Synthesis of a C-linked glycosylated thymine-based PNA monomer and its incorporation into a PNA oligomer

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Backbone modification of peptide nucleic acids (PNAs) by glycosylation has been shown to enhance selective biodistribution and cellular targeting of PNA oligomers based on sugar and cell surface lectin interactions. Here we report the synthesis of a new backbone-glycosylated thymine-based PNA monomer (T^{gal}). The sugar residue was attached to the backbone of PNA *via* a stable carbon–carbon linkage between the sugar and the PNA monomers. Also, incorporation of the modified monomer into a PNA decamer (H-Ala^{gal}-G-G-G-T^{gal}-C-A-G-C-T^{gal}-T-Lys-NH_2) was successfully performed. Melting temperature (UV-T_m) of the modified PNA against the complementary DNA was only slightly lower than unmodified PNA.

Introduction

Peptide nucleic acid (PNA) is a DNA analog in which the deoxyribose phosphate backbone of DNA is replaced with an ethylenediamine glycine backbone that has very similar spatial requirements.[1,2] PNA can bind to DNA in a sequence-specific manner with a higher affinity compared to double-stranded DNA (dsDNA) itself. Several *in vitro* and *in vivo* studies have already shown an antisense effect of PNAs that are targeted to specific mRNA regions.[3–6] PNA has a high stability towards cellular nucleases and proteases. This, together with the apparent lack of general toxicity and non-specific protein binding makes PNA an attractive agent in the development of gene targeted drugs.[7]

The success in developing PNA as a gene targeted drug also depends on its pharmacokinetic behaviour. Results from *in vivo* studies of McMahon *et al.* indicate that unmodified PNAs are rather poorly taken up and rapidly eliminated by the kidney after intravenous injection in rats. Most of the PNA was excreted unchanged in the urine after 24 hours.[8] Due to this, PNA modifications that lead to a more effective and specific biodistribution have to be invented. It has been shown that pharmacokinetic behaviour of PNA can be controlled by modification of PNA with various glycosyl substituents (backbone glycosylation). Effective liver targeting of glycosylated PNA has been reported due to galactose and *N*-acetylgalactosamine interactions with a sialoglycoprotein receptor (hepatic lectin) of liver cells.[9]

Glycopeptide synthesis strategies have been introduced as models in such work either for monomer or for oligomer synthesis of glycosylated PNAs. Glycosylation of peptides is accomplished by direct attachment of a sugar to an amino acid through a linker. The suitably protected monomer is then incorporated into the peptide chain as a building block in solid phase peptide synthesis (SPPS). The attachment usually links the α position of the amino acid and the anomeric carbon of the sugar. Synthetic *C*-glycosyl amino acids and *C*-glycosylated PNA derivatives are very interesting molecules because of the chemical and enzymatic stability of the carbon–carbon linkage compared to ester- or amide-linked derivatives. We now report the synthesis of a new class of *C*-glycosylated PNA monomers in which a C-bound glycosylated building block is integrated into a PNA monomer. We prepared a *C*-galactosylated alanine derivative (Ala^{gal}) that was used as a building block replacing glycine in the PNA backbone. The new synthetic route described herein requires fewer chemical synthesis steps and has a higher yield compared to the previously reported synthesis of a *C*-glycosylated PNA monomer.[9] Moreover, UV melting experiments with complementary DNA indicate similar stability for oligomers containing the new *C*-glycosylated PNA monomers compared to unmodified PNA oligomers.

Experimental procedures

Reagents and solvents were obtained from commercial sources and used without further purification. PNA monomers were obtained from Applied Biosystems, DNA oligomers (HPLC purified quality, checked in-house for purity and correct mass) were purchased from IBA. Mass spectra were recorded on a Mat 8200 instrument in FAB mode (positive ions, glycerol or 3-nitrobenzyl alcohol matrix), ESI mass spectra on a Finnigan TSQ 700 and MALDI-TOF mass spectra on a Bruker Biflex instrument. NMR spectra were obtained on a Bruker AM 360 instrument. Flash chromatography was carried out using Silica Gel 60 (Merck particle size 0.040–0.063 mm). HPL chromatograms were measured on a customized Varian Prostar instrument on reverse-phase Dynamax Microsorb 60-8 C_{18} columns. A linear gradient composed of A (0.1% TFA in water) and B (0.1% TFA in acetonitrile) was used for analytical and preparative HPLC. Analytical: Time 0–2 min 5% B. Time 30 min 50% B. Time 33 min 70% B. Time 40 min 5% B. Time 45 min 5% B (250 × 4.6 mm, 1 mL min^{-1}). Preparative: Time 0–10 min 5% B. Time 40 min 60% B. Time 45 min 5% B (250 × 10.0 mm, 5 mL min^{-1}).

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N-(Boc)-3-(2,3,4,6-tetra-*O*-acetyl-galactopyranose-1-yl)-2-aminopropionic acid benzyl ester (1)

Boc-protected (α,β-dehydroalanine) benzyl ester (2.5 g, 6.8 mmol) and 2,3,4,5-tetra-*O*-acetyl-galactosyl bromide (2.0 g, 7.2 mmol) were twice co-evaporated with toluene, dissolved in dry toluene (20 mL) and heated to 60 °C under nitrogen. nBu$_3$SnH (2.1 g, 11.7 mmol) and AIBN (150 mg, 0.9 mmol) in dry toluene (12 mL) were added. The reaction mixture was stirred for 1 hour. The solvent was evaporated and the residue was dissolved in acetonitrile (100 mL) and extracted with *n*-pentane (3 × 50 mL) in order to remove tin compounds. After evaporation of the solvents, the residue was dissolved in ethyl acetate and extracted with saturated aq. NaHCO$_3$. Volatiles were removed and the residue was purified by flash chromatography eluting with *n*-hexane–ethyl acetate (2 : 1 v/v). Compound **1** was obtained as a white solid (yield = 69.5%). MS (FAB) m/z 609 (M + H)$^+$. ^{1}H NMR (CDCl$_3$): δ 1.4 (s, 9H, Boc), 2.0–2.2 (4s, 12H, sugar acetyls), 4.1 (m, 3H, H-5 and H-6), 4.2 (dd, 1H, J = 11.0, 5.5 Hz, H-1), 4.4 (m, 2H, β-CH$_2$), 4.5 (m, 1H, α-CH$_2$), 5.1 (dd, 1H, J = 11.0, 3 Hz, H-3), 5.2 (overlapping, 3H, H-2 and -CH$_2$ of benzyl), 5.4 (t, 1H, J = 3 Hz, H-4), 5.5 (s, 1H, -NH-Fmoc), 7.4 (m, 5H, benzyl aromatics); ^{13}C NMR (CDCl$_3$): δ 20.6, 20.7, 20.8, 20.9, 28.2, 28.3, 28.4, 42.7, 50.6, 62.0, 66.7, 67.1, 67.3, 67.7, 68.3, 71.5, 74.9, 80.3, 128.3, 128.5, 128.6, 128.7, 128.8; Elemental analysis for C$_{29}$H$_{39}$NO$_{13}$·0.5 H$_2$O: calc. C 56.30, H 6.52, N 2.26; found C 56.47, H 6.51, N 2.21%.

N-(2-Fmoc-aminoethyl)-3-(2,3,4,6-tetra-*O*-acetyl-galactopyranose-1-yl)-2-aminopropionic acid benzyl ester (3)

Compound **1** (3.5 g, 5.75 mmol) was added to a mixture of water–TES–TFA (1 + 1 + 28 mL) at 0 °C and stirred for 1 hour. Toluene (50 mL) was added, and volatiles were removed under vacuum. The remaining solid was dissolved in ethyl acetate (100 mL) and extracted with saturated aq. NaHCO$_3$ (2 × 50 mL) and brine. After drying the organic phase with Na$_2$SO$_4$, all volatiles were removed under vacuum and 2-(2,3,4,6-tetra-*O*-acetyl-galactopyranosyl)alanine benzyl ester (**2**) was obtained as a white solid (yield = 90%). MS (FAB) m/z 509 (M + H)$^+$. Without further purification, compound **2** (2.0 g, 3.9 mmol) and Fmoc-aminoacetaldehyde (850 mg, 3.0 mmol) were dissolved in methanol (5 mL) and stirred for 10 minutes. Acetic acid (250 µl, 4 mmol) and NaBH$_3$CN (250 mg, 3.0 mmol) were added sequentially. The reaction mixture was stirred for 2 h. All volatiles were removed under vacuum and the residue was dissolved in ethyl acetate (100 mL) and extracted with sat. aq. NaHCO$_3$ (2 × 50 mL). The organic phase, after drying with MgSO$_4$, was removed under vacuum and the residue was purified by flash chromatography eluting with ethyl acetate–*n*-hexane (3 : 2 v/v). Compound **3** was obtained as a white solid. (yield = 38.5%). MS (FAB) m/z 775 (M + H)$^+$;^{1}H NMR (CDCl$_3$): δ 1.8 (m, 2H), 2.0–2.2 (4s, 12H, sugar acetyls), 2.6 (m, 1H), 2.8 (m, 1H), 3.4 (m, 2H), 4.1 (m, 1H), 4.2 (m, 2H), 4.3 (m, 2H), 4.5 (m, 4H), 5.2 (overlapping, 4H, H-2 and H-3 and -CH$_2$ of benzyl), 5.4 (s, 1H, H-4), 5.5 (broad s, 1H, -NH$_{Fmoc}$), 7.2–7.8 (m, 13H, benzyl and Fmoc). ^{13}C NMR (CDCl$_3$): δ 20.6, 20.7, 20.8, 20.9, 29.2, 40.8, 47.2, 47.6, 57.9, 60.6, 66.2, 66.6, 66.8, 66.9, 67.7, 68.3, 68.4, 69.4, 119.9, 125.1, 125.2, 127.0, 127.6, 128.3, 128.5, 128.6, 128.8, 130.9, 135.5, 141.3, 144.0, 156.1, 156.5, 156.9, 169.7, 169.8, 169.9, 170.9, 173.9, 174.0; HRMS (M + H)$^+$, calc. (found) for C$_{41}$H$_{47}$N$_2$O$_{13}$: 775.3078 (775.3071).

N-(2-Fmoc-aminoethyl)-*N*-(thymine-1-ylacetyl)-3-(2,3,4,6-tetra-*O*-acetyl-galactopyranose-1-yl)-2-aminopropionic acid benzyl ester (4)

Thymine-1-ylacetic acid (550 mg, 3 mmol), DhbtOH (520 mg, 3.2 mmol) and DCC (1.0 g, 5 mmol) were dissolved in DMF (20 mL) and stirred for 20 minutes. Compound **3** (1.2 g, 1.5 mmol) was added and the reaction mixture was stirred overnight. The volatiles were removed under vacuum and the remaining solid was dissolved in ethyl acetate (150 mL). Insoluble DCU was filtered off and the filtrate was extracted with sat. aq. NaHCO$_3$ (2 × 50 mL) and brine (50 mL). After drying over MgSO$_4$ the organic phase was evaporated to dryness. The residue was purified by flash chromatography eluting with ethyl acetate. Compound **4** was obtained as a white solid. (yield = 64%), MS (FAB) m/z 941 (M + H)$^+$. ^{1}H NMR (CDCl$_3$): δ 1.8 (m, 2H), 1.9 (s, 3H, thymine-CH$_3$), 2.0–2.4 (4s, 12H, sugar acetyls), 2.7 (m, 1H), 3.4 (m, 2H), 3.6 (m, 1H), 3.9 (m, 1H), 4.0 (dd, 1H, J = 11.0, 5.5 Hz, H-1), 4.1–4.2 (m, 3H), 4.3–4.5 (m, 4H), 4.6 (m, 1H), 5.2 (overlapping, 4H, H-2, H-3 and -CH$_2$ of benzyl), 5.4 (t, 1H, J = 3 Hz, H-4), 5.7 (br. s, 1H, NH$_{Fmoc}$), 6.8 (s, 1H, thymine aromatic), 7.3–7.7 (m, 13H, benzyl and Fmoc), 9.0 (br. s, 1H, NH$_{thymine}$); ^{13}C NMR (CDCl$_3$): δ 12.4, 20.5, 20.6, 20.7, 20.8, 24.9, 25.6, 33.9, 39.4, 47.2, 58.9, 61.2, 61.3, 66.6, 67.2, 67.3, 67.4, 67.6, 67.7, 67.8, 110.5, 120.0, 120.1, 125.0, 127.0, 127.1, 127.2, 127.7, 127.9, 128.4, 128.6, 128.7, 128.8, 128.9, 130.9, 141.1, 141.3, 164.1, 166.5, 166.6, 167.2, 167.7, 169.7, 169.8, 170.0; HRMS (M + H)$^+$, calc. (found) for C$_{48}$H$_5$N$_4$O$_{16}$: 941.3457 (941.3465).

N-(2-Fmoc-aminoethyl)-*N*-(thymine-1-ylacetyl)-3-(2,3,4,6-tetra-*O*-acetyl-galactopyranose-1-yl)-2-aminopropionic acid (5)

Compound **4** (900 mg, 0.96 mmol) was dissolved in methanol (20 mL) and a catalytic amount of palladium on charcoal (Pd/C) was added. The reaction mixture was hydrogenated under atmospheric pressure until TLC (ethyl acetate–*n*-hexane, 2 : 1 v/v) showed the absence of all starting material (approximately 2 h). The catalyst was filtered off with Celite and the filtrate was evaporated to dryness. The residue was triturated with diethyl ether until compound **5** precipitated as a white powder. (yield = 61.5%), MS (FAB) m/z 629 (M + H)$^+$. ^{1}H NMR (CD$_3$OD) resolved signals: δ 1.9 (s, 3H, thymine-CH$_3$), 2.0–2.2 (4s, 12H, sugar acetyls), 2.6 (m, 1H), 3.5 (m, 2H), 3.7 (m, 1H), 4.1 (m, 2H), 4.3 (m, 3H), 4.5 (m, 2H), 4.7 (m, 2H), 5.3 (overlapping s, 2H, H-2 and H-3), 5.4 (s, 1H, H-4), 7.3 (s, 1H$_{thymine}$), 7.4–7.9 (m, 8H, Fmoc); ^{13}C NMR (CDCl$_3$): δ 12.2, 20.5, 20.6, 20.8, 40.5, 62.6, 67.7, 69.0, 69.2, 110.9, 120.8, 120.9, 126.1, 126.3, 127.9, 128.1, 128.3, 128.8, 142.5, 142.6, 143.7, 143.8, 145.3, 171.3, 171.4, 171.5, 171.6, 171.8, 172.4; Elemental analysis for C$_{41}$H$_{46}$N$_4$O$_{16}$·3H$_2$O: calc. C 54.42, H 5.79, N 6.19; found C 54.77, H 5.83, N 6.66%.

N-(Boc)-3-(2,3,4,6-tetra-*O*-acetyl-galactopyranose-1-yl)-2-aminopropionic acid (6)

The same procedure for hydrogenation of compound **4** (described above) was used for hydrogenation of compound **1** (1.0 g, 1.6 mmol). After removal of the catalyst, the filtrate was

evaporated and the residue was precipitated from THF–*n*-hexane (1 : 1 v/v). The precipitate was filtered off and dried in vacuum (yield = 92%). MS (FAB) *m/z* 520 $(M + H)^+$;^{1}H NMR (CD_3OD): δ 1.4 (s, 9H, Boc), 2.0–2.2 (4s, 12H, sugar acetyls), 4.2 (m, 2H), 4.3 (m, 3H), 4.4 (m, 1H, α-CH_2), 5.1 (m, 1H), 5.3 (m, 2H), 5.4 (overlapping, 2H, H-4 and NH_{Fmoc}); ^{13}C NMR ($CDCl_3$): δ 20.6, 20.7, 20.8, 20.9, 28.7, 28.8, 28.9, 39.3, 61.4, 62.0, 63.1, 67.4, 70.4, 72.9, 75.9, 80.4, 171.3, 171.7, 172.2; Elemental analysis for $C_{22}H_{33}NO_{13}$ calc. C 50.86, H 6.40, N 2.70; found C 50.75 H 6.84 N 2.76%.

Synthesis of PNA oligomer conjugates

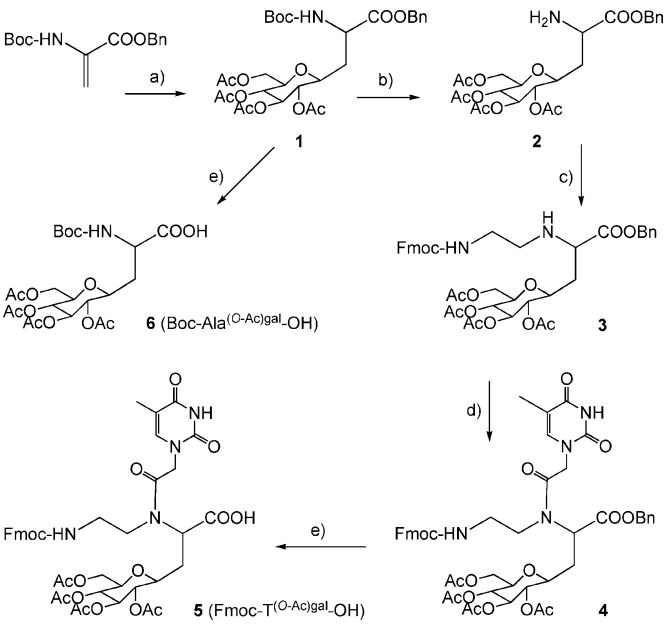
50 mg of Tentagel R-RAM resin (capacity = 0.2 mmol g^{-1}) with preloaded Fmoc-Lys(Boc) amino acid was used as solid support for the solid phase synthesis. A Teflon syringe with a frit at the bottom was used as reactor and all reactions were carried out on a shaker at 600 vibrations per minute. Fmoc/Bhoc protected PNA monomers (Applied Biosystems) and galactosylated derivatives (40 μmol, 4-fold access) were activated in an Eppendorf tube with TBTU (12.2 mg, 38 μmol dissolved in 270 μL DMF). DIPEA (30 μL, 4.7 eq.) was added and the acid pre-activations were performed by vibration for 2 minutes (7 minutes for C(Bhoc) monomer). Activated acids were transferred to the syringe containing the resin and the coupling was allowed to proceed for 40 minutes (120 minutes for glycosylated derivatives) under vibration. The resin was washed with DMF and DCM successively. Success of the coupling reactions was controlled by the Kaiser test. Piperidine (20%) in DMF (3 + 2 min) was used for double Fmoc deprotection. Prior to cleavage of the final product from the resin, acetyl groups at the galactosyl moieties were cleaved by vibration for 24 hours with 1 mL of a mixture of TEA–methanol–water (5 : 3 : 2 v/v). The oligomer was cleaved from the resin with 700 μL of a mixture of TFA–anisol–*m*-cresol (14 : 5 : 1 v/v) for 2 hours. The crude product was precipitated by draining the cleavage mixture into ice-cold diethyl ether. The precipitate was washed with cold diethyl ether (5 × 10 mL), centrifuged each time and collected and purified by preparative RP-HPLC. The molecular mass was determined by MALDI-TOF. *m/z* 3513.7 $(M + H)^+$, *m/z* 3536.6 $(M + H + Na)^+$.

UV-T_m measurements

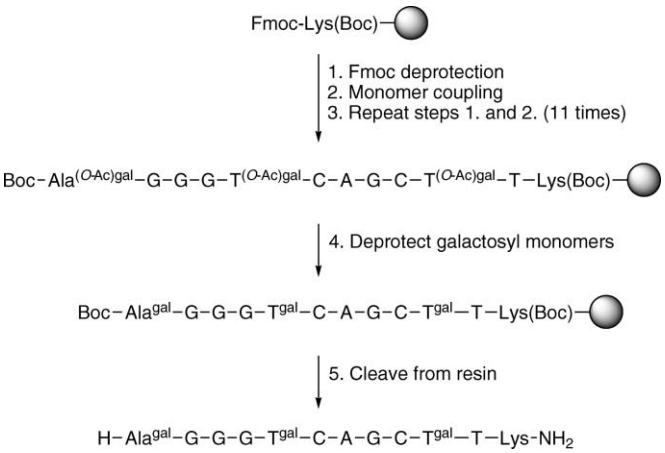
The UV-T_m values were measured in a phosphate buffer (10 mM, pH 7.0, containing 100 mM NaCl) using a DNA decamer with complementary sequence. Heating and cooling were performed at 0.5 °C min^{-1}. between 5 and 80 °C. The mixture was held at 80 °C for 5 min to ensure complete dissociation prior to starting the measurement at 5 °C. Temperature profiles were recorded at 260 nm and all profiles showed monophasing transitions from which the T_m was determined as the maximum of the first derivative.

Results and discussion

The monomer synthesis is outlined in Scheme 1. Orthogonal protection groups for the synthesis of *C*-galactosylated PNA are Fmoc, acetyl and benzyl for amine, hydroxyl and carboxylic acid groups, respectively. Initially, compound **1** was synthesized as described in the literature (analytical data are not given in that paper).[10] The glycosylated Ala derivative could be obtained in a higher yield (69.5%). As a radical donor, tetra-*O*-acetyl-galactosyl bromide was reacted with a protected dehydroalanine derivative in the presence of AIBN as radical initiator and nBu_3SnH as a hydrogen donor. This reaction represents the key step in the preparation of the *C*-linked glycosylated derivatives since it cleanly generates the C–C bond between sugar and PNA. The Boc protecting group of compound **1** was removed by TFA and a scavenger for the subsequent formation of the PNA backbone. The deprotection product **2** was used freshly for the reductive amination step with Fmoc-aminoacetaldehyde[9] without further purification. $NaBH_3CN$ and acetic acid were used as reducing agents and glycosylated PNA backbone **3** was obtained in good yield. The nucleobase thymine was introduced to the backbone *via* amide bond formation between thymine-1-ylacetic acid[3] and the secondary amino group of **3**. DCC and DhbtOH were used as coupling reagents to yield compound **4** in good yield. Finally, the benzyl groups of compounds **1** and **4** were removed by catalytic hydrogenation using Pd/C at atmospheric pressure. The glycosylated monomer **5** (Fmoc-$T^{(O\text{-Ac})gal}$-OH) and the glycosylated amino acid **6** (Boc-$Ala^{(O\text{-Ac})gal}$-OH) were obtained as white powders after trituration with ether and did not require further purification. Derivatives **5** and **6** were incorporated into a decamer PNA sequence (H-Ala^{gal}-G-G-G-T^{gal}-C-A-G-C-T^{gal}-T-Lys-NH_2) by Fmoc solid phase chemistry (Scheme 2). A Lys(Boc)-Fmoc preloaded Tentagel resin with RAM linker was used as the solid support. The couplings were carried out in a syringe using a TBTU–DIPEA mixture as coupling reagents. All coupling steps were monitored by the Kaiser test. By using an optimized synthetic protocol, capping reactions could be avoided. Acetyl protecting groups on sugars were removed by a TEA–methanol–water



Scheme 1 Synthesis of the C-linked glycosylated PNA monomer. (a) 2,3,4,5-tetra-*O*-acetyl-galactosyl bromide, nBu_3SnH, AIBN, 60 °C; (b) H_2O–TES–TFA, 1 : 1 : 28 v/v/v; (c) Fmoc-aminoacetaldehyde, acetic acid, $NaBH_3CN$, 2 h; (d) thymine-1-ylacetic acid, DhbtOH, DCC; (e) H_2, Pd/C in MeOH, 1 atm.



Scheme 2 Introduction of the glycosylated derivatives into a PNA oligomer. See Experimental section for details.

mixture prior to cleavage of the final product from the resin. The crude product was further purified by preparative RP-HPLC and the molecular mass was confirmed by MALDI-TOF.

In order to probe the interaction with complementary DNA, UV melting temperatures (UV-T_m) were measured.[11,12] For comparison, a non-glycosylated PNA oligomer with the same sequence (H-Ala-G-G-G-T-C-A-G-C-T-T-Lys-NH_2) was prepared from commercially available monomers. T_m values were recorded twice for glycosylated and control PNA. Only a slight decrease of the T_m value from (76 ± 0.5) °C to (73 ± 0.5) °C was observed due to the sugar modifications.

Conclusions

The synthesis of a *C*-linked glycosylated thymine-based PNA monomer and its incorporation into a PNA oligomer has been achieved in this work. A number of *O*-, *C*- and *N*-glycosylated PNA monomers were recently reported by Hamzavi *et al.*[9] In this work, it was shown that biologically and chemically very stable glycosylated PNA derivatives are obtained in the case of a carbon–carbon linkage between the sugar and the backbone. The number of synthetic steps for preparation of the new monomer described herein are fewer than for the previously reported analogues and the total yield is higher. By using optimized SPPS methods for PNA oligomer synthesis, the glycosylated monomer can be incorporated into a PNA oligomer in combination with a glycosylated alanine derivative which was attached to the PNA *N*-terminus. We observed that incorporation of both derivatives into a PNA decamer did not hamper binding to complementary DNA. On the contrary, glycosylated PNA oligomers are more soluble in water compared to normal PNA oligomers. This constitutes a significant advantage in biological application. In addition to metal–PNA derivatives, which are also studied in our group,[13–16] glycosylated PNA oligomers may enhance the applications of this promising new class of antisense agents.[7,17,18]

Abbreviations

AIBN, α,α′-azobisisobutyronitrile; Bhoc: benzhydryloxycarbonyl; Boc: *tert*-butyloxycarbonyl; DCC: *N*,*N*′-dicyclohexylcarbodiimide; DIPEA: diisopropylethylamine; DCU: *N*,*N*′-dicyclohexylurea; DhbtOH: 3-hydroxy-1,2,3-benzotriazine-4(3*H*)-one; ESI: electrospray ionization; FAB: fast atom bombardment; Fmoc: 9-fluorenylmethoxycarbonyl; MALDI-TOF: matrix-assisted laser desorption ionization time-of-flight; TBTU: 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TLC: thin layer chromatography.

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