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Oligonucleotide Synthesis by the Use of a 2-(Levulinyloxymethyl)-5-nitrobenzoyl Group as the Novel Base-labile Protecting Group for the 5'-Hydroxyl Groups of Ribonucleoside and 2'-Deoxyribonucleoside 3'-Phosphoramidites

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Abstract: A novel 2-(levulinyloxymethyl)-5-nitrobenzoyl (LMNBz) protecting group for the 5' position of nucleoside 3'-phosphoramidites was successfully applied to the solid-phase synthesis of both an oligodeoxyribonucleotide (TpTpT and TpTpTpT) and an octaribonucleotide in combination with a 2'-*O*-Thp protecting group (UpCpApGpUpUpGpG). The LMNBz group was simply unmasked due to its base-labile property by a two-step procedure, *i.e.*, treatments with 0.5 M hydrazine hydrate in 1:4 acetic acid - pyridine, and then with 0.5 M imidazole in acetonitrile.

A series of problems hampers the current methodology of automated solid-phase synthetic approaches on CPG-supports. Specifically, depurination occurs under the acidic conditions used for the removal of the 5'-*O*-(4,4'-dimethoxytrityl) (DMTr) protecting group in the synthesis of DNA-oligomers,[1] and the sterically bulky 2'-*O*-TBDMS protection tends to inhibit the coupling reactions of the ribonucleotide units in RNA-oligomers,[2] since the acid-labile 2'-*O*-(tetrahydropyran-2-yl) (Thp) protection is somewhat affected upon the removal of the 5'-*O*-DMTr group under acidic conditions.[3] Consequently, various protecting groups for the 5'-position of ribonucleoside and 2'-deoxyribonucleoside 3'-phosphoramidites have been reported,[4] as exemplified by the levulinyl group[5] and the modified 2-hydroxymethylbenzoyl groups,[6] which are removable under basic conditions. The former is characterized by facile unmasking by 0.5 M hydrazine hydrate in 1:4 acetic acid - pyridine at room temperature for 2 min (liquid-phase approach[5a,b]) or 10 - 15 min (solid-phase approach; no damage to the 2-cyanoethyl protecting group of phosphate[5c,d]), although the yield for its introduction is somewhat lower, ranging from 30% to 70%, depending on the structure of the nucleoside.

In view of this background, the authors addressed the potential utility of 2-(levulinyloxymethyl)benzoyl (LMBz) and LMNBz groups as novel base-labile protecting groups for the 5'-position of nucleoside 3'-phosphoramidites in solid-phase oligonucleotide synthesis. 2-(Levulinyloxymethyl)benzoic acid (**3**) and its 5-nitro derivative (**4**) were prepared from phthalide by a sequence of reactions, *i.e.*, alkaline hydrolysis,[7] crystallization of 2-hydroxymethylbenzoic acid (**2**) by adjusting the *p*H of the resulting mixture at ca. 2 with concentrated hydrochloric acid (91% yield), and esterification with levulinic anhydride (1.5 mol. equiv.) in the

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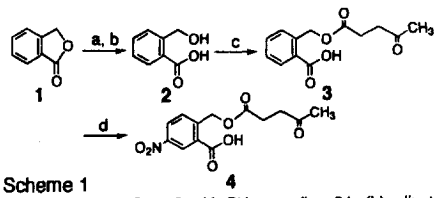
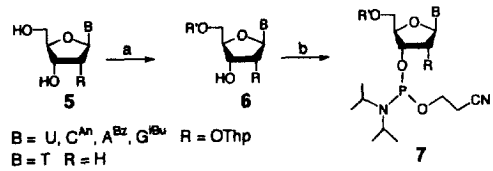
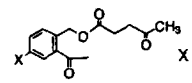
This communication is dedicated to the late Professor Tsujiaki Hata, Chiba Institute of Technology.



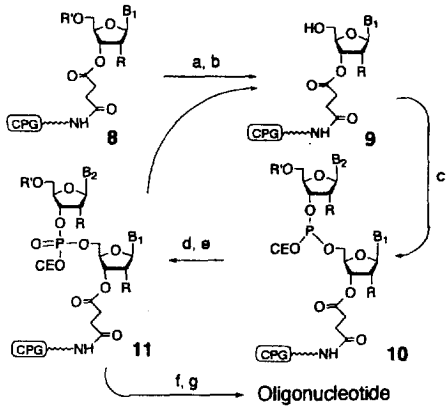
Scheme 1
Conditions; (a) KOH, 85% MeOH aq., reflux, 2 h; (b) adjust to *ca.* *p*H 2 with conc. hydrochloric acid; (c) levulinic anhydride, 1-methylimidazole, 1,4-dioxane, 1 h; (d) H_2SO_4 - HNO_3, 0 °C, 1 h.



B = U, C^{An}, A^{Bz}, G^{iBu} R = OThp
B = T R = H
R' = LMBz, LMNBz



Scheme 2
Conditions; (a) **3** or **4**, TPSCl, pyridine, 1 day (LMBz) or 2 - 3 h (LMNBz); (b) 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite, iPr_2NEt, CH_3CN, 0.5 - 1 h.



Scheme 3 Reaction Cycle for Assembly of Oligonucleotides
Conditions; (a) 0.5 M $NH_2NH_2 \cdot H_2O$,1:4 CH_3COOH - pyridine, 15 min;[5d] (b) 0.5 M imidazole, CH_3CN, 5 min;[12] (c) 0.05 M **7** (ca. 10 eq.), 0.25 M 1*H*-tetrazole, CH_3CN,10 min;[5c] (d) $(CH_3CO)_2O$, 2,6-lutidine,1-methylimidazole, THF,1 min; (e) 0.1 M I_2, H_2O - pyridine - THF,1 min; (f) conc. NH_4OH, r. t., 3 h - 55 °C, 6 h; (g) *p*H 2 HCl aq., r. t., 1 day.

presence of 1-methylimidazole (1.5 mol. equiv.), giving **3** (59% yield), which was then subjected to a nitration reaction with a mixture of concentrated nitric acid and sulfuric acid to afford **4** (62% yield) (Scheme 1).

The introduction of the LMBz and LMNBz protecting groups to the 5'-positions of 2'-*O*-Thp-uridine [**5** (B=U, R=OThp)],[8] N^4-anisoyl-2'-*O*-Thp-cytidine [**5** (B=C^{An}, R=OThp)],[8] N^6-benzoyl-2'-*O*-Thp-adenosine [**5** (B=A^{Bz}, R=OThp)],[8] N^2-isobutyryl-2'-*O*-Thp-guanosine [**5** (B=G^{iBu}, R=OThp)],[8] and thymidine [**5** (B=T, R=H)] was accomplished by treatment with **3** and **4** (1.1 mol. equiv.), respectively, in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride (TPSCl) (2.2 mol. equiv.) in pyridine at room temperature to give the 5'-*O*-LMBz- and -LMNBz-nucleoside derivatives (**6**) in 60% to 72% yields, which are comparable and/or superior to the yields of the 5'-*O*-levulinylation reaction[5] using levulinic acid (3 - 4 mol. equiv.) (Scheme 2). The consecutive treatments of 5'-*O*-LMBz- and -LMNBz-thymidine [**6** (B=T, R=H, R'=LMBz, LMNBz) with 0.5 M hydrazine hydrate in 1:4 acetic acid - pyridine at room temperature for 2 min,[5] acetone, and a 5% aqueous solution of sodium hydrogen carbonate[6] gave **5** (B=T, R=H), as expected.

The LMBz and LMNBz derivatives (**6**) were then subjected to phosphitylation reactions with 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite[9] (1.5 mol. equiv.) and *N*-ethyldiisopropylamine (1.5 mol. equiv.) in acetonitrile to give the corresponding 3'-(2-cyanoethyl) *N,N*-diisopropylphosphoramidites (**7**) in 65% - 91% yields (Scheme 2).

Prior to oligonucleotide synthesis, the controlled pore glass (CPG) support was functionalized with the 5'-*O*-LMBz and -LMNBz derivatives [**6** (B=T, R=H; B=G^{iBu}, R=OThp)] in the usual manner.[10] The CPG **8** (B=T, R=H, R'=LMBz), **8** (B=T, R=H, R'=LMNBz), **8** (B=G^{iBu}, R=OThp, R'=LMBz), and **8** (B=G^{iBu}, R=OThp, R'=LMNBz) thus obtained were used for oligonucleotide syntheses through the reaction cycle for their assembly, as shown in Scheme 3, by manual, and not automated, synthesis.[11] Starting with **8** (B=T, R=H,

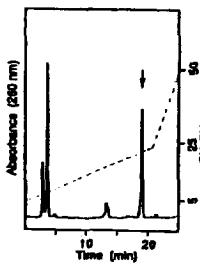


Fig. 1
Yield of TpTpT; 4.8 A_{260} units from **8** (B=T, R=H, R'=LMBz; 1.23 µmol)

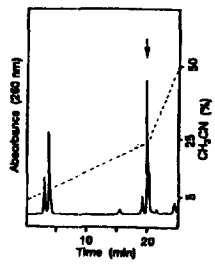


Fig. 2
Yield of TpTpTpT; 5.6 A_{260} units from **8** (B=T, R=H, R'= LMBz; 1.23 µmol)

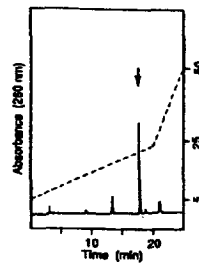


Fig. 3
Yield of TpTpT; 18 A_{260} units from **8** (B=T, R=H, R'=LMNBz; 1.07 µmol)

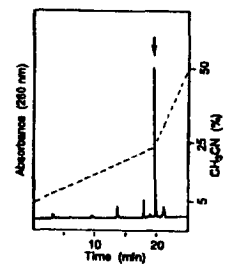


Fig. 4
Yield of TpTpTpT; 16.8 A_{260} units from **8** (B=T, R=H, R'= LMNBz; 1.07 µmol)

Reversed-phase HPLCs of crude products of TpTpT and TpTpTpT preparations using the 5'-*O*-LMBz-thymidine 3'-phosphoramidite derivative (Figs. 1 and 2) and the 5'-*O*-LMNBz-thymidine 3'-phosphoramidite derivative (Figs. 3 and 4), respectively.

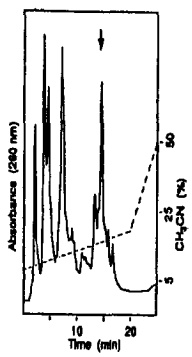


Fig. 5 Reversed-phase HPLC of crude products from the preparation of the octamer (UpCpApGpUpUpGpG) using 5'-*O*-LMBz-2'-*O*-Thp-ribonucleoside 3'-phosphoramidite derivatives.

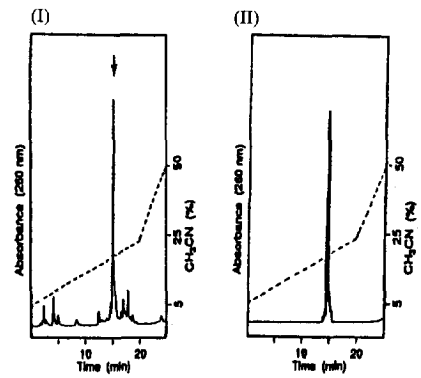


Fig. 6 (I) Reversed-phase HPLC of crude products from the preparation of the octamer (UpCpApGpUpUpGpG) using 5'-*O*-LMNBz-2'-*O*-Thp-ribonucleoside 3'-phosphoramidite derivatives. (II) Reversed-phase HPLC of the octamer isolated from the mixture of (I). Yield of the octamer; 41.1 A_{260} units from **8** (B=G^{iBu}, R=OThp, R'=LMNBz; 1.95 µmol)

Conditions of reversed-phase HPLCs (Figs. 1, 2, 3, 4, 5, and 6): column µBONDASPHERE 5µ C18 (3.9 mm ID x 150 mm L); elution buffer acetonitrile - 0.1 M TEAA (*p*H 7); flow rate 1 mL/min; detected by UV at 260 nm.

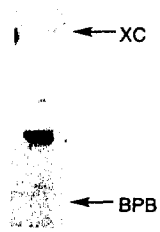


Fig. 7 Electrophoresis of the octamer (Fig. 6-II) on a 20% polyacrylamide gel containing 7 M urea, visualized by UV-shadowing.

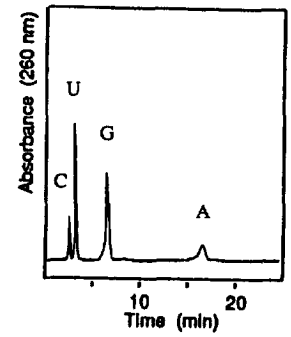


Fig. 8 Reversed-phase HPLC of products obtained by digestion of the completely unmasked octamer (Fig. 6-II) with snake venom phosphodiesterase and alkaline phosphatase.
Conditions of reversed-phase HPLC: column µBONDASPHERE 5µ C18 (3.9 mm ID x 150 mm L); elution buffer 2% acetonitrile - 0.04 M TEAA (*p*H 7); flow rate 1 mL/min; detected by UV at 260 nm.

R'=LMBz, LMNBz), TpTpT and TpTpTpT were synthesized, and HPLC tracings of each of the resulting mixtures from **8** (B=T, R=H, R'=LMBz) and **8** (B=T, R=H, R'=LMNBz) are shown in Figs. 1 - 4, respectively. Starting with **8** (B=G^{iBu}, R=OThp, R'=LMBz, LMNBz), an octaribonucleotide of UpCpApGp-UpUpGpG also was synthesized, and HPLC tracings of the mixtures, resulting from **8** (B=G^{iBu}, R=OThp, R'=LMBz) and **8** (B=G^{iBu}, R=OThp, R'=LMNBz) by consecutive treatments with concentrated ammoniacal water (for 3 h at room temperature and subsequently for 6 h at 55°C) and with hydrochloric acid (for 1 day at *p*H 2.0 at room temperature[13]) as usual, are shown in Figs. 5 and 6, respectively. Introduction of the nitro group to the 5-position of **3** is likely to have enhanced susceptibility of the LMNBz protecting group toward both processes of hydrazinolysis and deprotonation by imidazole, and excellence of the LMNBz group over the LMBz group was thus confirmed as could be seen from Figs. 1 - 6. The enzymatic degradation of these oligonucleotides gave satisfactory data for proving their structures (Fig. 8).

Therefore, it is clearly shown that the base-labile LMNBz protecting group for the 5'-position is of remarkably significant utility in oligonucleotide syntheses, judging from the above HPLC data.

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13. It was demonstrated by Reese and his co-workers [a) Griffin, B. E.; Reese, C. B. *Tetrahedron Lett.* **1964**, 2925 - 2931; b) Griffin, B. E.; Jarman, M.; Reese, C. B. *Tetrahedron* **1968**, *24*, 639 - 662.] that the Thp protecting group can be removed by treating with 0.01 M hydrochloric acid for 3 - 4 h at room temperature, and only 1 % of the 3'-5' phosphodiester linkage rearranged into the corresponding 2'-5' phosphodiester linkage after 216 h at 20°C.

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