Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 7, No. 15, pp. 2027-2032, 1997

0960-894X/97 $17.00 + 0.00

PII: S0960-894X(97)00351-X

Design and Synthesis of a Possible Mimic of a Thrombin-Binding DNA Aptamer

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Abstract: A synthesis is presented of the cyclic trimeric d-oligonucleotide 3'-isopropylphosphate **I**, comprising one formacetal and two (3'→ 5')-internucleosidic phosphodiester bonds. The ester linkages connect d-guanosine with the 3' and 5' ends of thymidine and 5-hydroxymethyl-2'-deoxyuridine-3'-isopropylphosphate (HMDUp*i*Pr), respectively. The 5'-end of the thymidine unit is anchored *via* the formacetal bond to the allylic hydroxyl group of HMDUp*i*Pr. The cyclic arrangement of the three d-nucleosides in **I** mimics, as based on molecular modeling, the key structural features of the conformationally constrained $T^7pG^8pT^9p$-domain of the thrombin-binding DNA aptamer d($G^1G^2T^3T^4G^5G^6\underline{T^7G^8T^9}G^{10}G^{11}T^{12}T^{13}G^{14}G^{15}$). Biological evaluation showed that compound **I** did not exhibit anti-thrombin activity. © 1997 Elsevier Science Ltd.

Introduction

The prominent role of serine protease thrombin in thrombosis and haemostasis stimulated extensive research towards the design and synthesis of effective thrombin inhibitors. Recently, Bock *et al.*[1] screened a pool of ~10^{13} synthetic 96-mer oligodeoxynucleotides for their interaction with thrombin using a novel *in vitro* selection/amplification technique. Comparison of the sequences having affinity for thrombin led to the identification of a consensus DNA-15 mer (*i.e.* d($G^1G^2T^3T^4G^5G^6T^7G^8T^9G^{10}G^{11}T^{12}T^{13}G^{14}G^{15}$)). This so-called aptamer inhibits thrombin activity at nanomolar concentrations. Since then a lot of research has been focused on the mode of binding of thrombin with the aptamer. Bock *et al.*[1] and Wu *et al.*[2] showed that the active site of thrombin is not involved in aptamer binding as the aptamer does not inhibit the cleavage of small chromogenic amide substrates. Recent biochemical[2-4] and physical experiments[5-8] revealed that the synthetic 15-mer interacts with the alleged anion-exosite of thrombin. Three-dimensional structure analysis of the aptamer by NMR spectroscopy[5-7] revealed that it adopts a unique folded structure, in which two stacked G-tetrads are connected through a TGT- and two TT-loops (see Fig. 1). A similar folding was reported in a preliminary study[8] on the crystal structure of the aptamer-thrombin complex. The crystallographic analysis also showed that the trimeric $T^7pG^8pT^9p$ domain solely interacts with the anion-exosite of thrombin and adopts a loop conformation in which the 5'-O-T^7 and the 7-C-T^9 are in close proximity (3.4 Å) (see Fig. 2). The latter information goaded us to devise a mimic in which the two

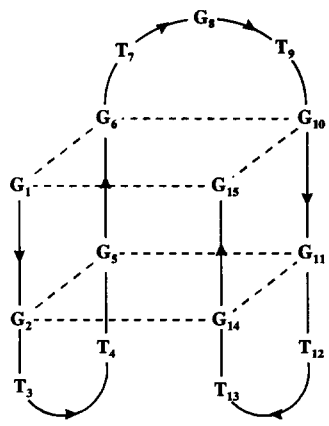

Figure 1: Schematic drawing of the folded 15-mer aptamer.

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thymidines in the trimeric $T^7pG^8pT^9p$-unit are connected by a suitable linker. Molecular modeling studies indicated that this objective could be achieved by replacing of T^9 by 5-hydroxymethyl-2'-deoxyuridine (HMDU) and anchoring the respective primary and allylic hydroxyl in T^7 and HMDU *via* a methylene acetal bond (see Fig. 3). In this way, a loop-structure is created covering a distance between the primary hydroxyl of T^7 and the allylic carbon of HMDU of approximately 2.8 Å. In addition, the (3'-5')-internucleosidic phosphodiester bond between T^9 and G^{10}, the presence of which is essential for thrombin interaction, was replaced by a 3'-isopropyl phosphate. We here report the synthesis of the novel cyclic oligonucleotide **I** (see Fig 3.) containing the key structural features of the aptameric TpGpTp-domain.

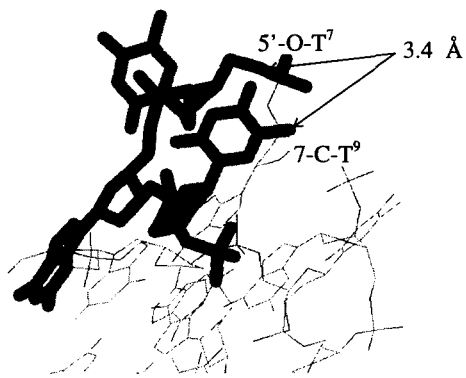


Figure 2: Detail of the crystal structure[8] of the aptamer in the aptamer-thrombin complex. The TpGpTp-domain, which binds with thrombin, is presented as a stick model

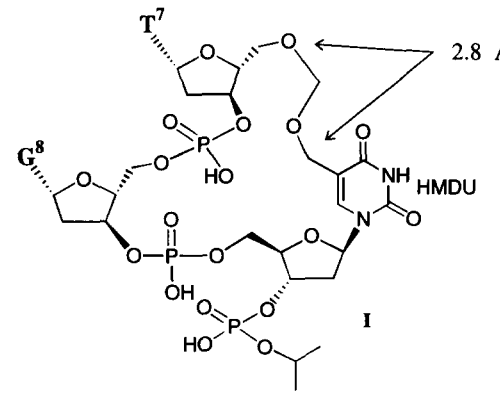
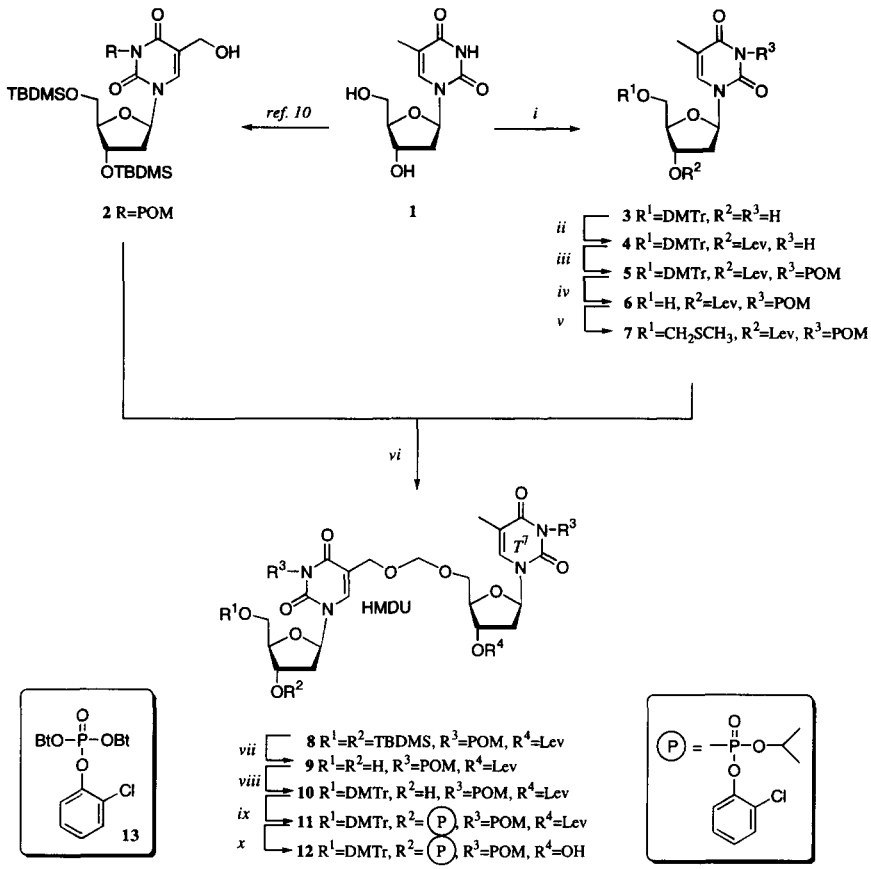


Figure 3. Proposed mimic of the aptameric TpGpTp-domain.

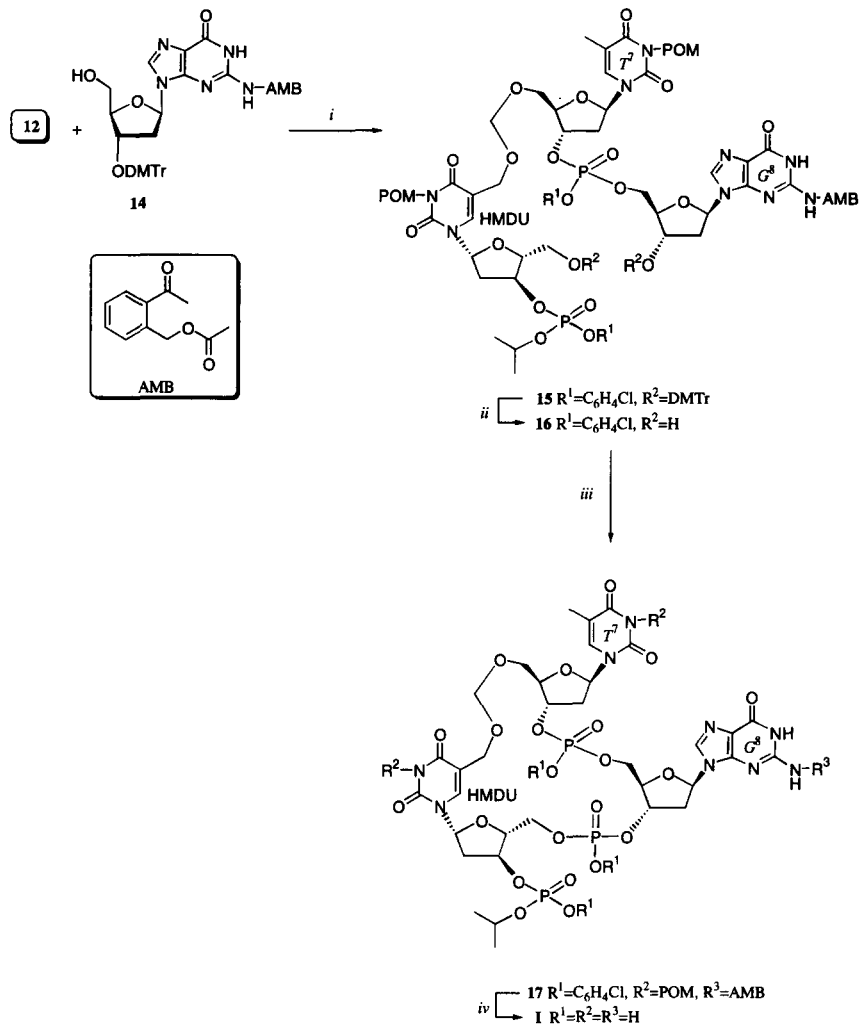
Results and discussion

One of the crucial steps in the assembly of cyclic oligonucleotide **I** entails the introduction of a methylene acetal linkage between the 5'-hydroxyl of T^7 and the allylic hydroxyl function of HMDU. It was established[9] that the formation of the purposive methylene acetal bond could be effected most conveniently starting from 5'-*O*-methylthiomethyl-3'-*O*-levulinoyl-N^3-pivaloyloxymethyl-thymidine (**7**) and known[10] 3',5'-di-*O*-*tert*-butyldimethylsilyl-N^3-pivaloyloxymethyl-5-hydroxymethyl-2'-deoxyuridine (**2**). The requisite donor **7** was readily accessible from **1** by the following five-step procedure. Tritylation of the primary hydroxyl in **1** with 4,4'-dimethoxytrityl chloride (DMTr-Cl) and subsequent acylation of resulting **3** with levulinic acid anhydride gave **4**. Alkylation[11] of **4** in DMF with pivaloyloxymethyl chloride (POM-Cl) in the presence of K_2CO_3, followed by acid treatment of the N-3 protected **5**, led to the isolation of **6**. Transformation of **6** into the thiomethyl ether derivative **7** proceeded as expected by subjecting **6** to dimethylsulfide and benzoylperoxide (BPO) in the presence of 2,6-lutidine[12]. Coupling of **7** with **2** under the agency of *N*-iodosuccinimide (NIS) and catalytic triflic acid (TfOH)[12,13] gave the desired methylene acetal linked dimer **8** in 84% yield. Dimer **8** was converted into **10** by desilylation with triethylamine trihydrofluoride in pyridine[14] and subsequent regioselective tritylation of the



Scheme 1: i) DMTr-Cl, pyridine, 1 h; ii) Lev_2O, pyridine, DMAP, 2 h, 96% (2 steps); iii) POM-Cl, K_2CO_3, DMF, 6 h; iv) 4% *p*-TosOH, $MeOH/CH_2Cl_2$ (1/1, v/v), 5 min, 72% (2 steps); v) Me_2S (10 equiv), BPO (4 equiv), 2,6-lutidine, CH_3CN/CH_2Cl_2 (1/1, v/v), 2 h, 65%; vi) NIS, *cat.*TfOH, THF, DCE, 5 min, 84%; vii) TEA•3HF, pyridine, 2 h, 89%; viii) DMTr-Cl, pyridine, 1 h, 84%; ix) **13**, 2-propanol, pyridine/dioxane, 3 h, 76%; x) NH_2NH_2, pyridine, AcOH, 5 min;

primary function with DMTr-Cl. Phosphorylation of **10** proceeded smoothly using the well-established bifunctional reagent O-2-chlorophenyl-O,O-*bis*-(benzotriazol-1-yl)phosphate[15] (**13**) to give, after work-up and purification, the homogeneous 3'-(*o*-chlorophenyl)(isopropyl) phosphate derivative **11** (δ p -8.14, -8.37 ppm). Removal of the levulinoyl group in **11** by short treatment with hydrazine in pyridine/acetic acid[16] afforded **12**. The introduction of the (3'→ 5')-internucleosidic linkage between **12** and partially protected d-guanosine derivative **14**, the N^2-2-(acetoxymethyl)benzoyl (AMB) group[17] of which can be removed under mild basic conditions[18], could be readily effected with bifunctional reagent **13** (see Scheme 2). Thus, phosphorylation of **12** with **13**, and coupling of **14** with the *in situ* formed benzotriazol-1-yl phosphate triester of **12**, led to the trimeric derivative **15** (δ p -8.14, -8.19, -8.28, -8.37 ppm). Acidolysis of both DMTr-groups in **15** with *p*-toluenesulfonic acid in dichloromethane/methanol furnished partially deprotected **16** in 85% yield. The linear oligonucleotide **16**



Scheme 2: i) **13**, pyridine, dioxane, 3 h, 63% (2 steps); ii) 4% *p*-TosOH, MeOH/CH_2Cl_2 (1/1, v/v), 5 min, 85%,; iii) **13**, pyridine (6 mM), 54%; iv) a. 0.25 M TBAF, pyridine, H_2O (1/1, v/v); b. 25% NH_4OH, room temperature, 16 h, 63%;

was now converted into the corresponding cyclic and fully protected oligonucleotide **17** according to a well-established protocol[19] devised for the preparation of cyclic oligonucleotides. Thus, **13** was added dropwise to a highly diluted solution (6 mM) of **16** in pyridine. Monitoring of the cyclization by TLC revealed the reaction to be complete (R_f 0 → 0.60, 5% MeOH/CH_2Cl_2) after one hour at 20 °C. Subsequent work-up and purification of the reaction mixture afforded **17** in a 54% yield. The cyclic oligonucleotide **17** was deblocked in a two-step process. Removal of the *o*-chlorophenyl protective groups with tetra-*n*-butylammonium fluoride (TBAF) and subsequent ammonolysis of the POM and AMB protective groups gave completely deblocked cyclic oligonucleotide **I**, which was purified by HW-40 gel filtration and isolated as the sodium salt. The homogeneity of **I** (Na^+-salt) was firmly established by HPLC analysis, ^1H and ^{31}P NMR-spectroscopy as well as ES-MS spectrometry[20]. The existence of the (3'-5')- internucleosidic phosphodiester bond, originating from the

cyclization of **16**, is supported by the two dimensional ^{1}H-^{31}P correlated NMR-spectrum of compound **I** (see Fig. 4). Moreover, the NOE-effects, observed after irradiation of the C5' primary protons of T^{7} in **I**, are in agreement with the presence of the methylene acetal between the primary hydroxyl of T^{7} and the allylic hydroxyl of HMDU[21]. In order to evaluate the extent of thrombin inhibition, compound **I** was tested in a fibrinogen dependent thrombin assay[22]. The outcome of these tests clearly indicated that compound **I** was not active. The biological inactivity of **I** may be explained by the recent findings[23,24] that the two TT-loops (see Fig. 1), instead of the earlier proposed TpGpTp-domain, interact with the anion-exosite of thrombin.

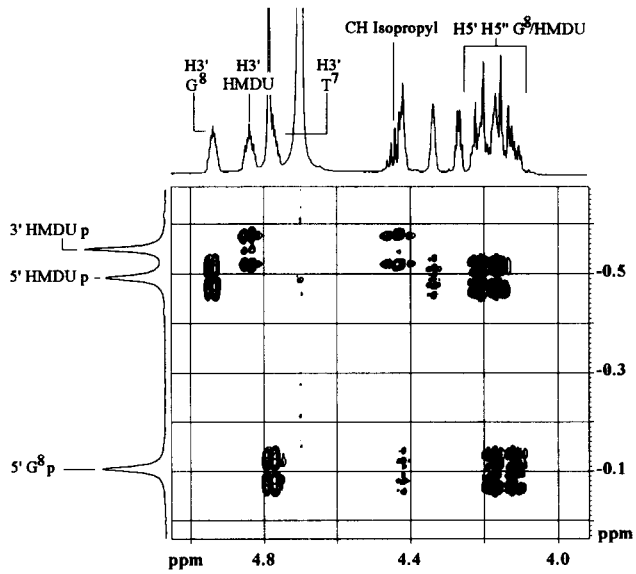


Figure 4: Part of the ^{1}H-^{31}P-NMR spectrum of compound **I**

The design and synthesis of mimics based on this recently attained insight into the mode of interaction of the aptamer with thrombin will be reported in due course.

References and notes

1. Bock, L.C.; Griffin, L.C.; Latham, J.A.; Vermaas, E.H.; Toole, J.J.; *Nature* **1992**, *355*, 564.
2. Wu, Q.; Tsiang, M.; Sadler, J.E.; *J. Biol. Chem.* **1992**, *267*, 24408.
3. Paborsky, L.R.; McCurdy, S.N.; Griffin, L.C.; Toole, J.J; Leung, L.L.K.; *J. Biol. Chem.* **1993**, *268*, 20806.
4. Tsiang, M.; Jain, A.K.; Dunn, K.E.; Rojas M.E.; Leung, L.K., Gibbs, C.S.; *J. Biol. Chem.* **1995**, *270*, 16854.
5. Wang, K.Y.; Krawczyk, S.H.; Bischofberger, N.; Swaminathan, S.; Bolton, P.H.; *Biochemistry* **1993**, *32*, 11285.
6. Macaya, R.F.; Schultze, P.; Smith, F.W.; Roe, J.A.; Feigon, J.; *Proc. Natl. Acad. Sci. USA*, **1993**, *90*, 3745.
7. Schultze, P.; Macaya, R.F.; Feigon, J.; *J. Mol. Biol.* **1994**, *235*, 1532.
8. Padmanabhan, K.; Padmanabhan, K.P.; Ferrara, J.D.; Sadler, J.E.; Tulinsky, A.; *J. Biol. Chem.* **1993**, *268*, 17651.
9. The NIS/*cat.* TfOH mediated condensation reaction between the corresponding N-3 unprotected donor and acceptor led to an intractable mixture of products.
10. De Kort, M., Wijsman, E.R.; Van der Marel, G.A.; Van Boom, J.H.; *manuscript in preparation.*

11. Rasmussen, M.; Leonard, N.J.; *J. Am. Chem. Soc.* **1967**, *89*, 5439.
12. Veeneman, G.H.; Van der Marel, G.A.; Van den Elst, H.; Van Boom, J.H.; *Recl. Trav. Chim. Pays-Bas* **1990**, *109*, 449.
13. Veeneman, G.H.; Van der Marel, G.A.; Van den Elst, H.; Van Boom, J.H.; *Tetrahedron* **1991**, *47*, 1547.
14. Westman, E.; Strömberg, R., *Nucl. Acids Res.* **1994**, *22*, 2430.
15. Van der Marel, G.A.; Van Boeckel, C.A.A.; Wille, G.; Van Boom, J.H.; *Tetrahedron Lett.* **1981**, *22*, 3887.
16. Van Boom, J.H.; Burgers, P.M.J. *Tetrahedron Lett.* **1976**, *17*, 4875.
17. Kuijpers, W.H.A.; Huskens, J.; Van Boeckel, C.A.A., *Tetrahedron Lett.* **1990**, *31*, 6729.
18. Prolonged ammonolysis (25% NH_4OH) at elevated temperature, necessary to remove conventional guanine-base protection (isobutyryl), may have a detrimental effect on target compound **I**
19. Capobianco, M.; Carcuro, A.; Tondelli, L.; Garbesi, A.; Bonara, G.M.; *Nucleic Acids Res.* **1990**, *18*, 2661.
20. ^{31}P NMR (D_2O): δ -0.12 (5' G^8p), -0.49 (5' HMDUp), -0.56 (3' HMDUp). ^1H NMR (D_2O, 600 MHz, HH-COSY): δ 8.04 (s, 1H, H8 G^8), 7.81, 7.60 (2 x s, 2H, H6 T^7, H6 HMDU), 6.31 (dd, 1H, H1' HMDU, $J_{1,2'} \approx J_{1,2''}$= 6.1 Hz), 6.30 (dd, 1H, H1' T^7, $J_{1,2'}$= 6.5 Hz, $J_{1,2''}$= 4.6 Hz), 6.25 (dd, 1H, H1' G^8, $J_{1,2'}$= 7.9 Hz, $J_{1,2''}$= 5.9 Hz), 4.95 (m, 1H, H3' G^8), 4.85 (m, 1H, H3' HMDU), 4.79 (s, 2H, OCH_2O), 4.78 (m, 1H, H3' T^7), 4.44 (dq, 1H, $OCH(CH_3)_2$), 4.43 (m, 1H, H4' G^8), 4.35 (m, 1H, H4' HMDU), 4.27 (ddd, 1H, H4' T^7, $J_{3,4}$= 3.7 Hz, $J_{4,5'}$= 4.5 Hz, $J_{4,5''}$= 3.3 Hz), 4.25-4.09 (m, 4H, H5', H5'' G^8, HMDU), 4.25, 4.23 (d, 2H, H7a, H7b, J_{AB}=12.3 Hz), 3.86 (dd, 1H, H5' T^7, $J_{4,5'}$= 4.5 Hz, $J_{5',5''}$= 11.5 Hz), 3.73 (dd, 1H, H5'' T^7, $J_{4,5''}$= 3.3 Hz, $J_{5',5''}$= 11.5 Hz), 2.77 (ddd, 1H, H2' G^8, $J_{1,2'}$= 7.9 Hz, $J_{2',2''}$= 13.8 Hz, $J_{2',3'}$= 5.8 Hz), 2.68 (ddd, 1H, H2'' G^8, $J_{1,2''}$= 5.9 Hz, $J_{2',2''}$= 13.8 Hz, $J_{2'',3'}$= 2.9 Hz), 2.58 (ddd, 1H, H2' T^7, $J_{1,2'}$= 6.5 Hz, $J_{2',2''}$= 14.2 Hz, $J_{2',3'}$= 7.1 Hz), 2.52 (ddd, 1H, H2' HMDU, $J_{1,2'}$= 6.1 Hz, $J_{2',2''}$= 14.2 Hz $J_{2',3'}$= 6.9 Hz), 2.46 (ddd, 1H, H2'' T^7, $J_{1,2''}$= 4.6 Hz, $J_{2',2''}$= 14.2 Hz, $J_{2'',3'}$= 6.6 Hz), 2.36 (ddd, 1H, H2'' HMDU, $J_{1,2''}$= 6.1 Hz, $J_{2',2''}$= 14.2 Hz, $J_{2'',3'}$= 2.7 Hz), 1.87 (s, 3H, CH_3 T^7), 1.27 (d, 6H, $OCH(CH_3)_2$, J = 6.2 Hz); ES-MS: $[M+H]^+$ 1026.3.
21. This assay was performed by T.H. van Dinther at N.V. Organon Oss, *personal communication.*
22. 300 MHz ^1H NOE-DIFF NMR (D_2O, 36 °C) showed, after irradiation of H5'-H5'' in T^7, two NOE resonances at δ 4.79 ppm (OCH_2O) and δ 4.25, 4.23 ppm (H7a,H7b).
23. Padmanabhan, K.; Tulinsky, A.; *Acta Cryst.* **1996**, *D52*, 272.
24. Kelly, J.A.; Feigon, J.; Yeates T.O.; *J. Mol. Biol.* **1996**, *256*, 417.

(*Received in Belgium* 5 *May* 1997; *accepted* 1 *July* 1997)