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Synthesis of Enantiopure γ-Glutamic Acid Functionalized Peptide Nucleic Acid Monomers

Hu Huang, Goon Ho Joe,†,* Sung Rok Choi,† Su Nam Kim,† Yong Tae Kim,† Chwang Siek Pak,† Joon Hee Hong, and Wonjae Lee*

*College of Pharmacy, Chosun University, Gwangju 501-759, Korea. *E-mail: wlee@chosun.ac.kr*
*†Panagene Incorporated, Daejeon 301-510, Korea. *E-mail: goonho.joe@panagene.com*
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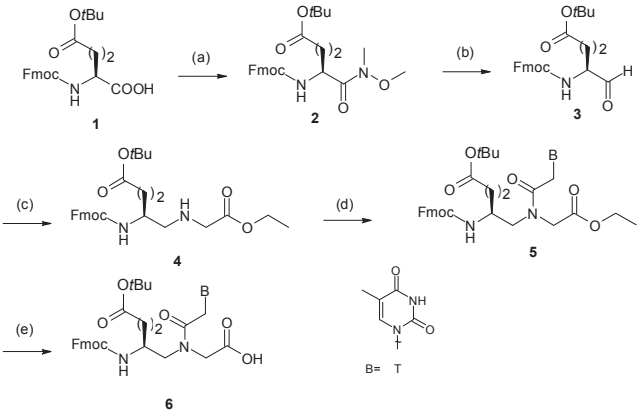
Peptide nucleic acids (PNAs) developed in the last decades, are particularly promising clue compounds of nucleic acid mimics, in which the natural sugar-phosphate backbone has been replaced by the *N*-aminoethylglycine units.[1] Recently, a number of modifications of the basic PNA monomeric units have been reported in order to improve the binding stability to the complementary nucleic acid and the specificity of complexation.[2-5] Among them, one of the most distinct modification is the introduction of stereogenic center at γ-carbon atom of the *N*-aminoethylglycine unit.[5] Compared to unmodified normal PNAs, the γ-substituted PNAs have several advantages of improved solubility, less self-aggregation, increased stability of PNA-DNA duplexes, and opportunities of further functionalization.[5,6] Also, it was reported that chiral PNAs have shown promising abilities in DNA recognition. In the case of γ-substituted PNAs, especially, L-isomer is more favored because D-isomer does not allow them to bind efficiently to DNA or RNA due to the geometric efficiency for α-helical duplex formation.[6(b)] Therefore, the preparation of chiral PNA monomers with high optical purity for PNA oligomers with high binding affinity to the complementary nucleic acids and the determination of their optical purity have become essential features. However, the direct enantiomer separation of PNA monomers to determine the optical purity has not been reported, although some indirect analytical methods using HPLC, GC and NMR were developed.[4(b),7] These indirect methods require additional processes like derivatization or hydrolysis of PNA monomers and, consequently, have a possibility of racemization during preparation of analytes. In this paper, we report the synthesis of enantiopure γ-glutamic acid functionalized fluorenylmethoxycarbonyl (Fmoc) PNA monomers and direct enantiomer separation method without any derivatization process for determination of the optical purity. This is the first report for direct enantiomer separation of PNA monomers by chiral HPLC.

For the chiral γ-functionalized PNA monomer, the γ-L-glutamic acid functionalized Fmoc protected PNA-thymine monomer was prepared according to the procedure outlined in Scheme 1. The commercially available Fmoc-L-Glu(OtBu)-OH (**1**) was selected as a starting material. The diprotected L-glutamic acid (**1**) was transformed into Fmoc-L-Glu(Boc)-*N*-methoxy-*N*-methyl amide (**2**) with *N*-methoxy-*N*-methylamine hydrochloride using HBTU activation in DMF. The amide (**2**) was then reduced with $LiAlH_4$ in THF at low temperature to prevent racemization and the subsequent hydrolysis with 5% HCl solution gave Fmoc-L-Glu(OtBu)-H (**3**). The crude aldehyde (**3**) was immediately used for reductive amination with ethyl glycine hydrochloride and $NaBH_3CN$ in the presence of acetic acid and DIEPA in methanol to make γ-L-Glu(OtBu)-PNA monomer backbone (**4**). After purification of the chiral PNA backbone by column chromatography, the pure backbone was converted into Fmoc-L-Glu(OtBu)-PNA-thymine-OEt monomer (**5**) by reaction with thymine acetic acid using DCC/DhbtOH coupling method in DMF. After hydrolysis with 1 M NaOH and $CaCl_2$ in THF, the desired γ-L-Glu(OtBu)-PNA-thymine-OH monomer (**6**) was obtained as a white solid by solidification, in which the further purification process was not performed. During hydrolysis of the ester (**5**), however, Fmoc deprotection occurred simultaneously with formation of five membered cyclic amide by coupling of the resulting free amine with the ester moiety of glutamic acid side chain. The yield of final step was affected by the hydrolysis conditions and, therefore, the Fmoc deprotection occurred unexpectedly during ester hydrolysis regardless of kinds and quantities of the used bases. By adding $CaCl_2$, Fmoc deprotection was minimized and the yield was increased up to 92%.



(a) (MeO)(Me)NH.HCl, HBTU, DIEPA in DMF; 98% (b) $LiAlH_4$ in THF
(c) $HCl.H_2NCH_2CO_2Et$, DIEPA, $NaBH_3CN$, CH_3COOH in MeOH; 58% (2 steps)
(d) $B\text{-}CH_2COOH$, DCC/DhbtOH in DMF; 82% (e) 1 M NaOH; 92%

Scheme 1. Synthesis of γ-L-glutamic acid functionalized PNA monomer

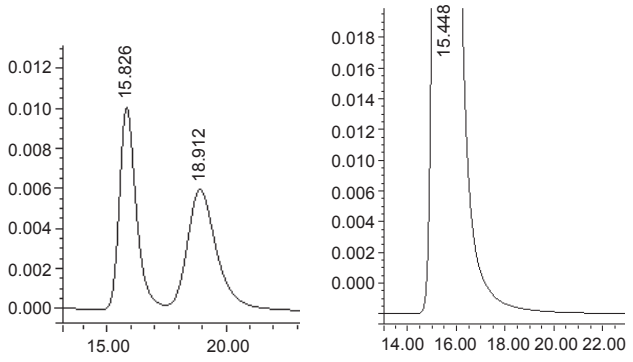


Figure 1. Chromatograms of enantiomer separation of racemic Fmoc-Glu(OtBu)-PNA-thymine-OH (the left) and Fmoc-L-Glu(OtBu)-PNA-thymine-OH (**6**) (the right, L : D = > 99.9 : < 0.1) on Chiralpak IB; Mobile phase: MeOH/EtOH/hexane/trifluoroacetic acid = 5/15/80/0.1 (v/v/v/v); Flow rate 1 mL/min; UV 254 nm.

For chiral analysis, Fmoc-D-Glu(OtBu)-PNA-thymine-OH monomer [D-(**6**)] from Fmoc-D-Glu(OtBu)-OH [D-(**1**)] as a starting material was synthesized, according to the same procedure described above (Scheme 1). Applying the modified direct enantiomer separation method developed in our group,[8] the optical purities of Fmoc-L-Glu(OtBu)-PNA-thymine-OEt (**5**) and D-(**5**) as well as Fmoc-L-Glu(OtBu)-PNA-thymine-OH (**6**) and D-(**6**) were determined. For chromatographic conditions of these analytes, the mobile phases of MeOH/EtOH/hexane = 5/15/80 (v/v/v) and MeOH/EtOH/hexane/trifluoroacetic acid = 5/15/80/0.1 (v/v/v/v) were used on Chiralpak IB, respectively. Not only (**5**) and (**6**) but also D-(**5**) and D-(**6**) analytes showed extremely high optical purities [(L : D = > 99.9 : < 0.1) and (L : D = < 0.1 : > 99.9)], respectively, which indicates clearly no racemization during all procedures of PNA monomer synthesis including the final basic hydrolysis step. Typical chromatograms of determination of the optical purity of Fmoc-L-Glu(OtBu)-PNA-thymine-OH (**6**) are presented in Figure 1.

In conclusion, we synthesized the significantly enantiopure Fmoc-L-Glu(OtBu)-PNA-thymine-OH (**6**) (L : D = > 99.9 : < 0.1) and D-(**6**) (L : D = < 0.1 : > 99.9) as the γ-functionalized PNA-thymine monomers starting from the commercially available Fmoc-L-Glu(OtBu)-OH (**1**) and D-(**1**). For determination of their optical purity, direct enantiomer separation of PNA-thymine monomers by chiral HPLC was developed and applied.

Experimental Section

Chromatography was performed at room temperature using an HPLC Breeze system consisting of a Waters model 1525 binary pump, a Rheodyne model 7125 injector with a 20 μL loop, and a dual absorbance detector (Waters 2487 detector). Chiralpak IB column (250 mm L × 4.6 mm I.D., 10 μm) was purchased from Daicel chemical company (Tokyo, Japan).

Fmoc-L-Glu(OtBu)-*N*,*O*-dimethyl (2). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.85 (d, 2H, Fmoc-*H*), 7.68 (m, 3H, Fmoc-*H* + Fmoc-N*H*), 7.37 (t, 2H, Fmoc-*H*), 7.28 (t, 2H, Fmoc-*H*), 4.46(m, 1H, NH-C*H*-CO), 4.14-4.22 (m, 3H, Fmoc-C*H*-CH_2-O), 3.66 (s, 3H, O-CH_3), 3.06 (s, 3H, N-CH_3), 2.23 (t, 3H, CH_2-CO_2*t*-Bu), 1.65-1.80 (m, 2H, CH_2-CH_2-CO_2*t*-Bu), 1.43 (s, 9H, *tert*-butyl-CH_3).

Fmoc-L-Glu(OtBu)-PNA backbone (4). ^{1}H NMR (600 MH, $CDCl_3$) δ 7.76 (d, 2H, Fmoc-*H*), 7.61 (d, 2H, Fmoc-*H*), 7.37 (t, 2H, Fmoc-*H*), 7.29 (t, 2H, Fmoc-*H*), 5.28 (d, 1H, Fmoc-N*H*), 4.39 (d, 2H, Fmoc-CH_2-O), 4.14-4.23 (m, 3H, Fmoc-C*H* + O-CH_2), 3.79 (m, 1H, N-C*H*-CH_2-N), 3.41-3.49 (m, 2H, N-CH_2-CO), 2.61-2.77 (m, 2H, N-CH-CH_2-N), 2.30 (t, 2H, CH_2-CO_2*t*-Bu), 1.70-1.86 (m, 2H, CH_2-CH_2-CO_2*t*Bu), 1.48 (s, 9H, *tert*-butyl-CH_3), 1.28 (t, 3H, O-CH_2-CH_3). ^{13}C NMR (150 MHz, DMSO-d_6) δ 172.76, 163.02, 156.62, 144.61, 144.50, 141.40, 128.27, 128.16, 127.67, 125.94, 125.74, 120.84, 120.76, 120.66, 80.07, 65.76, 60.55, 53.00, 50.80, 50.54, 47.55, 47.41, 28.40, 28.31, 14.83, 14.65. HRMS (ESI-MS *m/z*): mass calcd for $C_{28}H_{36}N_2O_6$ $[M+H]^+$, 497.26461; found, 497.26474.

Fmoc-L-Glu(OtBu)-PNA-thymine-OEt monomer (5). ^{1}H NMR (600 MHz, DMSO-d_6) δ major rotamer 11.26 (s, 1H, imide-N*H*), 7.86 (d, 2H, Fmoc-*H*), 7.66-7.68 (m, 2H, Fmoc-*H*), 7.28-7.39 (m, 5H, Fmoc-*H* + thymine-*H*), 5.5 (s, 1H, Fmoc-N*H*), 3.88-4.69 (m, 9H, thymine-CH_2-CO, N-CH_2-CO_2H + Fmoc-CH-CH_2-O + O-CH_2-CH_3 + Fmoc-C*H*-CH_2), 2.92-3.68 (m, 3H, Fmoc-NH-CH-CH_2-N, Fmoc-NH-C*H*-CH_2-N), 2.11-2.16 (m, 2H, CH_2-CH_2-CO_2*t*-Bu), 1.64 (s, 3H, thymine-CH_3), 1.45-1.56 (m, 2H, CH_2-CH_2-CO_2*t*-Bu), 1.34 (s, 9H, *tert*-butyl-CH_3), 1.22 (t, 3H, O-CH_2CH_3); minor rotamer 1.67 (s, 3H, thymine-CH_3), 1.32 (s, 9H, *tert*-butyl-CH_3). ^{13}C NMR (150 MHz, DMSO-d_6) δ 172.53, 169.77, 169.32, 168.56, 168.27, 165.04, 156.73, 156.59, 151.58, 144.63, 144.51, 144.44, 144.38, 141.41, 128.34, 128.22, 127.71, 125.92, 125.82, 125.72, 120.86, 120.73, 108.86, 108.82, 80.33, 80.19, 61.84, 61.15, 47.40, 32.16, 31.92, 28.41, 28.33, 27.80, 14.73, 14.69, 14.55, 12.57, 12.53. HRMS (ESI-MS *m/z*): mass calcd for $C_{35}H_{42}N_4O_9$ $[M+H]^+$, 663.30245; found, 663.30248.

Fmoc-L-Glu(OtBu)-PNA-thymine-OH monomer (6). ^{1}H NMR (600 MHz, DMSO-d_6) δ major rotamer 11.26 (s, 1H, imide-N*H*), 7.85 (d, 2H, Fmoc-*H*), 7.63 (m, 2H, Fmoc-*H*), 7.12-7.38 (m, 5H, Fmoc-*H* + thymine-*H*), 5.53 (s, 1H, Fmoc-N*H*), 3.83-4.70 (m, 6H, thymine-CH_2-CO, N-CH_2-CO_2H + Fmoc-CH-CH_2-O), 2.92-3.69 (m, 4H, Fmoc-C*H*-CH_2 + Fmoc-NH-C*H*-CH_2 + Fmoc-NH-CH-CH_2-N), 2.17 (m, 2H, CH_2-CH_2-CO_2*t*-Bu), 1.64 (s, 3H, thymine-CH_3), 1.50-1.57 (m, 2H, CH_2-CH_2-CO_2*t*-Bu), 1.35 (s, 9H, *tert*-butyl-CH_3); minor rotamer 11.24 (s, 1H, imide-N*H*), 1.67 (s, 3H, thymine-CH_3), 1.33 (s, 9H, *tert*-butyl-CH_3). ^{13}C NMR (150 MHz, DMSO-d_6) δ 172.52, 171.25, 170.84, 168.51, 168.12, 165.04, 156.74, 156.61, 151.61, 144.64, 144.53, 144.44, 144.38, 142.51, 141.40, 128.34, 128.22, 127.71, 125.92, 125.85, 125.73, 120.85, 120.68, 108.83, 108.79, 80.30, 80.17, 65.95, 65.83, 65.58, 65.34, 51.57, 49.82, 49.63, 49.51, 48.32, 47.55, 47.41, 32.17, 31.97, 28.40, 28.32, 27.79, 15.88, 15.73, 12.58, 12.54. HRMS (ESI-MS *m/z*): mass calcd for $C_{33}H_{38}N_4O_9$ $[M+H]^+$, 635.27115; found, 635.27111.

All characterization data of D-(**2**), D-(**4**), D-(**5**) and D-(**6**) matched (**2**), (**4**), (**5**) and (**6**), respectively.

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