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ORIGINAL ARTICLE

A practical synthesis of N^{α}-Fmoc protected L-*threo*-β-hydroxyaspartic acid derivatives for coupling via α- or β-carboxylic group

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Abstract A simple and practical general synthetic protocol towards orthogonally protected *t*HyAsp derivatives fully compatible with Fmoc solid-phase peptide synthetic methodology is reported. Our approach includes enantioresolution of commercially available D,L-*t*HyAsp racemic mixture by co-crystallization with L-Lys, followed by ion exchange chromatography yielding enantiomerically pure L-*t*HyAsp and D-*t*HyAsp, and their selective orthogonal protection. In this way N^{α}-Fmoc protected *t*HyAsp derivatives were prepared ready for couplings via either α- or β-carboxylic group onto the resins or the growing peptide chain. In addition, coupling of *t*HyAsp via β-carboxylic group onto amino resins allows preparation of peptides containing *t*HyAsn sequences, further increasing the synthetic utility of prepared *t*HyAsp derivatives.

Keywords Hydroxyaspartic acid · Enantioresolution · Orthogonal protection · Fmoc solid-phase peptide synthesis

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Abbreviations

ACN	Acetonitrile
Bn–OH	Benzyl alcohol
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCM	Dichloromethane
DHP	Dihydropyran
DIC	Diisopropylcarbodiimide
Dmab–OH	4-{*N*-[1-(4,4-Dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]-amino} benzyl alcohol
DMAP	Dimethylaminopyridine
DMSO	Dimethyl sulfoxide
EtOAc	Ethyl acetate
EtOH	Ethanol
Fmoc-OSu	*N*-(9-Fluorenylmethoxycarbonyloxy) succinimide
HOAc	Acetic acid
IPA	Isopropanol
MeOH	Methanol
PPTS	Pyridinium *p*-toluenesulfonate
TBAF	Tetrabutylammonium fluoride
TBDMS-Cl	*tert*-Butyldimethylsilyl chloride
TFA	Trifluoroacetic acid
THF	Tetrahydrofurane

Introduction

Stereoisomers of β-hydroxyaspartic acid (HyAsp) as well as its analog β-hydroxyasparagine (HyAsn) are found in both free form and as peptide constituents in many natural products. L-*threo*-β-hydroxyaspartic acid (L-*t*HyAsp) alone inhibits growth of various fungi (Ishiyama et al. 1975), and

its derivatives exhibit a series of biological activities such as inhibition of glutamate/aspartate transporters (Lebrun et al. 1997; Shimamoto et al. 2000), inhibition of tumor cell growth (Thomasset et al. 1991; Tournaire et al. 1994), and antiretroviral activity (Malley et al. 1994). HyAsp and HyAsn were found in sequences of antibacterial peptides such as ramoplanin (Cavalleri et al. 1984; Pallanza et al. 1984), katanosins (O'Sullivan et al. 1988; Kato et al. 1988), lanthiopeptin (Naruse et al. 1989), alterobactins (Reid et al. 1993), plusbacins (Shoji et al. 1992; Maki et al. 2001), and cormycin A (Scaloni et al. 2004). Among them, ramoplanin shows most promising clinical potential and is currently in Phase III trials for treatment of *Clostridium difficile*-associated disease (CDAD) and for the prevention of vancomycin-resistant enterococci bloodstream infections (Montecalvo 2003; Rogers and Leach 2004). Other examples of naturally occurring peptides containing HyAsp/HyAsn are theonellamide (Matsunaga et al. 1989) and cepacidine (Lee et al. 1994; Lim et al. 1994), exhibiting strong antifungal activity, and microviridins (Reshef and Carmeli 2006) that act as protease inhibitors.

However, limited synthetic access to these peptides and particularly their analogs hampered complete exploitation of their biological potential and utilization as lead compounds for drug development. Since Fmoc solid-phase peptide synthesis (Fmoc SPPS) represents a standard approach for the routine peptide synthesis, access to orthogonally protected HyAsp and HyAsn fully compatible with this methodology is of practical importance. Asymmetric syntheses of L-*t*HyAsn bearing standard protecting groups for Fmoc-solution or solid-phase peptide synthesis have been reported in the literature (Boger et al. 2000; Guzmán-Martínez and VanNieuwenhze 2007; Spengler et al. 2010). In contrast, a few synthetic protocols are reported for preparation of HyAsp, but none of the reported derivatives are compatible with the standard Fmoc SPPS methodology. Hanessian and Vanasse (1993) reported the synthesis of L-*threo*-HyAsp (L-*t*HyAsp) and L-*erythro*-HyAsp (L-*e*HyAsp) mixtures of diesters by hydroxylation of L-aspartic acid (L-Asp) diesters. Starting with *trans*-ethyl cinnamate, Sharpless asymmetric aminohydroxylation was used by Khalaf and Datta (2008) to prepare D-*t*HyAsp. In all cases reported, HyAsp derivatives possess unprotected α-amino and α-carboxylic groups. Boc-protecting strategy was chosen to synthesize N^{α}-protected HyAsp derivatives. Hansson and Kihlberg (1986) used tartaric acid to prepare protected L-*e*HyAsp, whereas N^{α}-Boc-L-Ser was used by Wagner and Tilley (1990) to prepare protected L-*t*HyAsp fully compatible with Boc peptide synthetic methodology. N^{α}-Boc-L-*t*HyAsp was also prepared by Deng et al. (1995) starting from methyl cinnamate and using Sharpless dihydroxylation reaction. In this case, synthesized L-*t*HyAsp derivative underwent additional deprotection and reprotection to obtain the desired N^{α}-Boc protected L-*t*HyAsp. All reported N^{α}-Boc-HyAsp derivatives possess a free α-carboxylic group, limiting somewhat their synthetic applicability.

We report here a practical and efficient synthetic approach towards orthogonally protected D- and L-*t*HyAsp fully compatible with the standard Fmoc SPPS methodology. Besides complete orthogonal protection, our approach allows synthetic exploitation of both HyAsp carboxylic groups expanding the versatility of these peptide building blocks.

Materials and methods

General methods

Unless otherwise specified, all chemicals and solvents were purchased from Fisher Scientific (Atlanta, GA) and were analytical reagent grade or better. All solvents used were dried over 4 Å molecular sieves prior to use, unless specified differently. D,L-*t*HyAsp and Marfey's reagent were purchased from TCI America (Portland, OR, USA). Thin-layer chromatography (TLC) used for monitoring reactions was performed on Whatman precoated silica gel F-254 plates (Whatman Inc., Piscataway, NJ, USA) and visualized by ultraviolet light and/or staining with ninhydrin solution. Silica gel (Dynamic Adsorbents Inc., Atlanta, GA; 32–63 μm) was used for flash chromatography. The ^{1}H and ^{13}C NMR spectra were obtained on Varian 400 MHz NMR spectrometer (Varian Inc., Palo Alto, CA, USA). Chemical shifts (δ) are reported in units of parts per million (ppm) downfield from tetramethylsilyl chloride. High resolution mass spectroscopy (HRMS) was performed on MALDI–TOF Voyager-DE^{TM} STR (Applied Biosystems, Foster City, CA, USA). Enantiomeric purity of resolved D- and L-*t*HyAsp was determined by derivatization with Marfey's reagent and RP-HPLC analyses of corresponding derivatives on a Thermo Electron Corporation SpectraSYSTEM (Thermo Fischer Scientific Inc., Waltham, MA, USA) liquid chromatography system. For RP-HPLC analysis, a C_{18} monomeric column (Grace Vydac, 250 × 4.6 mm, 5 μm, 120 Å), 1 mL/min flow rate, and elution method 100% A for 5 min followed by linear gradient of 0–100% B over 40 min, where A was 0.1% TFA in H_2O and B was 0.1% TFA in ACN, was used. Eluting products were detected by UV at 340 nm. Optical rotations were measured on Autopol III RA 7214 automatic polarimeter (Rudolph Research, NJ, USA) at 25°C, and 589 nm (Na-D).

Enantioresolution of D,L-*t*HyAsp

D,L-*t*HyAsp racemic mixture **1** was successfully separated by co-crystallization with L-Lys (equimolar ratio) followed

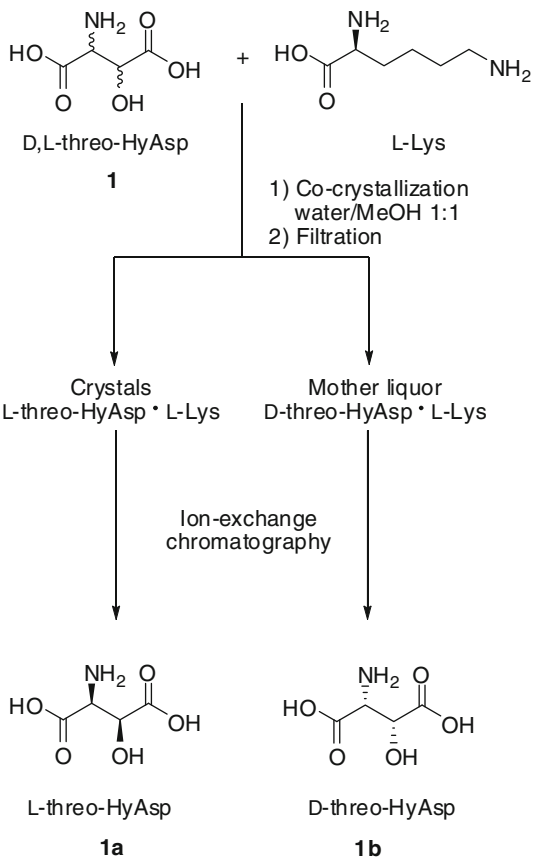
by ion exchange chromatography (Okai et al. 1967), Scheme 1. Enantiomerically pure L-Lys · L-*t*HyAsp salt crystallized from the H_2O:MeOH solvent mixture overnight at 4°C, whereas L-Lys · D-*t*HyAsp remained in the mother liquor (Supporting information). In both cases L-Lys was separated by ion exchange chromatography, and pure L- and D-*t*HyAsp, **1a** and **1b**, were obtained in 75% yields.

RP-HPLC analysis of D- and L-*t*HyAsp after derivatization with Marfey's reagent (Bhushan and Brückner 2004), Fig. 1, shows that in both cases enantiomeric purity was >99%. Specific optical rotations ($[\alpha]_D$) for the obtained D- and L-*t*HyAsp are in very good agreement with the literature data (Okai et al. 1967), further confirming the enantiomeric resolution efficiency, Table 1.

Compound synthesis and characterization

*(2S,3S)-2-amino-4-(benzyloxy)-3-hydroxy-4-oxobutanoic acid (**2**)*

L-*t*HyAsp **1a** (1 g, 6.71 mmol) was added to benzyl alcohol (10 mL) and concentrated HCl (1.2 mL), and the resulting mixture was stirred at 70°C for 4.5 h. Upon reaction completion, the pH was adjusted to 6 with NH_4OH, and cold EtOH was added to precipitate the product. Precipitate was recrystallized from hot water, and pure (2S,3S)-2-amino-4-(benzyloxy)-3-hydroxy-4-oxobutanoic acid (**2**) (1.4 g, 5.86 mmol, 87%) was obtained as white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.33–7.43 (m, 5H, Bn–ArH), 5.11 (dd, 2H, Bn–CH_2), 4.52 (d, 1H, Hβ), 3.47 (d, 1H, Hα) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.7, 167.7, 136.0, 128.4, 128.0, 69.7, 66.1, 55.9 ppm.



Scheme 1 General scheme of D,L-*t*HyAsp enantioresolution

*(2S,3S)-2-(((9H-fluoren-9-yl)methoxy)carbonylamino)-4-(benzyloxy)-3-hydroxy-4-oxobutanoic acid (**3**)*

A solution of Fmoc-OSu (0.141 g, 0.42 mmol) in acetone was added dropwise to a solution of compound **2** (0.1 g, 0.42 mmol) and $NaHCO_3$ (0.07 g, 0.84 mmol) in water:acetone mixture (1:2, 15 mL). The reaction was completed within 2 h, as determined by TLC. The reaction mixture was concentrated via rotary evaporation, and the residual water was acidified with 2 N HCl until pH 4 and extracted two times with EtOAc. EtOAc was evaporated and the crude residue was loaded on a silica gel column (solvent system toluene:EtOAc:HOAc, 5:5:1) yielding the product (0.185 g, 0.4 mmol, 96%) as a white solid. HRMS calculated for $C_{26}H_{23}NO_7$ *m/z* $(M + Na)^+$ 484.1372; found 484.4560. ^{1}H NMR (400 MHz, CD_3OD) δ 7.12–7.82 (m, 13H, Fmoc–ArH, Bn–ArH), 5.16 (dd, 2H, Bn–CH_2), 4.83 (d, 1H, Hα), 4.76 (d, 1H, Hβ), 4.27 (m, 2H, Fmoc–CH_2), 4.19 (t, 1H, Fmoc–CH) ppm; ^{13}C NMR (100 MHz, CD_3OD) δ 173.0, 172.8, 158.6, 145.4, 145.3, 142.7, 137.1, 129.6, 129.6, 129.5, 128.9, 128.4, 128.3, 126.53, 126.49, 121.1, 72.5, 68.5, 68.5, 58.5 ppm.

*(2S,3S)-2-(((9H-fluoren-9-yl)methoxy)carbonylamino)-4-(benzyloxy)-4-oxo-3-(tetrahydro-2H-pyran-2-yloxy)butanoic acid (**4**)*

PPTS was added (0.008 g, 0.0325 mmol) to a solution of **3** (0.15 g, 0.325 mmol) in dry DCM (15 mL).To the stirred reaction mixture, DHP was added dropwise over 10 min (0.045 mL, 0.487 mmol). Stirred was continued at room temperature for 72 h with occasional addition of PPTS and DHP (total 0.3 equiv. PPTS and 2.5 equiv. DHP). Reaction mixture was then extracted with water. DCM was evaporated and crude product purified using silica gel chromatography (solvent system toluene:EtOAc:HOAc, 10:1:1) yielding 0.115 g of product (0.211 mmol, 65%). HRMS calculated for $C_{31}H_{31}NO_8$ *m/z* $(M + Na)^+$ 568.1947; found 568.4458. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.28–7.77 (m, 13H, Fmoc–ArH, Bn–ArH), 5.69 (d, 1H, Hα), 5.17 (s, 2H, Bn–CH_2), 5.01 (m, 1H, Hβ), 4.86 (t, 1H, THP), 4.33 (m, 2H, Fmoc–CH_2), 4.22 (t, 1H, Fmoc–CH), 3.49–3.75 (m, 2H, THP), 1.50–1.77 (m, 6H, THP) ppm; ^{13}C NMR

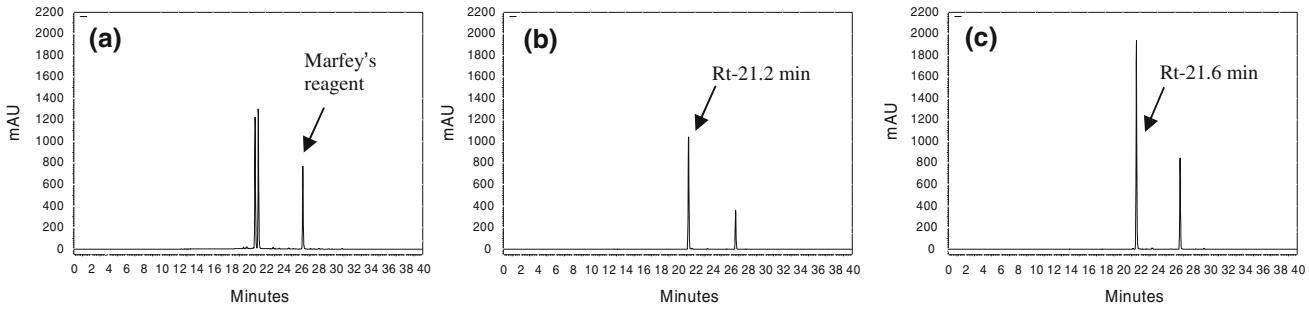


Fig. 1 RP-HPLC traces after derivatization with Marfey's reagent **a** D,L-*t*HyAsp, **b** D-*t*HyAsp, **c** L-*t*HyAsp

Table 1 Specific optical rotations for resolved *t*HyAsp enantiomers

[α]D	Literature values[a]		Observed values[b]	
	5 N HCl	H_2O	5 N HCl	H_2O
L-*t*HyAsp (**1a**)	+6.4°	−8.5°	+6.0°	−8.2°
D-*t*HyAsp (**1b**)	−6.5°	+8.9°	−6.0°	+8.4°

[a] Okai et al. 1967

[b] For the detailed method see Supporting Information

(100 MHz, $CDCl_3$) δ 173.0, 169.1, 156.5, 143.9, 141.4, 135.2, 128.8, 128.7, 128.6, 127.9, 127.3, 125.4, 120.2, 97.6, 73.5, 67.8, 67.7, 62.6, 56.2, 47.1, 30.0, 25.2, 18.9 ppm.

*(2S,3S)-1-allyl 4-benzyl 2-(((9H-fluoren-9-yl)methoxy)carbonylamino)-3-(tetrahydro-2H-pyran-2-yloxy)succinate (**5**)*

Aliquat 336 (0.084 mL, 0.183 mmol) and allyl–Br (0.017 mL, 0.183 mmol) were dissolved in DCM (15 mL). This mixture was added to a stirred solution of **4** (0.1 g, 0.183 mmol) and $NaHCO_3$ (0.016 g, 0.183 mmol) in water (15 mL). Reaction was stirred at room temperature for 72 h with occasional addition of 0.5 equiv. of allyl–Br (total added was 2.5 equiv.). DCM was separated from the aqueous layer and extracted one more time with water, evaporated, and crude product was purified on a silica column (solvent system toluene:EtOAc:HOAc, 10:1:1) yielding 0.109 g (85%) of pure product **5**. HRMS calculated for $C_{34}H_{35}NO_8$ *m/z* $(M + Na)^+$ 608.2260; found 608.6071. ^1H NMR (400 MHz, $CDCl_3$) δ 7.25–7.77 (m, 13H, Fmoc–ArH, Bn–ArH), 5.91 (m, 1H, Allyl), 5.65 (d, 1H, Hα), 5.36 (dd, 1H, Allyl), 5.27 (dd, 1H, Allyl), 5.16 (s, 2H, Bn–CH_2), 5.02 (m, 1H, Hβ), 4.87 (t, 1H, THP), 4.67 (m, 2H, Allyl–CH_2), 4.33 (m, 2H, Fmoc–CH_2), 4.22 (t, 1H, Fmoc–CH), 3.43–3.62 (m, 2H, THP), 1.46–1.79 (m, 6H, THP) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.2, 169.1, 156.3, 144.0, 143.9, 141.4, 135.2, 131.5, 128.8, 128.7, 128.7, 127.9, 127.3, 125.5, 120.2, 119. 5, 101.0, 96.9, 73.2, 67.7, 67.7, 67.5, 66.8, 66.8, 61.9, 56.4, 47.2, 30.2, 29.9, 25.3, 25.2, 19.0, 18.4 ppm.

*(2S,3S)-2-amino-4-(benzyloxy)-3-(tert-butyldimethylsilyloxy)-4-oxobutanoic acid (**7**)*

To a stirred solution of **2** (0.56 g, 2.33 mmol) in dry ACN (300 mL), DBU was added (0.351 mL, 2.33 mmol) to obtain pH approximately 9 after which TBDMS-Cl was added in half equimolar portions (0.175 g, 1.17 mmol). Occasionally DBU was added to correct the pH of the solution. Reaction mixture was refluxed at 50°C. Total 2 equiv. of TBDMS-Cl and 3.5 equiv. of DBU were used. The reaction was monitored by TLC and was completed within 3–5 h. The solvent was evaporated; residue redissolved in water, acidified with 2 N HCl until pH 4 and extracted two times with EtOAc. Crude product (yellow oil, 0.674 g, 82% yield) was used in the next step without further purification.

*(2S,3S)-2-(((9H-fluoren-9-yl)methoxy)carbonylamino)-4-(benzyloxy)-3-(tert-butyldimethylsilyloxy)-4-oxobutanoic acid (**8**)*

To a solution of **7** (0.674 g, 1.91 mmol) and $NaHCO_3$ (0.321 g, 3.82 mmol) in water:acetone mixture (1:2, 100 mL), a solution of Fmoc-OSu (0.644 g, 1.91 mmol) in acetone was added dropwise. The reaction was monitored by TLC and was completed within 2 h. Reaction mixture was concentrated, residual water acidified with 2 N HCl until pH 4 and extracted two times with EtOAc. EtOAc was evaporated and crude residue was loaded on a silica gel column and purified (solvent system toluene:EtOAc:HOAc, 10:1:1) yielding pure product **8** (1.06 g, 1.84 mmol, 96% yield) as a white solid. HRMS calculated for $C_{32}H_{37}NO_7Si$ *m/z* $(M + Na)^+$ 598.2237; found 598.2075. ^1H NMR (400 MHz, $CDCl_3$) δ 7.3–7.76 (m, 13H, Fmoc–ArH, Bn–ArH), 5.58 (d, 1H, Hα), 5.16 (dd, 2H, Bn–CH_2), 4.93 (d, 1H, Hβ), 4.33 (m,2H, Fmoc–CH_2), 4,23 (t, 1H, Fmoc–CH), 0.89 (s, 9H, TBDMS-*t*Bu), 0.11 (s, 3H, TBDMS–CH_3), 0.03 (s, 3H, TBDMS–CH_3) ppm; ^{13}C NMR

(100 MHz, $CDCl_3$) δ 174.3, 169.9, 156.1, 143.7, 141.2, 141.2, 135.0, 128.57, 128.55, 128.50, 127.7, 127.1, 125.2, 119.9, 72.2, 67.6, 67.5, 57.3, 46.9, 25.5, 25.4, 18.7, −4.9, −5.8 ppm.

*(2S,3S)-1-(4-(1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutylamino)benzyl) 4-benzyl 2-(((9H-fluoren-9-yl)methoxy)carbonylamino)-3-(tert-butyldimethylsilyloxy)succinate (**9**)*

To a solution of **8** (0.3 g, 0.52 mmol) in DCM (30 mL), DMAP (0.013 g, 0.1 mmol) and Dmab–OH (0.205 g, 0.624 mmol) were added. DIC (0.097 mL, 0.624 mmol) was added dropwise over 15 min. Reaction was stirred at room temperature for 4–6 h with monitoring using TLC. The solvent was removed and silica gel chromatography (solvent system toluene:EtOAc:HOAc, 10:5:1) afforded product **9** (0.319 g, 0.36 mmol, 70%) as a yellowish solid. HRMS calculated for $C_{52}H_{62}N_2O_9Si$ *m/z* $(M + Na)^+$ 909.4122; found 909.1248. ^1H NMR (400 MHz, $CDCl_3$) δ 7.07–7.78 (m, 17H, Fmoc–ArH, Bn–ArH, Dmab–ArH), 5.63 (d, 1H, Hα), 5.10–5.15 (m, 4 H, Bn–CH_2,Dmab–CH_2), 4.97 (d, 1H, Hβ), 4.31 (m, 2H, Fmoc–CH_2), 4.23 (t, 1H, Fmoc–CH), 2.49 (s, 2H, Dmab–CH_2), 2.40 (s, 2H, Dmab–CH_2), 2.09 (d, 2H, Dmab–CH_2 isopropyl), 1.82 (m, 1H, Dmab–CH isopropyl), 1.08 (d, 6H, 2 Dmab–CH_3), 0.88 (s, 9H, TBDMS-*t*Bu), 0.76 (s, 3H, Dmab–CH_3 isopropyl), 0.74 (s, 3H, Dmab–CH_3 isopropyl), 0.09 (s, 3H, TBDMS–CH_3), −0.04 (s, 3H, TBDMS–CH_3) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 200.2, 196.5, 176.5, 170.0, 169.8, 169.2, 169.5, 156.0, 155.5, 143.74, 143.71, 143.6, 141.2, 137.1, 136. 9, 135.1, 135.1, 134.4, 134.3, 129.2, 129.2, 128.64, 128.58, 128.53, 127.7, 127.1, 126.7, 126. 6, 120.0, 107.8, 72.4, 67.6, 67.4, 67.0, 66.8, 66.8, 57.7, 53.7, 52.3, 47.0, 46.9, 38.4, 30.0, 29.6, 28.3, 25.6, 22.6, 18.2, −4.8, −5.8 ppm.

*(2S,3S)-4-(4-(1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutylamino)benzyloxy)-3-(((9H-fluoren-9-yl)methoxy)carbonylamino)-2-(tert-butyldimethylsilyloxy)-4-oxobutanoic acid (**10**)*

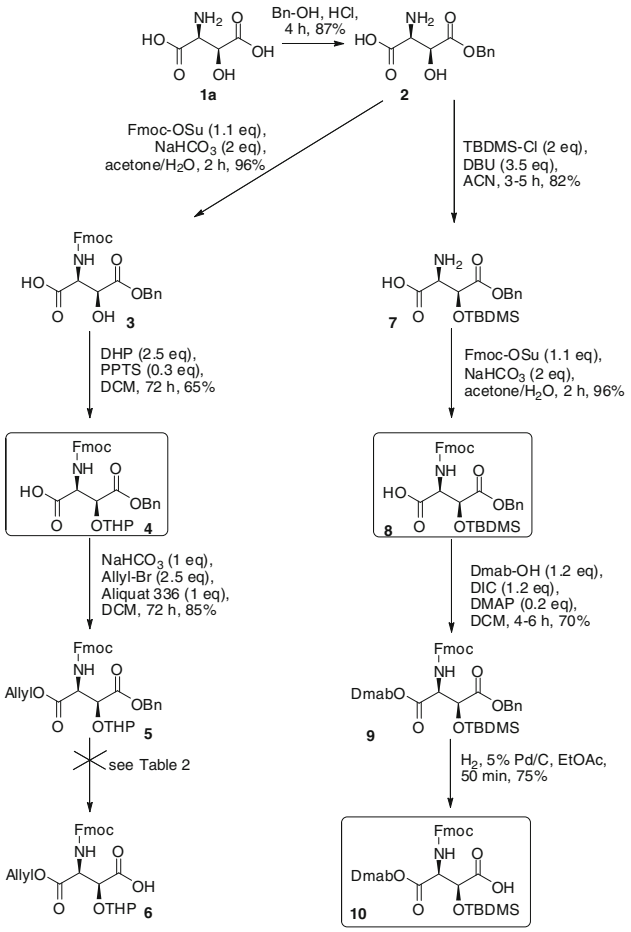
Fully protected amino acid **9** (0.27 g, 0.3 mmol) was dissolved in EtOAc, and 5% Pd/C (270 mg) was added. The reaction vessel was set up at 1 atm of H_2 and shaken at room temperature for 50 min. Reaction mixture was filtered through Celite and evaporated. Product was precipitated with petroleum ether yielding pure **10** (0.184 g, 0.23 mmol, 75%). HRMS calculated for $C_{45}H_{56}N_2O_9Si$ *m/z* $(M)^+$ 795.3677; found 795.6108. ^1H NMR (400 MHz, $CDCl_3$) δ 7.04–7.76 (m, 12H, Fmoc–ArH, Dmab–ArH), 5.71 (d, 1H, Hα), 5.10–5.13 (dd, 2 H, Dmab–CH_2), 4.98 (d, 1H, Hβ), 4.33 (m, 2H, Fmoc–CH_2), 4.23 (t, 1H, Fmoc–CH), 2.49 (s, 2H, Dmab–CH_2), 2.41 (s, 2H, Dmab–CH_2), 2.06 (d, 2H, Dmab–CH_2 isopropyl), 1.81 (m, 1H, Dmab–CH isopropyl), 1.06 (d, 6H, 2 Dmab–CH_3), 0.90 (s, 9H, TBDMS–*t*Bu), 0.75 (s, 3H, Dmab–CH_3 isopropyl), 0.73 (s, 3H, Dmab–CH_3 isopropyl), 0.14 (s, 3H, TBDMS–CH_3), 0.01 (s, 3H, TBDMS–CH_3) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 203.2, 197.0, 176.8, 176.5, 173.3, 169.2, 167.4, 156.3, 144.3, 143.7, 143.6, 141.5, 141.2, 136.9, 134.4, 129.2, 127.7, 127.6, 127.1, 126.7, 125.2, 125.2, 124.7, 120.0, 107.6, 71.9, 67.7, 66.8, 61.4, 57.6, 53.5, 49.3, 46. 9, 39.0, 38.5, 30.0, 29.6, 28.2, 25.6, 22.7, 22.6, 20.7, 18.2, −4.8, −5.7 ppm.

Results and Discussion

The goal of this work was to develop a practical and efficient synthesis of *t*HyAsp derivatives suitable for Fmoc SPPS. Selection of orthogonal protecting groups allows activation and subsequent coupling of N^{α}-Fmoc protected derivatives via either α- or β-carboxylic group onto the free hydroxyl or amine of the resin linker or of the growing peptide chain. Synthesis commences with enantioresolution of commercially available D,L-*t*HyAsp racemic mixture, Scheme 1, thus avoiding the multi-step process of enantioselective synthesis. Selective orthogonal protections of the resulting enantiomers **1a** and **1b**, Scheme 2, make these amino acid derivatives versatile building blocks in Fmoc solid phase peptide synthesis.

Enantioresolution of D,L-tHyAsp

The enantioselective enzyme-catalyzed hydrolysis of N^{α}-acyl amino acids had been a widely used method in the resolution of amino acid racemic mixtures. In particular, acylase I (*N*-acetylamino-acid amidohydrolase) was shown to be very efficient in the preparation of large quantities of enantiopure L-amino acids (Švedas and Galaev 1983; Bommarius et al. 1992; Sato and Tosa 1993). However, several studies demonstrated that acyl derivatives of aspartic acid are not substrates for acylase I due to unfavorable charge interactions (Chenault et al. 1989; Liljeblad et al. 2001), thereby eliminating this method as an option for enantioresolution of D,L-*t*HyAsp. Instead, we have focused on their chemical resolution. Attempts to separate the D,L-mixture of compound **3** via co-crystallization with (−)-ephedrine hydrochloride (Oki et al. 1970; Wong and Wang 1978), a method traditionally used for resolution of N^{α}-benzyloxycarbonyl-D,L-amino acids, were unsuccessful. Limited solubility of D,L-**3** in a variety of solvents including $CHCl_3$, MeOH, EtOH, ACN, acetone, THF, EtOAc, EtOAc:MeOH 1:1, EtOAc:petroleum ether 2:3, EtOAc: diethyl ether 1:1, EtOAc:$CHCl_3$ 1:1, EtOAc:DCM 2:3,



Scheme 2 General protection scheme for HyAsp

EtOAc:ACN 1:3 resulted in poor resolution of the corresponding diastereomeric ephedrine salts. Here, D,L-*t*HyAsp was successfully enantioresolved by modified method previously described by Okai et al. (1967) via co-crystallization with L-Lys followed by ion-exchange chromatography. Both enantiomers were obtained with satisfactory yield (75%) and enantiomeric purity (e.e. >99%).

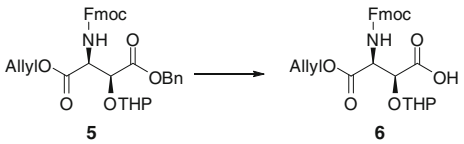
Design of a protection scheme

Appropriate selection of orthogonal protecting groups and their selective manipulation is an essential requirement in peptide and amino acid synthesis. In order to find the optimal combination of orthogonal protecting groups for *t*HyAsp, we have tested benzyl (Bn), allyl (Allyl), and (4{N-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]-amino} benzyl (Dmab) for protection of carboxyl groups, 9-fluorenylmethylmethoxycarbonyl (Fmoc) for protection of amino, and *tert*-butyldimethylsilyl (TBDMS) and tetrahydropyranyl (THP) for protection of the hydroxyl group. Considering this reaction conditions for the standard Fmoc SPPS and the requirement for the *t*HyAsp coupling via either α- or β-carboxylic group, combination of the Fmoc, Dmab, TBDMS and Bn protecting groups gave the best results. The general protection scheme for L-*t*HyAsp **1a** is shown in Scheme 2. Selective protection of β-carboxylic group was achieved by Fischer-type esterification, resulting in compound **2** (87% yield). Due to the presence of acid catalyst, protonation of the α-amino group determined the esterification selectivity (Okonya et al. 1995). In order to prepare *t*HyAsp derivative with a free α-carboxylic group, we have devised two protecting approaches. One includes Fmoc protection of the α-amino group resulting in compound **3**, 96% yield (Cudic et al. 2005), followed by β-hydroxyl group protection with THP (Miyashita et al. 1977) with a 65% yield to obtain compound **4**.

In another approach, due to the basic reaction conditions requirements for TBDMS introduction, order of protections was reversed. The β-hydroxyl group was protected first with TBDMS (Orsini et al. 1989) (**7**, 82% yield), followed by α-amino group protection with Fmoc (**8**, 96% yield). Although both THP and TBDMS protecting groups fulfill the requirement for orthogonal protection, we gave preference to the TBDMS due to better reaction yields, the lack of need for chromatographic purification, less complex reaction monitoring and final product characterization. Introduction of the THP group added an additional stereocenter to the *t*HyAsp molecule, resulting in two diastereomers of **4** with distinguishable R_f values on the silica-gel TLC plates (0.35, 0.33; toluene:EtOAc:AcOH 10:1:1), and complex NMR spectra (Green and Wuts 1999). On the other hand, preparation of the orthogonally protected L-*t*HyAsp derivate with a free β-carboxyl group turned out to be more challenging. After successful introduction of an allyl protecting group (**5**, 85% yield, Friedrich-Bochnitschek et al. 1989), selective removal of benzyl ester using various approaches was unsuccessful. We have tested several reaction conditions including mild base, Lewis acids, catalytic hydrogenation, neutral reagents, or lipase C from *Candida Antarctica*, and in all cases either no Bn removal or more that one protecting group removal was observed. The reaction conditions used for benzyl ester removal are summarized in Table 2.

Replacement of the allyl protecting group with the Dmab, compound **9**, allowed preparation of the desired L-*t*HyAsp derivative **10** over five reaction steps in a satisfactory yield of 36%. Among several previously published methods for coupling of Dmab-OH to amino acid's carboxyl group, the most satisfactory results were obtained using DIC/DMAP coupling reagents (Berthelot et al. 2006). However, this method had to be modified for optimal Dmab-OH coupling to compound **8**. If catalytic amounts of DMAP were to be used, the required reaction time is significantly prolonged resulting in a poor yield for compound **9**. On the other hand, use of equimolar amount

Table 2 Tested conditions for selective removal of benzyl protecting group from **5**



Conditions	Observed[a]
20% K_2CO_3/H_2O, EtOH, 1 h (Verbeure et al. 2002)	Removal of Fmoc, Bn and Allyl
15% K_2CO_3/H_2O, THF, 4 h (Huffman et al. 1978)	No deprotection
4 mM NaOH, 0.5 M $CaCl_2$, IPA/H_20 (7:3), 2 h (Pascal and Sola 1998)	Removal of Fmoc and Bn
1 N LiOH, THF, 2 h (Chauhan et al. 2007)	Removal of Fmoc and Bn
1 N BCl_3, DCM, −10°C to r.t., 1 h (Schmidt et al. 1991)	Removal of THP first
$Pd(OAc)_2$, Et_3SiH, Et_3N, DCM, 1 h-overnight (Coleman and Shah 1999)	Short time-no deprotection, longer time-all protecting groups removed except THP
H_2, 10% Pd/C, EtOAc, 1 h (Broddefalk et al. 1998)	Removal of Bn and Allyl or Allyl reduction
TBAF, THF, 1 h (Namikoshi et al. 1991)	Removal of Fmoc first
$HCOONH_4$/Mg, MeOH, 3 h (Channe Gowda et al. 2002)	Removal of Bn and Allyl
Enzymatic removal, 24 h (Barbayianni et al. 2005)	No deprotection

[a] Based on ^{1}H NMR analysis and TLC comparison of reaction mixtures with compounds **1**–**5**

of DMAP causes Fmoc deprotection. The best results were obtained with 0.2 equiv. of DMAP. In this case compound **9** was obtained in 70% yield, with no detectable epimerization as indicated by RP-HPLC and NMR analysis (see Supporting Information). Final benzyl protecting group removal was successfully achieved by hydrogenation of compound **9** with 5% Pd/C in ethyl-acetate (Broddefalk et al. 1998). Yield for this final step was 75%. The outlined protection scheme is also expected to be useful for the preparation of orthogonally protected D-*t*HyAsp, as well as D- and L-*e*HyAsp derivatives.

Conclusions

We have successfully devised a simple and practical general synthetic protocol towards orthogonally protected HyAsp derivatives, fully compatible with Fmoc solid-phase peptide synthetic methodology. This approach allows utilization of the commercially available D,L-*t*HyAsp racemic mixture and preparation of multi-gram quantities of enantiomerically pure N^{α}-Fmoc protected *t*HyAsp derivatives for coupling via either α- or β-carboxylic group onto the resins or the growing peptide chain. In addition, the coupling of HyAsp via free β-carboxylic group onto amino resins enables the preparation of peptides containing HyAsn sequences, further increasing the utility of the designed protection scheme.

Considering the increasing demand for peptides containing unusual sequences due to their interesting biological activities, the described synthesis of N^{α}-Fmoc protected *t*HyAsp building blocks is of particular practical value allowing multiple possibilities for their incorporation into a peptide backbone.

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