One step synthesis of biotinylated amino acids

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A rapid method is reported for the preparation of biotinylated 4-amino-D-phenylalanine(D-Aph) with Fmoc as the protecting group at the α-amino. Different coupling reagents and conditions were studied to get the biotinylated compound. Coupling reagents like 1-[bis(dimethylamino)methylene]-1*H*-benzotriazolium 3-oxide hexafluorophosphate and BOP were found to be very efficient. Biotinylated Boc-L-Aph and biotinylated Fmoc-L-lysine were also successfully prepared by this method.

Keywords: biotin, 4-aminophenylalanine, lysine, 1-[bis(dimethylamino)methylene]-1*H*-benzotriazolium 3-oxide hexafluorophosphate

Vitamin transporters have been reported to facilitate intestinal peptide uptake,[1,2] and the bio-formulations using this transport system have shown promising results.[3,4] Glucagon-like peptide-1 was recently modified with biotin (vitamin H), which actively traverses the intestine membrane via sodium-dependent multivitamin transportation (SMVT).[5] The biotinylated products displayed higher Caco-2 cell monolayer permeability and higher oral hypoglycemic efficacy than the corresponding compounds without biotin.

The introduction of biotin to a peptide could be accomplished via direct peptide biotinylation at the amino group of the N-terminal or the side chain of Lys,[5] or synthesis of a peptide with biotinylated amino acids as building blocks. The latter was more convenient for the preparation of a peptide compared with the former. 4-aminophenylalanine (Aph) is an unnatural amino acid with an amino group on its side chain and is often used in bioactive peptides research. So we believed that biotinylated 4-aminophenylalanine (Aph (Bio)) should be another good choice to introduce a biotin into a bioactive peptide from its side chain. Here we reported a one-step-synthesis of fluorenylmethyloxycarbonyl (Fmoc) and -butyloxycarbonyl (Boc) protected Aph (Bio) from biotin and α-amino protected Aph. Fmoc and Boc protected amino acids were mostly used in solid phase peptide synthesis. Our method provides an efficient way to introduce biotin at desired position in synthesised peptides.

Generally, a biotinylated amino acid was prepared by reaction the reactive intermediate, biotin hydroxysuccinimide ester (biotin-OSu), with amino acid bearing a free carboxyl group.[6,7] In another way, the carboxyl group of the amino acid was first protected and then hydrolyzed after biotinylation with a coupling reagent like dicyclohexylcarbodiimide.[8] However, we found that biotin-OSu would not react with Fmoc-D-Aph-OH under reported conditions.[6,7] The reaction might be inhibited by the lesser activity of the aromatic amino group and greater steric hindrance of the phenyl ring on the amino acid compared with lysine. Moreover, the second method is very inconvenient and maybe unsuitable since the carboxyl group must be protected first and then removed with NaOH solution after introduction of biotin, which may cause hydrolysis of Fmoc. To get the biotinylated amino acid, different coupling reagents and conditions were studied. 1-[bis(dimethylamino)methylene]-1*H*-benzotriazolium 3-oxide hexafluorophosphate (HBTU) worked better than others at room temperature (Scheme 1). The results were presented in Table 1. Boc-L-Aph(Biotin)-OH (**3b**) and Fmoc-L-Lys(Biotin)-OH (**3c**) were also obtained by this way (Scheme 2 and Table 2) and the yield of the latter was moderate higher than that reported with biotin-OSu method.[7] ^{1}H NMR and high resolution mass spectrums were used to identify the structures of the three biotinylated compounds.

Table 1 Synthesis of biotinylated Fmoc-D-Aph under different conditions

Reaction no.	CR	T /°C	Reaction time /min	HPLC /%	t_R /min	Yield /%
1	Biotin-OSu	25	—[a]	—	—	—
2	Biotin-OSu	50	—[a]	—	—	—
3	Biotin-OSu	75	—[b]	—	—	—
4	Biotin/EDC·HCl/ HOBt	25	90[c]	95.1	9.663	68
5	Biotin/EDC·HCl/ HOBt	50	70[c,d]	—	—	—
6	Biotin/HBTU/DIEA	25	60[e]	95.4	9.688	76
7	Biotin/HBTU/DIEA	50	40[d,e]	—	—	—
8	Biotin/BOP/DIEA	25	60[e]	96.2	9.681	75
9	Biotin/BOP/DIEA	50	40[d,e]	—	—	—

EDC·HCl: *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride; HOBt: 1-hydroxy-1H-benzotriazole; BOP: benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate.
[a] No product observed in 48 hours.
[b] Decomposition in 30 minutes.
[c] Including 30 minutes for preactivating the carboxyl group.
[d] New byproduct observed.
[e] Including 5 minutes for pre-activating the carboxyl group; t_R: retention time; HPLC conditions: Phase A: 0.1% trifluoroacetic acid/water(v/v); Phase B: 0.1% trifluoroacetic acid/70% acetonitrile/water(v/v/v). Phase B: 0min, 30%; 5min, 100%; 15min, 100%; 17min, 30%; phase A + Phase B = 100%. The wavelength of the detector was set at 210 nm. The flow rate was 1mL min^{-1}.

1a 3a

Scheme 1 Synthesis of Fmoc-D-Aph(Bio) under different conditions. CR represented coupling reagent(s), either biotin hydroxysuccinimide ester or biotin with a coupling reagent.

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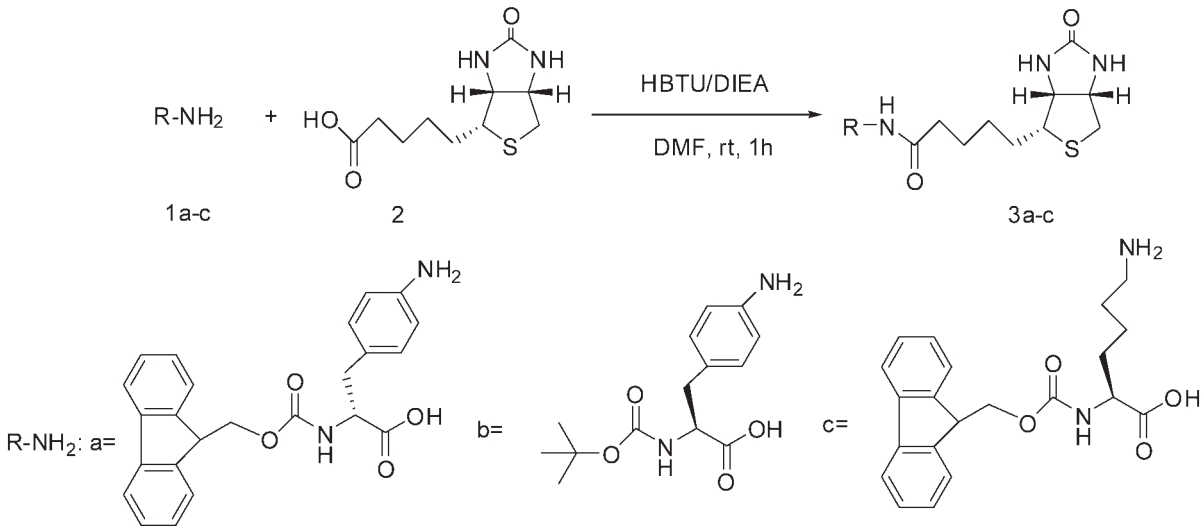

Scheme 2 Synthesis of biotinylated amino acids with HBTU method.

Table 2 Synthesis of biotinylated amino acids with HBTU method

Compd no.	Reaction time /min	HPLC /%	t_R /min	Yield /%	M.p. /°C
3a	60[a]	95.4	9.688	76	201–203 (dec.)
3b	60[a]	93.3	8.494	82	136–138 (dec.)
3c	60[a]	94.6	9.163	85	178–180
3c[b]	>600	NA	NA	72	181–182

[a] Including 5 minutes for pre-activating the carboxyl group.
[b] Data reported in ref. 7 with biotin-OSu method; NA: not available; t_R: retention time; HPLC conditions: Phase A: 0.1% trifluoroacetic acid/water(v/v); Phase B: 0.1% trifluoroacetic acid/70% acetonitrile/water(v/v/v). Phase B: 0min, 30%; 5min, 100%; 10min, 100%; 17min, 30%; phase A + Phase B = 100%. The wavelength of the detector was set at 210nm. The flow rate was 1mL min^{-1}.

In conclusion, a one-step-synthesis for rapid preparation of biotinylated amino acids was reported. Fmoc-D-Aph (Biotin)-OH(**3a**), Boc-L-Aph(Biotin)-OH(**3b**) and Fmoc-L-Lys(Biotin)-OH (**3c**) were successfully synthesised in this manner. To the best of our knowledge, the preparation both of Fmoc-D-Aph(Biotin)-OH(**3a**) and Boc-L-Aph(Biotin)-OH(**3b**) have not been reported previously. We found that biotin-OSu did not work on preparing Fmoc-D-Aph(Biotin)-OH(**3a**) due to the less activity of the aromatic amino group. The synthesis of Fmoc-L-Lys(Biotin)-OH (**3c**) by the way described above was much faster and gave moderate higher yield than that reported method using biotin-OSu method. Our work provide a rapid and convenient method for the preparation of biotinylated amino acids.

Experimental

Melting points were measured on a type YRT-3 melting point apparatus. The ^{1}H NMR (400 MHz) spectra were recorded on a JNM-ECA-400 spectrometer in DMSO-d_6 with tetramethylsilane as internal standard. Mass spectra were performed on a type JMS-700 instrument. HPLC was performed on a Shimadzu LC-10AT VP Plus liquid chromatograph system with a Wondasil 4.6 mm × 150 mm C18 column. Optical rotations were measued by a Polaar 3005 polarimeter (Optical Activity Limited). Fmoc-D-Aph and Boc-L-Aph were prepared according to ref. 9 from D-Phe and L-Phe respectively. Fmoc-L-Lys were purchased from Chengnuo Biochem (Chengdu) Ltd.. HBTU, BOP, DCC, HOBt, EDC·HCl and DIEA were purchased from GL Biochem (Shanghai) Ltd.

Preparation of **3a–c** *with HBTU method at room temperature; general procedure*

Diisopropylethylamine (DIEA, 0.17 mL, 1.0 mmol) was added to N-protected amino acid, **1a–c**, (1.0 mmol) in a dimethylformamide (DMF 5 mL) solution and this was stirred at room temperature for 5 minutes. DIEA (0.33 mL, 2.0 mmol) was added to D-Biotin (244 mg, 1.0 mmol) DMF (10 mL) solution in the same way. The solutions were mixed and the reaction was monitored by TLC. After about 50 minutes, the reaction was completed and DMF was removed under vacuum. Saturated $NaHCO_3$ (50 mL) solution was poured into the reaction bottle and the mixture was stirred vigorously. A white precipitate appeared in about 1 hour. The solid was collected by suction filtration and washed first with water, and then with cold methanol. The powder was suspended in water(50 mL) and acidified with saturated solution of citric acid solution. The precipitate was collected by filtration under vacuum and washed with water and cold methanol sequentially and was dried. The crude product was recrystallised from methanol-DMF to give a pure product. The structures of the compounds **3a–c** were identified by ^{1}H NMR and MS.

Fmoc-D-Aph(Biotin)-OH (**3a**): Yield: 76%; m.p. 201–203 °C (dec.); ^{1}H NMR (DMSO-d_6): δ 1.28–1.72 (m, 6H), 2.28 (t, J = 7.4 Hz, 2H), 2.58 (d, J = 12.4 Hz, lH), 2.76–2.85 (m, 2H), 2.96–3.16 (m, 2H), 4.06–4.32 (m, 6H), 6.35 (s, 1H), 6.43 (s, 1H), 7.10–7.20 (d, J = 8.4 Hz, 2H), 7.22–7.72 (m, 7H), 7.82–7.92 (m, 2H), 9.81 (s, 1H), 12.72 (s, 1H); M-1: 627.22892, exact $mass_{cal.}$: 628.23556; $[\alpha]_D^{25}$ +24.0 (c=1.0, DMSO).

Boc-L-Aph(Biotin)-OH (**3b**): Yield 82%; m.p. 136–138 °C (dec.); ^{1}H NMR (DMSO-d_6): δ 1.23–1.72 (m, 15H), 2.28 (t, J = 7.4 Hz, 2H), 2.58 (d, J = 12.4 Hz, lH), 2.76–2.85 (m, 2H), 2.96–3.16 (m, 2H), 3.95–4.32 (m, 3H), 6.39 (s, 1H), 6.48 (s, 1H), 7.04–7.20 (m, 3H), 7.48 (d, 2H), 9.84 (s, 1H), 12.57 (s, 1H). M-1: 505.21256; exact $mass_{cal.}$: 506.21991; $[\alpha]_D^{25}$ +31.6 (c=1.0, DMSO).

Fmoc-L-Lys(Biotin)-OH (**3c**): Yield 85%; m.p. 178–180 °C (Ref[8]: 181–182 °C) ; ^{1}H NMR (DMSO-d_6): δ 1.25–1.36 (m, 6H), 1.37–1.50 (m, 3H), 1.55–1.66 (m, 2H), 1.65–1.72 (m, 1H), 2.04 (t, J = 7.6 Hz, 2H), 2.56 (d, J = 12.4 Hz, lH), 2.81 (dd, J = 12.2, 5.2 Hz, 1H,), 2.96–3.04 (m, 2H), 3.04–3.11 (m, 1H), 3.86–3.94 (m, 1H), 4.08–4.14 (m, 1H), 4.18–4.32 (m, 4H), 6.35 (s, 1H), 6.41 (s, 1H),7.29–7.45 (m, 4H), 7.58–7.92 (m, 6H), 12.55 (s, 1H); $[\alpha]_D^{25}$ +15.6 (c=1.0, DMSO).

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