidine substituted quinolone acid. *Heterocycles* 1990; **31**: 87–95.

48. Stammer CH, Kartha CC, Chaturvedi NC, McKinney JD. Cycloserine derivatives. *J. Med. Chem.* 1970; **13**: 1013–1015.

49. Dolence EK, Lin CE, Miller MJ. Synthesis and siderophore activity of albomycin-like peptides derived from $N^5$-acetyl-$N^5$-hydroxy-L-ornithine. *J. Med. Chem.* 1991; **34**: 956–968.

50. Niese D, Gilsdorf K, Hiester E, Dressen P, Michels S, Dengler HJ. Immuno-modulating properties of the uremic pentapeptide H-Asp-Leu-Trp-Gly-Lys-OH. *Klin. Wochenschr.* 1986; **64**: 642–647.

51. Chang CD, Meinhofer J. Solid-phase peptide synthesis using mild base cleavage of *N*-fluorenylmethyloxycarbonyl amino acids. *Int. J. Pep. Prot. Res*. 1978; **11**: 246–249.

52. Atheron E, Fox H, Harkiss D, Logan CJ, Sheppard RC, Williams BJ. A mild procedure for solid-phase peptide synthesis: use of fluorenylmethyloxycarbonyl amino acids. *J. Chem. Soc., Chem. Commun.* 1978; 537–539.

53. Rink H. Synthesis of protected peptide fragments using a trialkoxy-diphenyl-methyl ester resin. *Tetrahedron Lett.* 1987; **28**: 3787–3790.

54. Carpino LA. 1-Hydroxy-7-azabenzotriazole – an efficient peptide coupling additive. *J. Am. Chem. Soc.* 1993; **115**: 4397–4398.

55. Krchnak V, Vagner J, Safar S, Lebl M. Noninvasive continuous monitoring of solid phase peptide synthesis by acid–base indicator. *Collect. Czech. Chem. Commun.* 1988; **53**: 2542–2548.

56. Gordeev MI, Luehr GW, Hui HC, Gordon EM, Patel DV. Combinatorial chemistry of natural products: solid phase synthesis of D- and L-cycloserine derivatives. *Tetrahedron* 1998; **54**: 15879–15890.