Synthetic Communications: An International Journal for Rapid Communication of Synthetic Organic Chemistry
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/lsyc20

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Published online: 04 Dec 2007.

To cite this article: John Scheigetz & Bruno Roy (2000) Synthesis of Fluorescein Phosphorotriesters Using Photolabile Protecting Groups, Synthetic Communications: An International Journal for Rapid Communication of Synthetic Organic Chemistry, 30:8, 1437-1445, DOI: 10.1080/00397910008087172

To link to this article: http://dx.doi.org/10.1080/00397910008087172

SYNTHETIC COMMUNICATIONS, 30(8), 1437–1445 (2000)

SYNTHESIS OF FLUORESCEIN PHOSPHOROTRIESTERS USING PHOTOLABILE PROTECTING GROUPS

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Abstract: Fluorescein phosphorotriesters **5**, each having four identical photoactivatable adducts have been synthesized to investigate their applications as intracellular fluorescence indicators.

The design and applications of photoprotecting groups for phosphates[1] generated our interest in developing a whole cell assay based on the controlled release of a photolabile precursor. The possibility of combining this feature with a highly sensitive fluorescence technique[2] led us to investigate the synthesis of photoprotected fluorescein-3,6-diphosphate (FDP). In an earlier publication,[3] we described the synthesis of FDP and related analogs but those products did not penetrate cell membranes. Using different photochemically labile protecting groups for phosphates, both Nerbonne[4] and Iwamura[5] have demonstrated cell-permeability. A selection of suitable protecting groups for phosphates and for photolabile properties desired was made based on a review of the literature. The 2-nitrobenzyl (NB) and the 1-(2-nitrophenyl)ethyl (NPE) groups can be photolysed at ≤ 360 nm. 4,5-dimethoxy-2-nitrobenzyl (DMNB) and 1-(4,5-dimethoxy-2-nitrophenyl)ethyl (DMNPE) have a longer wavelength absorption maximum ≥ 360

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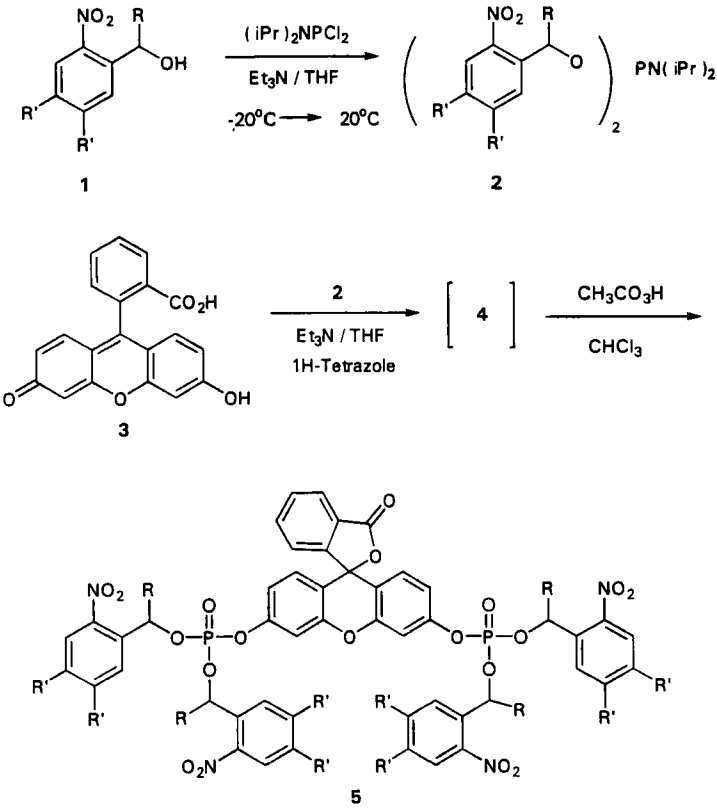
nm. The NPE adducts are known to photolyse 20 times more rapidly than the NB adducts[6] and also, the photo-by-product 2-nitrosoacetophenone generated from an NPE adduct is known to be less toxic to biological preparations than the 2-nitrosobenzaldehyde arising from an NB adduct.

RESULTS AND DISCUSSION

Due to the sensitive nature of FDP, its phosphates cannot be functionalized directly using any known methodology. The general sequence requires preparations of the appropriate phosphitilating agents containing two identical photolabile adducts resulting in FDP analogs having four identical photoprotecting groups each (Scheme 1). Alcohols 1-(2-nitrophenyl) ethanol **1b** was prepared in 89% yield by the reduction of 2-nitroacetophenone with DIBAL-H and 1-(4,5-dimethoxy-2-nitrophenyl) ethanol **1c** was obtained in 61% yield by the addition of methyl lithium to 4,5-dimethoxy-2-nitrobenzaldehyde. The syntheses of **2** were based on literature procedures.[7,8,9] Fluorescein **3** lactonized under the reaction conditions and underwent a bisphosphitilation. A subsequent oxidation of the bisphosphite intermediate **4** to the more stable bisphosphate **5** enabled isolation and purification of the desired photoprotected FDP. Benzyl phosphites have been reported to be sensitive to certain oxidation systems[9] and here, 3-chloroperoxybenzoic acid was found to be unsuitable for the oxidation of the phosphites of the 1-(2-nitrophenyl)ethyl **b** and 1-(4,5-dimethoxy-2-nitrophenyl)ethyl **c** groups. Introduction of the α-methyl group to the 2-nitrobenzyl group would have greatly increased electropositivity at the benzylic carbon such that groups **b** and **c** were activated to cleavage on generation of the phosphonium intermediate and especially in the presence of 3-chlorobenzoic acid. Product yields increased from 5% to 60% when 32% peracetic acid was used as an oxidant. Although other oxidants were not investigated here, *bis* (trimethylsilyl)peroxide and t-butyl hydroperoxide should also be suitable for the oxidation of α-methyl benzyl phosphites since these oxidants do not generate an acidic by-product.[10]

Scheme 1



a, R =H, R' =OMe; b, R =Me, R' =H; c, R =Me, R' =OMe

In conclusion, the present method provides a route to some photoprotected fluorescein diphosphates but is not general. Other photolabile adducts such as benzoins and coumarins failed to provide the phosphoramidites necessary to convert the fluorescein to their disubstituted phosphorotriesters. The unique substrates **5** that were synthesized here were found to be cell-permeable and the detail of these results will be reported in a future publication.

EXPERIMENTAL SECTION

Melting points were measured by use of a Buchi B-540 apparatus in open capillary tubes and are uncorrected. NMR spectra were obtained on Bruker AMX500 and Bruker ARX400 spectrometers and proton chemical shifts are relative to TMS as an internal standard. Analytical thin-layer chromatography (TLC) was routinely monitored on precoated Analtech glass sheets (Silica Gel GF, 0.25 mm thick) and detection was effected using an 8% cerium molybdate solution in 15% sulfuric acid. Elemental analyses were performed at the University of Montreal Laboratories, Montreal, Quebec. Mass spectral analyses were provided by Chun Li of these laboratories. Diisopropylphosphoramidous dichloride was prepared following a literature procedure.[7,8] 4,5-dimethoxy-2-nitrobenzyl alcohol and fluorescein (95%) were purchased from Aldrich Chemical Co. and used without further purification.

1-(2-Nitrophenyl)ethanol (1b)

To a -20°C cooled solution of 2-nitroacetophenone (2 g, 12.1 mmol) in THF (50 mL) and under nitrogen atmosphere was added dropwise DIBAL-H (18.2 mL of 1M in toluene, 18.2 mmol). The mixture was stirred at 0°C for 15 min. and then quenched dropwise with saturated NH_4Cl (40 mL) followed by the addition of EtOAc (100 mL). The organic layer was separated, dried (Na_2SO_4), filtered and concentrated to obtain 1.8 g (89%) of **1b** as a yellow oil.

^1H NMR (500 MHz, $CDCl_3$): δ 7.88 (d, 1H), 7.82 (d, 1H), 7.63 (t, 1H), 7.40 (t, 1H), 5.42 - 5.37 (m, 1H), 2.32 (d, 1H), 1.56 (d, 3H).

1-(4,5-Dimethoxy-2-nitrophenyl)ethanol (1c)

To a -70°C cooled solution of 4,5-dimethoxy-2-nitrobenzaldehyde (4.9 g, 23.2 mmol) in THF (250 mL) and under nitrogen atmosphere was added dropwise methyl lithium (22.3 mL of 1.25 M in diethyl ether, 27.9 mmol). The mixture was stirred at -70°C for 15 min. and then

quenched dropwise with saturated NH_4Cl (50 mL), followed by the addition of EtOAc (200 mL). The organic layer was separated, dried (Na_2SO_4), filtered, concentrated and the residue purified by chromatography on silica gel (EtOAc/Hex 2:3) to obtain 2.7 g (61%) of **1c**; m.p. 150-153°C.

^{1}H NMR (500 Mz, acetone-d_6): δ 7.53 (s, 1H), 7.45 (s, 1H), 5.47 - 5.42 (m, 1H), 4.49 (d, 1H), 3.94 (s, 3H), 3.90 (s, 3H), 1.43 (d, 3H). Anal. Calcd (Found) for $C_{10}H_{13}NO_5$: C, 52.86 (53.29); H, 5.77 (5.96); N, 6.16 (6.21).

***bis*(4,5-Dimethoxy-2-nitrobenzyl)-N,N-diisopropylphosphoramidite (2a)**

To a -20°C cooled suspension of 4,5-dimethoxy-2-nitrobenzyl alcohol **1a** (10 g, 46.9 mmol) and triethylamine (7.4 mL, 51.7 mmol) in dry THF (170 mL) was added dropwise a solution of diisopropylphosphoramidous dichloride (4.7 g, 23.5 mmol) in dry THF (10 mL). The mixture was allowed to warm to 20°C, stirred for 18 h, and a saturated solution of aq. $NaHCO_3$ (75 mL) added. The solid was filtered, washed with water (20 mL) and dried to give 8.3g (64%) of **2a**.

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.72 (s, 2H), 7.39 (s, 2H), 5.3 - 5.1 (m, 4H), 3.95 (d, 12H), 3.85 - 3.70 (m, 2H), 1.27 (d, 12H).

***bis*[1-(2-Nitrophenyl)ethyl]-N,N-diisopropylphosphoramidite (2b)**

A mixture of 1-(2-nitrophenyl)ethanol **1b** (1.8 g, 10.8 mmol), dry THF (10 mL), triethylamine (2.0 mL, 13.8 mmol) and diisopropylphosphoramidous dichloride (1.2 g, 6.2 mmol) in dry THF was reacted as described for the preparation of **2a**. After the addition of a saturated solution of aq. $NaHCO_3$ (15 mL), the product was extracted with ethyl acetate (20 mL), dried (Na_2SO_4), filtered and concentrated. Purification by chromatography on silica gel (Et_3N/EtOAc/Toluene 1:2.5:96.5) gave 2 g (80%) of **2b** as a yellow solid; m.p. 78-81°C.

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.82 - 7.55 (m, 3H), 7.52 - 7.36 (m, 3H), 7.32 - 7.18 (m, 2H), 5.42 - 5.23 (m, 2H), 3.6 - 3.5 (m, 1H), 3.46 - 3.35 (m, 1H), 1.47 (d, 2H), 1.34 (d, 4H), 1.07 (d, 6H), 1.03 (d, 3H), 0.82 (d, 3H). Anal. Calcd (Found) for $C_{22}H_{30}N_3O_6P$: C, 57.02 (56.84); H, 6.48 (6.50); N, 9.07 (8.98).

***bis*[1-(4,5-Dimethoxy-2-nitrophenyl)ethyl]-N,N-diisopropylphosporamidite (2c)**

To a –40°C cooled solution of diisopropylphosphoramidous dichloride (1.2 g, 5.9 mmol) in dry THF (2.5 mL) was added dropwise a solution of 1-(4,5-dimethoxy-2-nitrophenyl)ethanol **1c** (2.6 g, 11.4 mmol) and triethylamine (1.8 mL, 12.7 mmol) in dry THF (15 mL). The cooling bath was then removed and the mixture stirred for 18 h. Suspended triethylamine hydrochloride was filtered off and washed with THF (5 mL). To the filtrate was added silica gel (30 g) and the solvent evaporated to preadsorb the mixture. The product was isolated by chromatography on silica gel (Et_3N/EtOAc/Toluene 2:5:93) and gave 2.7 g (78%) of **2c** as a yellow solid; m.p. 151-154°C.

^{1}H NMR (500 MHz, Acetone-d_6): δ 7.43 (s, 2H), 7.11 (s, 2H), 5.60 - 5.52 (m, 2H), 3.91 (s, 6H), 3.87 - 3.78 (m, 2H), 3.80 (s, 6H), 1.42 (d, 6H), 1.24 (d, 12H). Anal. Calcd (Found) for $C_{26}H_{38}N_3O_{10}P$: C, 53.51 (53.36), H, 6.56 (6.71), N, 7.20 (7.25).

3′,6′-*bis*[*bis*(4,5-Dimethoxy-2-nitrobenzyloxy)phosphoryloxy]spiro[isobenzofuran-1(3H),9′-[9H]xanthen]-3-one (5a)

A mixture of fluorescein **3** (332 mg, 1 mmol), THF (45 mL), *bis* (4,5-dimethoxy-2-nitrobenzyl)N,N-diisopropylphosphoramidite **2a** (1.66 g, 3 mmol), triethylamine (0.56 mL, 4 mmol), and 1H-tetrazole (700 mg, 10 mmol) was refluxed for 5 h in a darkened hood. An aliquot was added to a soln of 2 drops of 32% peracetic acid in chloroform and stirred for 15 min. TLC eluting with 3% methanol in dichloromethane showed the non highly fluorescent

fluorescein *bis*(dibenzylphosphate) **5a** at R_f 0.7. The reaction mixture was cooled to r.t. and concentrated. The residue was diluted in $CHCl_3$ (50 mL), cooled to 5°C and 32% peracetic acid (1.0 mL, 4.6 mmol) added dropwise. The cooling bath was removed and after stirring for 1h, saturated $NaHCO_3$ (40 mL) was added. The partitioned organic phase was washed with more saturated $NaHCO_3$ (2x20 mL), dried ($NaSO_4$), filtered and concentrated. Purification obtained by successive chromatography on silica gel (MeOH/CH_2Cl_2 3:97) R_f 0.7 and (EtOAc/Hex 3:1) R_f 0.5 gave 753 mg (60%) of **5a** as a white foam after stripping with ether.

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.02 (d, 1H), 7.69 - 7.64 (m, 2H), 7.68 (s, 4H), 7.19 (d, 2H), 7.14 (d, 1H), 7.10 (s, 4H), 6.95 (dd, 2H), 6.7 (d, 2H), 5.64 (d, 8H), 3.92 (s, 12H), 3.89 (s, 12H). MS (FAB$^+$): m/z = 1273 $(M+H)^+$, 1078 $(M+H - C_9H_9NO_4)^+$, 493 $(M+H-4C_9H_9NO_4)^+$. Anal. Calcd (Found) for $C_{56}H_{50}N_4P_2O_{27}$: C, 52.84 (52.35); H, 3.96 (4.10); N, 4.40 (4.11).

3′,6′-*bis*[*bis*(1-(2-Nitrophenyl)ethyloxy)phosphoryloxy]spiro[isobenzofuran-1(3H), 9′-[9H]xanthen]-3-one (5b)

A mixture of fluorescein **3** (406 mg, 1.22 mmol) THF (55 mL), *bis* [1-(2-nitrophenyl)ethyl]-N,N-diisopropylphosphoramidite **2b** (1.7 g, 3.67 mmol) triethylamine (0.7 mL, 4.88 mmol) and 1H-tetrazole (8.54 mg, 12.2 mmol) was reacted and oxidized as described for the preparation of **5a**. Purification obtained by successive chromatography on silica gel (CH_2Cl_2/EtOAc/Toluene 2:5:17.5:5:80) R_f 0.33 and (EtOAc/Hex 1/1) R_f 0.35 gave 757 mg (57%) of **5b** as a white foam.

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.91 (d, 1H), 7.87 - 7.77 (m, 3H), 7.71 (d, 1H), 7.61 - 7.42 (m, 10H), 7.37 - 7.23 (m, 4H), 7.02 - 6.97 (m, 1H), 6.95 - 6.88 (m, 1H), 6.78 - 6.63 (m, 2H), 6.60 - 6.44 (m, 3H), 6.10 - 5.96 (m, 4H), 1.63 - 1.46 (m, 12H). MS (FAB$^+$): m/z = 1089 $(M+H)^+$, 940 $(M+H - C_8H_7NO_2)^+$, 493 $(M+H-4C_8H_7NO_2)^+$. Anal. Calcd (Found) for $C_{52}H_{42}N_4P_2O_{19}$: C, 57.36 (57.24); H, 3.89 (4.02); N, 5.15 (5.08).

3′,6′-*bis*[*bis*(1-(4,5-Dimethoxy-2-nitrophenyl)ethyloxy)phosphoryloxy]spiro[isobenzofuran-1(3H), 9′-[9H]xanthen]-3-one (5c)

A mixture of fluorescein **3** (436 mg, 1.31 mmol), THF (60 mL), bis [1-(4,5-dimethoxy-2-nitrophenyl)ethyl]-N,N-diisopropylphophoramidite **2c** (2.3 g, 3.94 mmol), triethylamine (0.74 mL, 5.2 mmol) and 1H-tetrazole (917 mg, 13.1 mmol) was reacted and oxidized as described for the preparation of **5a**. Purification obtained by successive chromatography on silica gel (CH_2Cl_2/EtOAc/Toluene 5:35:60) R_f 0.30 and (EtOAc/Hex 2:1) R_f 0.35 gave 1.04 g (60%) of **5c** as a white foam.

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.91 (d, 1H), 7.63 - 7.50 (m, 2H), 7.45 - 7.38 (m, 2H), 7.35 - 7.28 (m, 2H), 7.10 - 7.02 (m, 1H), 6.97 - 6.72 (m, 6H), 6.7 - 6.52 (m, 4H), 6.26 - 6.11 (m, 4H), 3.85 - 3.70 (m, 24H), 1.6 - 1.41 (m, 12H). MS (FAB^+): m/z = 1329 $(M+H)^+$, 1120 $(M+H-C_{10}H_{11}NO_4)^+$, 493 $(M+H-4C_{10}H_{11}NO_4)^+$. Anal. Calcd (Found) for $C_{60}H_{58}N_4O_{27}P_2$: C, 54.20 (53.70); H, 4.37 (4.60); N, 4.22 (4.12).

Acknowledgement.: The authors gratefully acknowledge Dr. Quingping Wang of the department of Biochemistry and Molecular Biology at Merck Frosst for helpful discussions.

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(Received in the USA 13 August 1999)