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Stereoselective Synthesis of a Pyridoxamine Coenzyme–Amino Acid Chimera: Assembly of a Polypeptide Incorporating the Pyridoxamine Moiety

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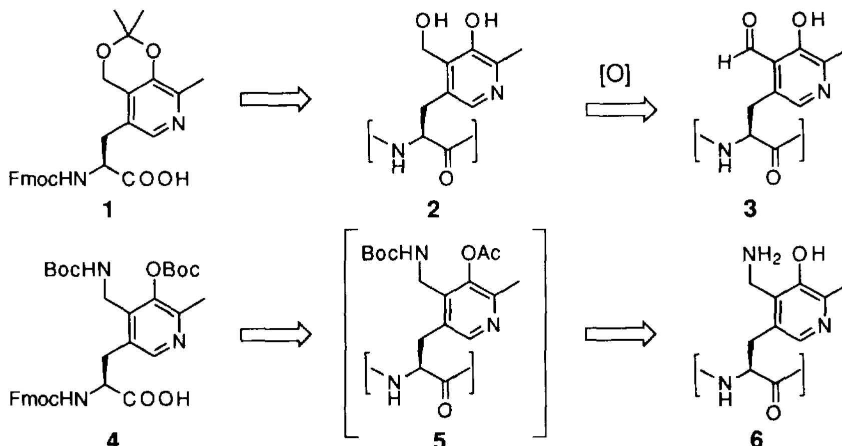
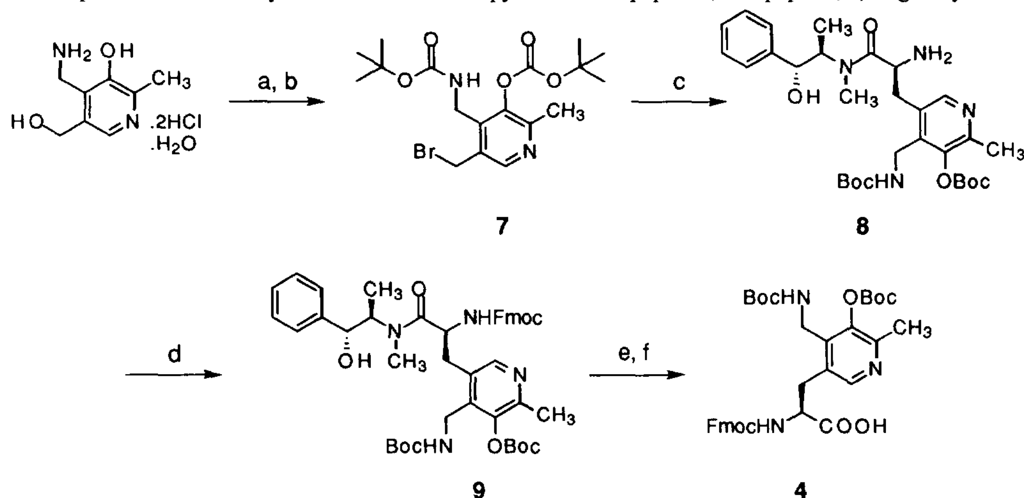
Abstract: The stereoselective synthesis of (*S*)-2-amino-N^{α}-(9-fluorenylmethoxycarbonyl)-3-(α^4,3-*O*-*bis*-*tert*-butoxycarbonylpyridoxamin-5-yl)propanoic acid (**4**) was accomplished from pyridoxamine dihydrochloride. A key step is the stereoselective alkylation of (-)pseudoephedrine glycinamide with the pyridoxamine bromide **7**. Amenability of the amino acid derivative **4** to solid phase methodology was demonstrated by its incorporation into a synthetic hexapeptide. Critical to the orthogonal protection of the pyridoxamine moiety is the *in situ* acetylation of the C3-hydroxyl group during peptide assembly.

The coenzyme–amino acid chimeras represent a powerful new class of amino acids for the *de novo* design of functional polypeptides and proteins.[1-4] We have previously reported the semisyntheses of pyridoxal coenzyme–amino acid (Pal, **3**) containing proteins, which mimic the native pyridoxal-dependent transaminases and mediate the transamination of α-amino acids in the absence of metal ions.[1] These Pal-polypeptides were obtained by selective oxidation of the corresponding pyridoxol precursors (**2**).[2] The low yields of this solution phase oxidation (10-29%), and an inability to purify the reactive aldehyde-peptides prevented the investigation of catalytic turnover within these systems. In addition, the thermodynamics and kinetics of the transamination process favor a reaction flux in the direction: pyridoxamine + α-keto acid → pyridoxal + α-amino acid. It would therefore be advantageous to initiate transamination within synthetic polypeptides with a pyridoxamine coenzyme–amino acid chimera.



Scheme 1. Protected amino acid derivatives (**1**, **4**), and key intermediates (**2**, **5**) in the assembly of peptides incorporating the pyridoxal and pyridoxamine coenzyme–amino acid chimeras.

Herein, we present the stereoselective synthesis of a protected pyridoxamine coenzyme–amino acid (**4**), which was incorporated into a polypeptide by solid phase Fmoc methodology without the need for solution phase modification. *In situ* conversion of the *bis*-Boc derivative to the aryl acetate (**5**), followed by solid phase deacetylation, release of the peptide from the solid support, and purification of the crude product by reversed-phase HPLC directly afforded the reactive pyridoxamine-peptide (Pam-peptide, **6**) in good yields.



Scheme 2. Synthesis of the protected pyridoxamine coenzyme–amino acid chimera (**4**).
a. di-*tert*-butyldicarbonate 2.7 equiv, 1:1 2N NaOH/*p*-dioxane, pH 9.5, 0 °C → rt, 12 h (100%); b. *N*-bromosuccinimide 1.1 equiv, triphenylphosphine 1.2 equiv, CH_2Cl_2, 0 °C → rt, 25 min (85%); c. LDA 4.88 equiv, LiCl 15.0 equiv, (-)pseudoephedrine glycinamide 2.5 equiv, THF, 0 °C, 90 min (60%); d. FmocOSu 1.2 equiv, Na_2CO_3 2.0 equiv, 1:1 H_2O/*p*-dioxane, 0 °C → rt, 90 min (79%); e. 1:1 9N H_2SO_4/*p*-dioxane, reflux, 6.5 h, neutralize with 50% (w/w) NaOH (100%); f. di-*tert*-butyldicarbonate 4.4 equiv, 1:1 10% Na_2CO_3/*p*-dioxane, pH 9.5, 0 °C → rt 16 h (93%).

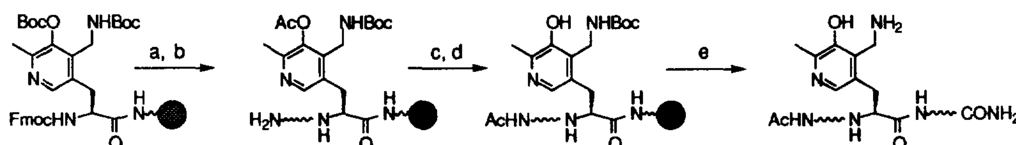
Stereoselective synthesis of the coenzyme–amino acid chimera **4** was challenging because of the heterocyclic and multifunctional nature of the target. Appropriate protection of the pyridoxamine moiety was required for the subsequent stereoselective alkylation. Several protecting schemes for the C3-hydroxyl and C4′-amine (including the cyclic carbamate, boroxazolidinone,[5] and di-*tert*butylsilylene[6] derivatives) suffered from low yields, or the generation of intermediates with unacceptable solubility properties. Ultimately, the *bis*-Boc protected bromide (**7**) was obtained in excellent yield from commercially available pyridoxamine dihydrochloride monohydrate, by treatment of the latter with excess di*tert*-butyldicarbonate and subsequent bromination of the *bis*-Boc protected alcohol with NBS and triphenylphosphine. This compound could be stored at -80 °C for a few weeks, being slowly converted to the dimer.

A key step in the assembly of the chiral coenzyme–amino acid was the utilization of (-)pseudoephedrine as a chiral auxiliary for setting the stereochemistry at the C^{α} position. Myers and co-workers have recently introduced this methodology for the stereoselective syntheses of α-amino acids in good yields with excellent enantioselectivities.[7] Their original procedure was modified to accommodate the highly functionalized bromide **7**. Thus, treatment of (-)pseudoephedrine glycinamide[8] with 1.95 equiv LDA in the presence of 6 equiv LiCl at 0 °C generated the corresponding enolate with minimal side products. Addition of **7** (pre-

neutralized by treatment with basic alumina) to *excess* enolate (2.5 equiv) at 0 °C afforded the alkylated product **8** in 60% isolated yield (no reaction was observed at lower temperatures). The alkylation proceeded with excellent diastereoselectivity (>10:1), and the minor diastereomer was separable by flash chromatography (eluant: 90:5:5 $CHCl_3/CH_3OH$/triethylamine).

Attempts to release the chiral auxiliary by basic (2 equiv NaOH) or aqueous hydrolysis resulted in partial loss of one or both Boc groups. An exhaustive acidic hydrolysis at this stage was not desirable, since the fully deprotected coenzyme–amino acid chimera is not easily amenable to selective reprotection of the pyridoxamine moiety. The Fmoc derivative **9** was therefore prepared by treatment of **8** with Fmoc-succinimidylcarbonate. The N^{α}-Fmoc amino acid derivative was obtained in quantitative yield upon subsequent refluxing in 1:1 9N H_2SO_4/*p*-dioxane for 6.5 h. Although this procedure resulted in a complete deprotection of the pyridoxamine moiety, the Boc groups were easily re-introduced in the final step of the synthesis. Thus, neutralization of the acid hydrosylate with 50% (w/w) NaOH and adjustment of the pH to 9.5 with 5% Na_2CO_3 allowed the extraction of the chiral auxiliary into CH_2Cl_2. Subsequent treatment of the aqueous phase with di-*tert*-butyldicarbonate in *p*-dioxane directly afforded the protected Pam derivative **4** in 93% overall yield from **9**.[9]

The hexapeptide Ac-Thr-Pyr-Pro-DAla-Pam-Gly-NH_2 (Pyr = 3-(3-pyridyl)-L-alanine) was assembled using solid phase peptide synthesis to demonstrate compatibility of the Pam chimera with Fmoc methodology. The peptide was synthesized on an automated peptide synthesizer using Fmoc-PAL-PEG-PS® resin (PerSeptive Biosystems, MA) and HOBT/DIPCDI chemistry as described previously.[2] Residual terminal amines were capped at the end of each coupling cycle with a 0.3 M acetic anhydride/HOBT solution. Unexpectedly, ^1H NMR and FAB-MS analyses of the purified peptide revealed the incorporation of an extra acetyl group. *O*-acetylation was ruled out by stability of the product to $K_2CO_3/H_2O/CH_3OH$.[10] Apparently, HOBT-mediated deprotection of the labile C3-Boc carbonate during coupling of the Pam residue and subsequent exposure to capping reagent led to the formation of an aryl acetate (**5**). A 1,3-acyl shift to the C4′-amine during acidolytic cleavage of the peptide from the solid support subsequently resulted in the N-α^4-acetylated product.



Scheme 3. Solid phase synthesis of the Pam-peptide subsequent to incorporation of the Pam residue.
a. 0.3 M 1-hydroxybenzotriazole/acetic anhydride, DMF, 10 min; b. Coupling of additional residues (HOBT/DIPCDI amino acid activation and subsequent Fmoc deprotection with 20% piperidine in DMF); c. Acetic anhydride 21 equiv, triethylamine 6 equiv, DMF, 2 h; d. 1:4:5 satd. $K_2CO_3/H_2O/CH_3OH$, 12 h; e. 90:5:3:2 trifluoroacetic acid/thioanisole/ethanedithiol/anisole, 3 h.

Formation of the *bis*-acetylated peptide was prevented by deprotection of the *O*-acetate prior to release of peptide from the resin. Accordingly, the peptide synthesis protocols were modified to include a deacetylation step (Scheme 3, d). Treatment of the resin with 1:4:5 satd. $K_2CO_3/H_2O/CH_3OH$ for 12 h and subsequent cleavage afforded only the desired mono-acetylated hexapeptide.[11] The transaminase activity of this peptide is currently being evaluated.

Acknowledgments

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9. The identities of all compounds were confirmed by NMR and HRMS analyses. Data for key compounds are presented: *N*-([1*R*,2*R*]-2-hydroxy-1-methyl-2-phenylethyl)-*N*-methyl-(*S*)-2-amino-3-(α^4,3-*O*-bis-*tert*-butoxycarbonylpyridoxamin-5-yl)-propionamide (**8**): ^{1}H NMR (500 MHz, $CDCl_3$, 27 °C, 2:1 rotameric forms, * denotes minor rotamer) δ 0.8 (d, 2H), 0.91* (d, 1H), 1.37-1.51 (overlapping s, 18H), 2.34 (s, 3H), 2.71 (s, 2H), 2.88* (s, 1H), 3.00 (d, 1H), 3.01-3.28 (broad, 3H) 3.30 (d, 1H), 4.09 (overlapping m, 1H), 4.25 (overlapping m, 2H), 4.44 (d, 1H), 4.65 (broad m, 1H), 5.55 (broad t, 1H), 7.23-7.32 (overlapping m, 5H), 7.80* (s, 0.15 H) 7.95* (s, 0.05 H), 8.17* (s, 0.6 H), 8.32* (s, 0.2 H); ^{13}C NMR (75 MHz, $CDCl_3$, * denotes minor rotamer) δ 14.0, 15.4*, 18.7, 18.8*, 27.4, 27.7, 28.1, 30.7, 30.8*, 34.4, 35.5*, 35.8, 51.9*, 52.2, 57.0*, 57.9*, 74.8, 75.5, 79.5, 84.1, 126.6, 126.7*, 127.6, 128.1, 128.4*, 130.4, 137.9, 141.1*, 141.5, 144.8, 148.3, 148.8*, 150.6, 150.9*, 155.3, 174.6; FAB-HRMS [MH^+] calcd for $C_{30}H_{45}O_7N_4$ 573.3288, obsd 573.3283.
   (*S*)-2-amino-N^{α}-9-fluorenylmethoxycarbonyl-3-(α^4,3-*O*-bis-*tert*-butoxycarbonylpyridoxamin-5-yl)-propionic acid (**4**): ^{1}H NMR (500 MHz, $CDCl_3$, 27 °C) δ 1.26 (s, 9H), 1.46 (s, 9H), 2.27 (s, 3H), 2.97 (broad, 1H), 3.37 (broad, 1H), 3.93 (broad, 1H), 4.01 (broad, 1H), 4.22 (overlapping m, 3H), 4.41 (broad, 1H), 5.39 (broad, 1H), 7.14 (d, 2H), 7.25 (t, 2H), 7.42 (m, 2H), 7.61 (d, 2H), 8.24 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 18.6, 27.3, 28.0, 32.5, 35.6, 46.8, 57.1, 66.7, 79.7, 83.9, 119.3, 125.0, 126.7, 127.1, 132.1, 137.7, 140.8, 143.9, 148.4, 149.5, 151.1, 156.6, 177.3; FAB-HRMS [MH^+] calcd for $C_{35}H_{42}O_9N_3$ 647.2843, obsd 647.2828.
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11. Identity of the peptide was confirmed by NMR and HRMS analyses. In addition, the UV signature of the Pam residue closely matches that of the pyridoxamine cofactor.

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