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Synthesis and Properties of Chiral Peptide Nucleic Acids with a *N*-Aminoethyl-D-proline Backbone

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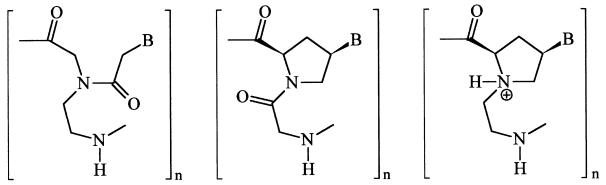
Abstract—A synthon of D-proline substituted at the 4-position by thymine and at *N* by a flexible aminoethyl linker, has been used to prepare a novel chiral peptide nucleic acid (cPNA) with (2*R*,4*R*) stereochemistry using solid phase methodology. The homothymine decamer cPNA binds to complementary polyadenylic acid to form a 2:1 hybrid with high affinity and specificity according to UV and CD studies, whereas no binding to the corresponding polydeoxyadenylic acid was observed. © 2000 Published by Elsevier Science Ltd.

Since the discovery by Nielsen and co-workers in 1991,[1–4] peptide nucleic acids (PNA) (**1**)[5–7] have attracted much attention due to the exceptionally high affinity and specificity towards complementary oligoribonucleotides and oligodeoxyribonucleotides. In addition they are stable towards nucleases and proteases and can be easily synthesized via solid phase techniques. Such a combination of properties, not found in natural oligonucleotides, make PNA potentially useful as a tool in antisense technology[8] as well as other areas of research. The stronger binding properties of PNA compared to natural oligonucleotides were attributed to the lack of electrostatic repulsion between the uncharged PNA backbone and the negatively charged sugar phosphate backbone of the oligonucleotides. The three-dimensional structure of hybrids between PNA and oligonucleotides were studied by X-ray crystallography[9] and NMR spectroscopy[10] which revealed that the conformation of the oligonucleotides in such hybrids are quite different from A or B-DNA conformations. Modification to PNA could provide a system which might exhibit superior properties such as water solubility and the ability to penetrate cell membranes, without loss of binding affinity and specificity. Many such modifications have been made in order to achieve these properties with varying degrees of success. Recent examples include: introduction of substituents into (**1**) to create chirality,[11] introduction of positively-charged groups onto the PNA backbone,[12] restricting the movement of the backbone or the side chain of PNA, e.g. by replacing the amide bond with a double bond,[13] or by making the backbone or side chain part of a ring system.[14–22]



(**1**) (**2**) (**3**)

We have recently reported a synthesis of chiral PNA (**2**) based on a rigid glycyl-D-proline skeleton with the nucleobases attached to the 4-position on the proline ring.[20–22] These chiral PNAs were shown to interact strongly with complementary DNA and RNA.[23] This chiral PNA and its hybrid with oligonucleotides, however, has poor solubility in aqueous media, making biological studies difficult. Attempts to improve the solubility of PNAs have so far met with variable success.[24–26] Here we report the synthesis and binding studies of the deoxy-analogue of (**2**), i.e. the glycine being replaced by an aminoethyl linker (**3**).

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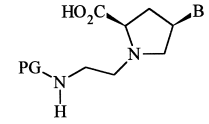
Replacing the glycine carbonyl group in the glycylproline backbone of cPNA with a methylene group ('aminoethylprolyl cPNA') should create a more conformationally flexible backbone while the conformation of the side chain is still restricted. Increasing conformational flexibility of the backbone might decrease the binding affinity due to the increased entropy loss upon hybridization, but it should allow the cPNA to adopt a wider range of conformations than the cPNA (**2**). Combination of the two factors may decrease or increase the binding affinity of the resulting cPNA to its complementary oligonucleotide, depending on how close the conformation of the cPNA in the hybrid is to that in its native state. The basic proline-nitrogen atom should be at least partially protonated under physiological conditions and should attract the negatively charged phosphate group of DNA so providing further stabilization of the hybrid formed with natural DNA. This idea has been exploited in Nielsen's type PNA by replacing the methylenecarbonyl linker which joined the base and the backbone to an ethylene linker[12] so that the α-nitrogen atom became basic. Unfortunately, the resulting modified PNA had lower affinity towards DNA presumably due to the increased flexibility of the linker. Very recently a system related to (**3**), with different stereochemistry around the proline ring, has been described.[27]

A synthetic route towards the *N*-aminoethylproline synthon carrying suitable protecting groups (**7a** and **7b**) for solid phase peptide synthesis was therefore developed. *N*-*p*-Nitrobenzenesulfonyl ('Nosyl') aziridine, obtained by treatment of *N*-nosylethanolamine with Ph_3P/DEAD in THF at low temperature, was employed as the electrophilic *N*-aminoethylating agent.[28] Protected *cis*-4-thyminyl-D-proline (**4**) was synthesized as described previously.[20–22] The Boc group was selectively removed in the presence of diphenylmethyl (Dpm) ester using *p*-toluenesulfonic acid in acetonitrile under conditions previously reported. Nucleophilic ring opening of *N*-nosylaziridine by this free amine proceeded efficiently to give the desired *N*-aminoethylproline derivative (**5**). Reaction of (**5**) with Boc_2O was achieved in the presence of 4-dimethylaminopyridine (DMAP) to give the *N*-Boc derivative. Treatment with thiophenol in the presence of potassium carbonate in DMF at room temperature gave the *N*-Boc derivative (**6a**) as an amorphous solid [50% yield from (**4**)].[29] Deprotection of the carboxyl group by catalytic transfer hydrogenolysis (cyclohexene, Pd–C) gave the free *N*-Boc amino acid (**7a**) in quantitative yield.[29]

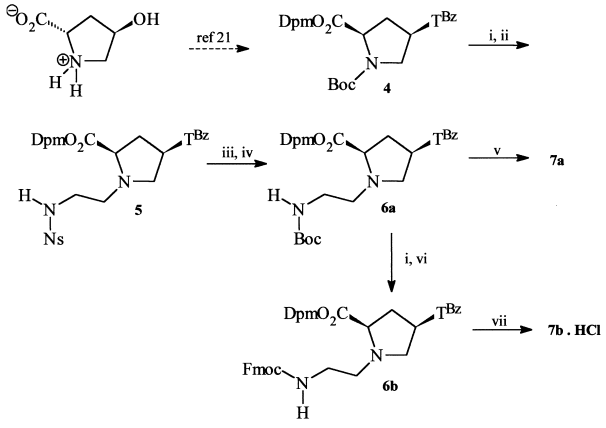
The Boc protecting group in (**6a**) was converted to the Fmoc group in order to take advantage of the milder conditions of peptide synthesis employing Fmoc protection strategy. When (**6a**) was treated with *p*-toluenesulfonic acid in acetonitrile followed by 9-fluorenylmethyl chloroformate (Fmoc-Cl) in the presence of DIEA, the Fmoc derivative (**6b**) was obtained in 80% yield.[30] The carboxyl protecting group was removed by HCl/dioxane to give the free acid (**7b**) (71%) as its hydrochloride salt with the N^3-benzoyl protecting group of thymine intact.[30] The Fmoc protected monomer (**7b**) was oligomerized on Novasyn TGR resin, previously loaded with Fmoc-Lys(Boc) on a 5 μmol scale using standard HBTU/DIEA activation protocol with capping at the end of each cycle. Quantitative monitoring of the coupling efficiency, by measurement of the absorbance of dibenzofulvene-piperidine adduct at 264 nm, after the deprotection cycle revealed the average coupling yield of approximately 92%. Using the Fmoc-ON purification strategy,[31] it was possible to isolate the Fmoc-(**3a**) together with its partially debenzoylated product without difficulty using reverse phase HPLC. Subsequent treatment with 20% aqueous piperidine followed by HPLC gave the fully deprotected (**3a**). ESI mass spectrometric analysis revealed a single peak corresponding to the expected product ($M_r = 2787.27$, calcd for $M_r = 2788.06$). In contrast to the analogous glycylproline PNA (**2**) with the same T_{10} sequence, the product is freely soluble in aqueous solvents and a concentration exceeding 5 mg/mL could be achieved (Scheme 1).



7a : B = T^{Bz}; PG = Boc
7b : B = T^{Bz}; PG = Fmoc

H-[(ψ-CH_2)-Gly-D-Pro(T)]$_{10}$-$LysNH_2$ **(3a)**

Interaction between the decathymine PNA oligomer (**3a**) with poly(rA), poly(dA) and $(dA)_{10}$ was investigated by T_m measurement. Melting was observed only when the nucleic acid component is poly(rA) with a single transition at $T_m = 53\,^{\circ}C$ (150 mM NaCl). A typical melting curve is shown in Figure 1 and the T_m values are as shown in Table 1. Control experiments with poly(rU) and poly(rI) showed no observable melting curve, suggesting that the interaction is specific for A·T base-pairing. UV titration between (**3a**) and poly(rA) revealed a 2:1 stoichiometry of T:A, indicating the formation of a triple helical complex, probably via Watson–Crick and Hoogsteen-type T·A·T pairing similar to those observed with other homopyrimidine PNAs.[1–4] The interaction of cPNA and poly(A) was further studied by CD spectroscopy. While single stranded poly(rA) exhibited a characteristic CD signal in the region of 200–300 nm, single-stranded cPNA (**3a**)



Scheme 1. Synthesis of the monomer for aminoethylproline PNA. Reagents and conditions: (i) 2.5 equiv *p*TsOH/MeCN; (ii) *N*-nosylaziridine/DIEA/MeCN, rt; (iii) Boc_2O/Et_3N/DMAP in CH_2Cl_2; (iv) $PhSH/K_2CO_3$ in DMF, rt; (v) cyclohexene, cat Pd/C in MeOH, reflux; (vi) FmocCl/DIEA; (vii) 4 M HCl/dioxane.

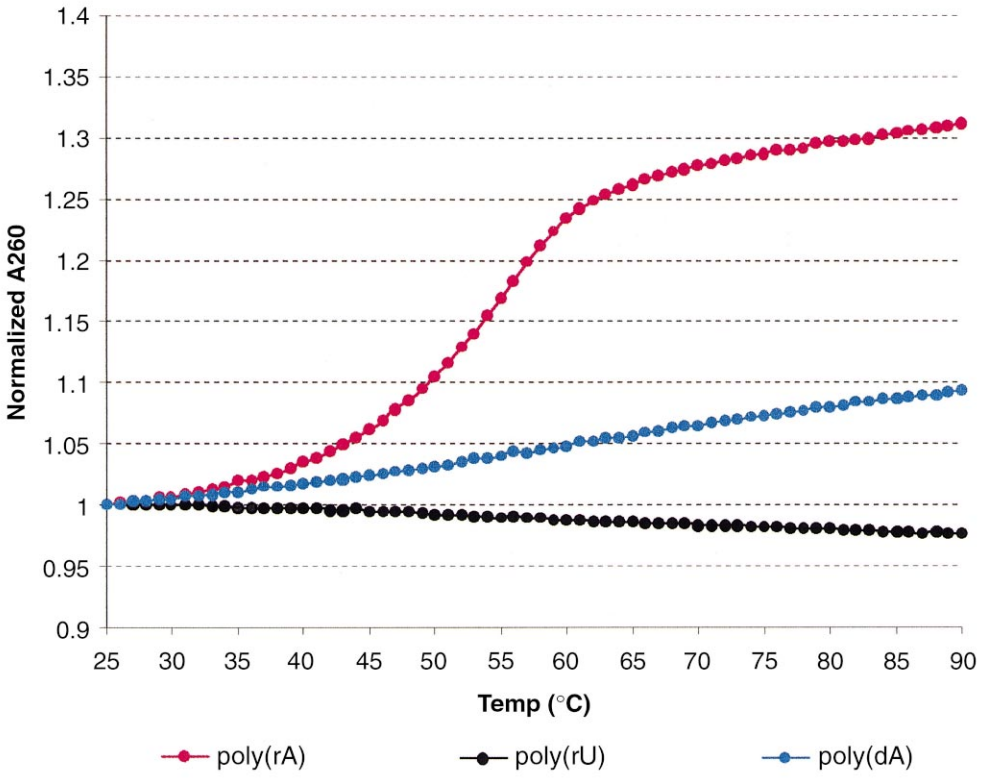


Figure 1. Melting curve of (**3a**) and poly(rA), poly(rU) and poly(dA). Conditions: 10 mM sodium phosphate buffer pH 7.0; 150 mM NaCl; 1.0 μM (**3a**); ratio of T:A = 1:1; 20% hypochromicity.

exhibited very weak CD spectra in this region. Any change in CD signal of the mixture between (**3a**) and poly(rA) would therefore indicate the formation of new species. As expected, addition of (**3a**) to poly(rA) resulted in a strong induced CD signal with a minima at 275 nm (Fig. 2a) whereas no significant change was observed when (**3a**) was added to poly(dA) under the same conditions (Fig. 2b). The shape of the CD curve is, however, significantly different from those of conventional DNA-DNA and DNA-RNA complexes thus possibly indicating a new type of helical structure. By following the CD signal as a function of amount of (**3a**) added, the stoichiometry of binding of PNA:RNA was found to be 2:1, consistent with the result obtained from UV titration. Gel electrophoresis experiments between (**3a**) and fluorescent labelled $(dA)_{10}$ also confirmed the lack of binding between (**3a**) and $(dA)_{10}$.

Table 1. Comparison of T_m of hybrids between PNA and oligonucleotides[a]

Hybrids	T_m (°C)	Stoichiometry (PNA:DNA/RNA)
(**1a**):poly(dA)	73	2:1
(**2a**):poly(dA)	70	1:1
(**3a**):poly(dA)	—[b]	—
(**3a**):poly(rA)	53	2:1
(**3a**):poly(rU)	—[b]	—
(**3a**):poly(rI)	—[b]	—

(**1a**): H-[Aeg(T)]$_{10}$-LysNH$_2$; (**1b**): H-[Gly-D-Pro(*cis*-T)]$_{10}$-LysNH$_2$
[a]Conditions: see Fig. 1.
[b]No melting above 20 °C was observed.

Although the hybrid between PNA oligomer (**3a**) and poly(rA) exhibited lower T_m value compared to Nielsen's type PNA (**1a**) or glycylproline cPNA (**2a**), the T_m is still remarkably high compared to the natural oligodeoxyribonucleotide $(dT)_{10}$ (T_m < 20 °C under comparable conditions). The lowered T_m compared to the glycylproline cPNA (**2a**) emphasizes the importance of restricting the conformation. The electrostatic interaction between the protonated form of the proline nitrogen atom and the negatively charged phosphate group of the nucleic acid may be a factor that provides a positive contribution to the binding. This is supported by the fact that no binding of (**3a**) to poly(rA) could be observed at high salt concentration (1.0 M NaCl). The selective recognition of polyribonucleotide in preference to polydeoxyribonucleotide is, however, of great interest with regard to its use in antisense research. Furthermore, the results are in sharp contrast to recent work[27] on related aminoethylproline PNA systems but with different stereochemistry around the proline ring [i.e. (2*S*,4*S*) and (2*R*,4*S*)], in which both stereoisomers were reported to bind strongly with complementary oligodeoxyribonucleotides according to T_m studies. At present we do not have a satisfactory model to explain the difference observed, but clearly stereochemistry of the proline ring must play an important role in determining the specificity.

In conclusion, we have demonstrated that a novel cPNA carrying a hydrophilic *N*-aminoethylproline backbone can be readily synthesized using standard peptide chemistry. The cPNA is readily soluble in aqueous

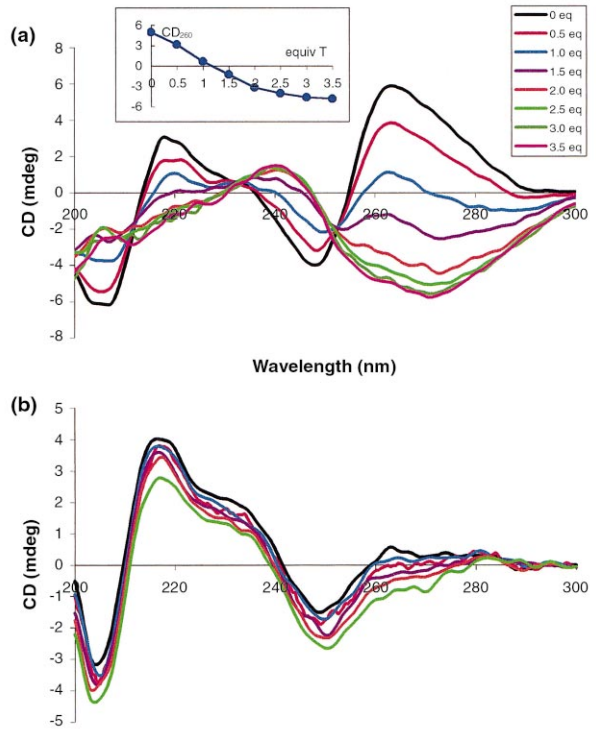


Figure 2. (a) CD spectra of mixture of poly(rA) and (**3a**) at different equivalents of T; inset: plot of ε_{260} versus equivalents of T; (b) CD spectra of mixture of poly(dA) and (**3a**) at different equivalents of T. Conditions 10 mM sodium phosphate buffer pH 7.0. The concentration of poly(rA) and poly(dA) was 10 μM based on AMP.

solvents and exhibit strong interaction with oligoribonucleotides but not with oligodeoxyribonucleotides. Such high selectivity suggests it has potential as an antisense agent where selective targeting of mRNA would be beneficial.

Acknowledgements

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29. *N*-2-(*N*-*tert*-Butoxycarbonylamino)ethyl-*cis*-4-(N^3-benzoylthymin-1-yl)-D-proline diphenylmethyl ester (**6a**) δ_H (500 MHz; $CDCl_3$) 1.43 [9H, s, ^{t}Bu C$\underline{H}_3$], 1.87 [3H, s, thymine C$\underline{H}_3$], 1.92 [1H, m, 1×C$\underline{H}_2$(3′)], 2.52–2.59 [1H, m, 1×BocNHCH$_2$C$\underline{H}_2$N], 2.71–2.80 [2H, m, 1×BocNHC$\underline{H}_2$-C$\underline{H}_2$N and 1×C$\underline{H}_2$(5′)], 2.85 [1H, ddd, J=19.0, 15.0, 9.5 Hz, 1×C$\underline{H}_2$(3′)], 3.03–3.12 [1H, m, 1×BocNHC$\underline{H}_2$CH$_2$N], 3.18–3.21 and 3.21–3.26 [2H, m, 1×BocNHC$\underline{H}_2$CH$_2$N and 1×CH$_2$(5′)], 3.40 [1H, dd, J=7.0, 9.5 Hz, C$\underline{H}$(2)], 5.09 [1H, br s, Boc N$\underline{H}$], 5.17–5.24 [1H, m, C$\underline{H}$(4′)], 6.96 [1H, s, C$\underline{H}$Ph$_2$], 7.24–7.38 [10H, m, Dpm aromatic C$\underline{H}$], 7.45 [2H, t, J=8.0 Hz, benzoyl *m*-C$\underline{H}$], 7.61 [1H, t, J=8.0 Hz, benzoyl *p*-C$\underline{H}$], 7.87 [2H, d, J=8.0 Hz, benzoyl *o*-C$\underline{H}$], 7.96 [1H, s, thymine C(6)$\underline{H}$]; *m*/*z* (APCI+) 676 ($M+Na^+$, 18.5%), 653 ($M+H^+$, 59), 597 ($M-C_4H_8^+$, 25), 554 ($M-Boc^+$, 9), 475 (26.5), 167 (Ph_2CH^+, 100); $[\alpha]_D^{22}$ +6.5 (c=1.06, $CHCl_3$).
N-2-(*N*-*tert*-Butoxycarbonylamino)ethyl-*cis*-4-(N^3-benzoylthy-

min-1-yl)-D-proline (**7a**) δ_H (200 MHz; $CDCl_3$) 1.42 [9H, s, ^{t}Bu CH_3], 1.95 [3H, s, thymine CH_3], 2.05 [1H, m, 1×CH_2(3′)], 2.60–3.50 [m, CH_2(5′) and 2×aminoethyl CH_2 and CH(2′)], 4.65 [1H, br m, OH], 5.18 [1H, m, CH(4′)], 5.60 [1H, br m, NH]; 7.36 [10H, m, Dpm aromatic CH], 7.48 [2H, t, Bz *m*-CH], 7.60 [1H, t, Bz *p*-CH], 7.89 [2H, d, Bz *o*-CH], 8.01 [1H, s, thymine C(6)H]; *m*/*z* (APCI+) 509 ($M+Na^+$, 38%), 487 ($M+H^+$, 56), 431 ($M-C_4H_8^+$, 45), 309 (100), 155 (82); $[\alpha]_D^{23}$ +12.2 (c = 1.04, $CHCl_3$).

30. *N*-2-(9-Fluorenylmethoxycarbonylamino)ethyl-*cis*-4-(N^3-benzoylthymin-1-yl)-D-proline diphenylmethyl ester (**6b**) (80%), mp 94–98 °C (Found C, 72.3; H, 5.7; N, 7.4%; $C_{47}H_{42}N_4O_7$ requires C, 72.3; H, 5.5; N, 7.2%); δ_H (500 MHz; $CDCl_3$) 1.73 [3H, s, thymine CH_3], 1.85–1.93 [1H, m, 1×CH_2(3′)], 2.50–2.59 [1H, m, 1×Fmoc $NHCH_2CH_2N$], 2.65–2.76 [2H, m, 1×CH_2(5′) and 1×Fmoc $NHCH_2CH_2N$], 2.77–2.88 [1H, m, 1×CH_2(3′)], 3.02–3.11 [1H, m, 1×Fmoc $NHCH_2CH_2N$], 3.14–3.24 [1H, m, 1×CH_2(5′)], 3.25–3.33 [1H, t, 1×Fmoc $NHCH_2CH_2N$], 3.35–3.42 [1H, m, CH(2′)], 4.14 [1H, t, *J* = 7.0 Hz, 1×Fmoc aliphatic CH_2], 4.24 [1H, t, *J* = 8.5 Hz, Fmoc aliphatic CH_2], 5.14–5.20 [1H, br m, CH(4′)], 5.40 [1H, br s, Fmoc NH], 6.92 [1H, s, $CHPh_2$], 7.18–7.30 [10H, m, Dpm aromatic CH], 7.30–7.36 [4H, m, Fmoc aromatic CH], 7.40 [2H, t, *J* = 7.5 Hz, benzoyl *m*-CH], 7.52–7.58 [3H, m, benzoyl *p*-CH, 2×Fmoc CH aromatic], 7.71 [2H, d, *J* = 7.5 Hz, benzoyl *o*-CH], 7.81–7.87 [3H, m, thymine C(6)H, 2×Fmoc aromatic CH]; *m*/*z* (APCI+) 775 ($M+H^+$, 98%), 553 (18.5), 265 (21), 167 (Ph_2CH^+, 74), 137 (100); ν_{max} (KBr)/cm^{-1} 1746s, 1722s, 1697s and 1653s (C=O); $[\alpha]_D^{23}$ +7.03 (c = 0.92, $CHCl_3$).

N-2-(9-Fluorenylmethoxycarbonylamino)-ethyl-*cis*-4-(N^3-benzoylthymin-1-yl)-D-proline hydrochloride (**7b**) (71%) mp 144–146 °C (Found C, 60.9; H, 5.0; N, 8.2%; $C_{34}H_{33}N_4O_7Cl+2H_2O$ requires C, 59.9; H, 5.5; N, 8.2%); δ_H (500 MHz; DMSO-d_6 + 1 drop D_2O) 2.50 [3H, s, thymine CH_3], 2.34–2.44 [1H, m, 1×CH_2(3′)], 2.83 [1H, m, 1×CH_2(3′)], 3.12–3.22, 3.26–3.36, 3.37–3.42 and 3.44–3.53 [4H, 4×m, FmocNHCH_2CH_2N], 3.55–3.75 [1H, m, 1×CH_2(5′) overlap with D_2O peak], 3.93–4.00 [1H, m, 1×CH_2(5′)], 4.20 [1H, t, *J* = 6.5 Hz, Fmoc aliphatic CH], 4.25–4.36 [2H, m, Fmoc aliphatic CH_2], 4.46–4.54 [1H, m, CH(2′)], 5.27–5.35 [1H, m, CH(4′)], 7.24–7.42 [6H, m, Fmoc aromatic CH], 7.57 [2H, t, *J* = 7.5 Hz, benzoyl m-CH], 7.62–7.68 [3H, m, Fmoc aromatic CH], 7.74 [1H, t, *J* = 7.5 Hz, benzoyl *p*-CH], 7.86 [2H, d, *J* = 7.5 Hz, benzoyl *o*-CH], 7.96 [2H, d, *J* = 7.5, Fmoc aromatic CH], 8.03 [1H, br s, thymine C(6)H]; *m*/*z* (APCI+) 631 ($M+Na^+$, 6%), 609 ($M+H^+$, 8), 333 (7), 265 (12), 179 (26), 137 (100); ν_{max} (KBr)/cm^{-1} 1748s, 1733s, 1694s and 1645s (C=O); $[\alpha]_D^{23}$ +13.5 (*c* = 1.00, DMF).

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