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A novel phosphate protection for oligonucleotide synthesis: the 2-[(1-naphthyl)carbamoyloxy]ethyl (NCE) group

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Abstract

The utility of the 2-(arylcarbamoyloxy)ethyl group for protection of internucleosidic phosphate linkages in oligonucleotide synthesis was studied. Of the three protecting groups tested, the 2-[(1-naphthyl)carbamoyloxy]ethyl demonstrated high coupling yields, favorable deprotection kinetics and the highest hydrolytic stability of the thymidine phosphoramidite building block. The mechanism of deprotection was confirmed by deprotecting a model phosphate triester and synthetic dodecathymidylate. © 2000 Elsevier Science Ltd. All rights reserved.

For the past three decades, oligonucleotide synthesis on solid support has made tremendous progress from manual synthesis on a small-scale to the manufacturing of the first antisense drug, an anti-*CMV* 21-mer phosphorothioate oligonucleotide. Currently, oligonucleotide synthesis employs a phosphoramidite method[1] and is carried out automatically by stepwise coupling of nucleoside building blocks to a solid support. This allows an efficient preparation of natural[2] and modified[3] DNA fragments on a routine basis. Phosphoramidite building blocks are most often protected with a 2-cyanoethyl group at the phosphite moiety.[4] Treatment of the oligonucleotide with ammonia effects β-elimination in the 2-cyanoethyl group releasing internucleosidic phosphodiester moieties and acrylonitrile as a side product. Recently, acrylonitrile has been demonstrated to alkylate nucleic bases under conditions of deprotection.[5] Increasing interest in manufacturing oligonucleotides as drugs motivated our search for alternate protecting groups, whose removal is governed by mechanisms different from β-elimination.

More than 40 years ago, Winstein et al. studied base-catalyzed cyclization of 2-bromoethyl *N*-arylcarbamates to 3-aryl-1,3-oxazolidine-2-ones.[6] We hypothesized that this reaction might also occur with less efficient leaving groups e.g. the phosphodiester anion, which would provide a novel type of protection for the internucleosidic phosphate, via the 2-(arylcarbamoyloxy)ethyl group. Here we report the use of this protecting strategy in DNA synthesis by the phosphoramidite method.

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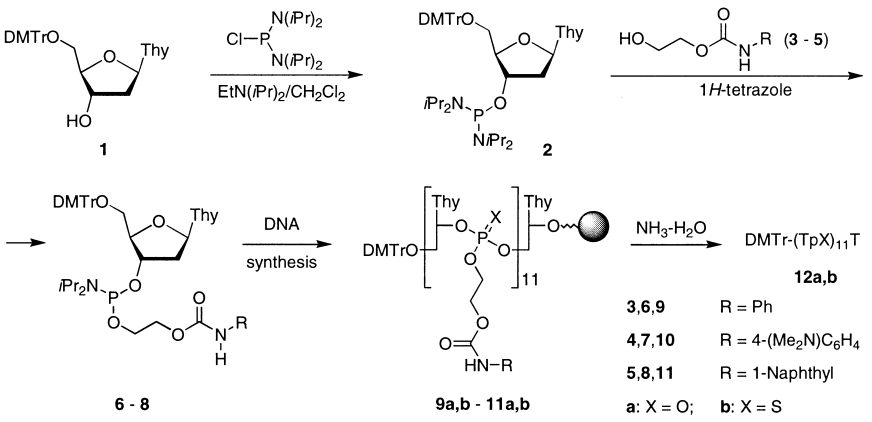


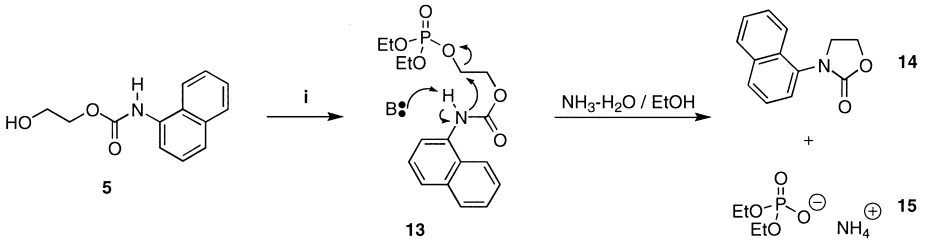
Scheme 1.

For the preparation of novel phosphoramidite building blocks, **1** was converted to a bisamidite **2**.[7] Without isolation, **2** was treated with **3**, **4**, or **5**[8] in the presence of 1*H*-tetrazole to give, after aqueous work-up, **6**–**8** (Scheme 1). These were isolated by column chromatography in 75–89% yield and characterized.[9] The phosphoramidites **6** and **8** were stable compounds whose shelf life exceeded 6 months. Moreover, as measured by ^{31}P NMR, the half-life of **8** in 95% aqueous MeCN was 235 h, which indicated its high hydrolytic stability. In contrast, **7** was light-sensitive and demonstrated a limited shelf life.

Next, the utility of the proposed protecting strategy for synthesis of oligonucleotides was studied. The model oligonucleotides **9**–**11** were assembled on a commercial solid support (2 μmol) employing 0.1 M of **6**–**8** in MeCN and the standard synthetic protocol (Scheme 1). When the synthesis was completed, aliquots of **9**–**11** were treated with conc. aqueous ammonium hydroxide at 25 or 55°C. On removal of the ammonia and extraction of the aqueous solution with CH_2Cl_2, the deprotection mixtures were analyzed by reverse phase HPLC–ESMS. The following observations are relevant to note. As judged by the DMTr-assay, all phosphoramidites demonstrated high coupling efficiency (>98%). Regardless of the oxidation protocol (I_2 for phosphodiester and 3*H*-1,2-benzodithiol-3-one 1,1-dioxide[10] for phosphorothioate oligonucleotides; X = O and S, respectively), all protecting groups demonstrated excellent stability towards the conditions of the chain assembly. Under treatment with ammonium hydroxide, the stability of the protecting groups increased as follows: 4-dimethylaminophenyl < 1-naphthyl < Ph (deprotection times of 14, 18, and 64 h at 25°C; 1, 1.5, and 6 h at 55°C for **10**, **11**, and **9**, respectively). The deprotection led exclusively to **12**, with no appreciably detectable nucleic base or backbone modifications occurring.

Taking into account the fact that the deprotection conditions for **10** and **11** were fairly similar and that the phosphoramidite **7** was less stable than **8**, the 2-[(1-naphthyl)carbamoyloxy]ethyl group (NCE) was therefore considered as the protection of choice. Indeed, the deprotection time for NCE is shorter than that for the standard base protecting groups (6 h at 55°C) and matched that of the *N*,*N*-dimethylformamidino group (2 h at 50°C or 1 h at 65°C).[11]

In order to illustrate the deprotection mechanism of the NCE group, a model compound **13** was synthesized (Scheme 2). Compound **5** (1 equiv.) was reacted with diethyl chlorophosphate (1.2 equiv.) in the presence of excess pyridine to give **13**, which was isolated by column chromatography in 86% yield.[12]



Scheme 2. (i) Diethyl chlorophosphate/Py/CH_2Cl_2

The reactivity of **13** under basic conditions was next studied. When treated with a mixture of conc. aqueous ammonium hydroxide and EtOH (4:1) at 55°C, **13** was completely consumed in 5 h. The reaction mixture was extracted with ethyl acetate and water, and **14** and **15** were isolated in quantitative yields from the organic and aqueous layers, respectively.[13] Similarly, when **11a** was synthesized and deprotected on a 5 μmol scale, the aqueous solution was extracted with CH_2Cl_2 to give **14** (10.5 mg, 90%), which was identical with the sample prepared according to Scheme 2 by HPLC and ^1H NMR. In both examples, formation of **14** demonstrated that the deprotection resulted from intramolecular cyclization of NCE where a phosphodiester group, either **15** or the internucleosidic phosphate in **11**, served as the leaving group.

To confirm the neutral character of NCE protection towards nucleic bases, mixtures of **13** (10 equiv.) and each of the 5′-DMTr protected nucleosides (dA, dC, dG, and T; 1 equiv.) were treated with ammonium hydroxide as described above for **13** alone. As evidenced by HPLC, both **14** and each of the nucleosides remained unchanged for at least 48 h at 55°C.

In conclusion, the 2-[(1-naphthyl)carbamoyloxy]ethyl (NCE) is a novel, convenient phosphodiester protecting group that is stable towards ancillary reagents used in DNA synthesis. NCE is quantitatively removed under standard deprotection conditions to release an internucleosidic phosphate and a neutral, inert by-product.

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8. Ethylene glycol (6.45 g, 10.4 mmol) and 1-naphthyl isocyanate (4.40 g, 26 mmol) were reacted overnight in MeCN. On evaporation, water (20 mL) was added, and the product was extracted with ethyl acetate (5×20 mL), dried, and evaporated. The residue was re-crystallized to give **5** (5.59 g 93%): m.p. 102.5–103°C (toluene). ^{1}H NMR (DMSO-d_6): δ 9.59 (1H, s), 8.10 (1H, m), 7.93 (1H, m), 7.74 (1H, m), 7.6–7.4 (4H, m), 4.87 (1H, t, J = 5.3 Hz), 4.16 (2H, t, J = 5.2 Hz), 3.67 (2H, m). ^{13}C NMR ($CDCl_3$): δ 155.1, 134.1, 132.3, 128.7, 126.3, 126.1, 125.8, 125.5, 120.7, 67.2, 61.5. FAB HRMS: calcd for $C_{17}H_{23}NO_6$ (M+Na$^+$): 254.0793; found: 254.0795.
9. Chloro bis[(*N*,*N*-diisopropyl)amino]phosphite (1.28 g, 4.8 mmol) in CH_2Cl_2 (5 mL) was added to **1** (2.18 g, 4.0 mmol) and Et*N*(*i*Pr)$_2$ (1.0 mL, 5.2 mmol) in CH_2Cl_2 (10 mL) at –20°C. This was kept at rt for 1 h and then treated with **5** (1.11 g, 4.8 mmol) and 1*H*-tetrazole (140 mg, 2.0 mmol). After 2 h, 5% aqueous $NaHCO_3$ and brine were added. The product was extracted with ethyl acetate (3×50 mL), dried, and evaporated. Separation on a silica gel column using a gradient from 20:75:5 to 50:45:5 ethyl acetate:hexane:Et_3N gave **8**, fast diastereomer (0.51 g), **8**, slow diastereomer (0.45 g), and their mixture to total in 3.21 g (88.8%) of **8**. Compound **8**, fast diastereomer: ^{1}H NMR ($CDCl_3$): δ 8.89 (1H, br. s), 7.92–7.20 (17H, m), 7.07 (1H, s), 6.84–6.76 (4H, m), 6.42 (1H, m), 4.71 (1H, m), 4.45–4.16 (3H, m), 3.75 (6H, s), 3.90–3.30 (6H, m), 2.55 (1H, m), 2.36 (1H, m), 1.42 (3H, s), 1.19 (12H, d, J = 5.5 Hz). ^{13}C NMR ($CDCl_3$): δ 163.9, 158.7, 154.3, 150.4, 144.4, 135.7, 135.5, 134.1, 132.5, 130.2, 128.7, 128.2, 128.0, 127.2, 126.2, 126.0, 125.8, 125.2, 120.7, 113.3, 111.2, 86.9, 85.8, 84.9, 73.6, 73.3, 65.4, 65.3, 63.1, 61.8, 61.5, 55.3, 43.3, 43.1, 40.1, 24.7, 24.6, 11.8. ^{31}P NMR ($CDCl_3$): δ 148.9. FAB HRMS: calcd for $C_{50}H_{57}N_4O_{10}P$ (M+Na$^+$): 927.3710; found: 927.3716. Compound **8**, slow diastereomer: ^{1}H NMR ($CDCl_3$): δ 7.94–7.76 (3H, m), 7.70–7.18 (16H, m), 6.88–6.76 (4H, m), 6.42 (1H, dd, J = 7.9, 5.9 Hz), 4.67 (1H, m), 4.50–4.15 (3H, m), 3.77 (6H, s), 4.0–3.25 (8H, m), 2.63 (1H, ddd, J = 13.5, 4.0, ≈1 Hz), 2.32 (1H, m), 1.41 (3H, s), 1.17 (6H, d, J = 6.8 Hz), 1.08 (6H, d, J = 6.8 Hz). ^{13}C NMR ($CDCl_3$): δ 163.8, 158.7, 154.5, 150.4, 144.4, 135.7, 135.5, 135.4, 134.1, 132.7, 130.1, 128.6, 128.2, 128.0, 127.2, 126.2, 126.0, 125.8, 125.2, 120.9, 113.3, 111.2, 86.9, 85.7, 85.5, 85.0, 74.1, 73.8, 65.4, 65.2, 63.4, 61.8, 61.5, 55.3, 43.2, 43.0, 40.1, 24.7, 24.5, 11.8. ^{31}P NMR ($CDCl_3$): δ 148.5. FAB HRMS: calcd for $C_{50}H_{57}N_4O_{10}P$ (M+Na$^+$): 927.3710; found: 927.3714.
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12. Compound **13**: m.p. 52–53°C. ^{1}H NMR ($CDCl_3$): δ 7.88 (3H, m), 7.69 (1H, m), 7.54 (3H, m), 7.11 (1H, s), 4.44 (2H, m), 4.31 (2H, m), 4.13 (4H, sept., J = 7.2 Hz), 1.33 (6H, t, J = 7.2 Hz). ^{13}C NMR ($CDCl_3$): δ 154.1, 134.1, 132.4, 128.7, 127.7, 126.3, 126.1, 125.8, 125.4, 120.7, 65.5, 65.4, 64.1, 16.2, 16.1. ^{31}P NMR ($CDCl_3$): δ –1.3. FAB HRMS: calcd for $C_{17}H_{22}NO_6P$ (M+H$^+$): 368.1263; found: 368.1262.
13. Compound **14**: m.p. 125–126°C (water). ^{1}H NMR ($CDCl_3$): δ 7.88 (3H, m), 7.48 (4H, m), 4.66 (2H, m), 4.11 (2H, m). ^{13}C NMR ($CDCl_3$): δ 157.5, 134.7, 134.1, 130.0, 128.8, 127.0, 126.6, 125.7, 124.6, 122.4, 62.5, 49.1. FAB HRMS: calcd for $C_{13}H_{11}NO_2$ (M+H$^+$): 214.0868; found: 214.0870. Compound **15** (ammonium salt): ^{1}H NMR (D_2O): δ 3.79 (4H, sept., J = 7.2 Hz), 1.12 (6H, t, J = 7.2 Hz). ^{31}P NMR (D_2O): δ –1.8.