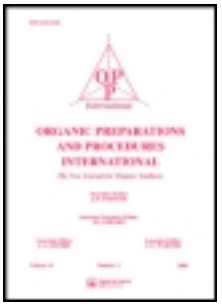

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SYNTHESIS OF FLUORESCEIN PHOSPHATES AND SULFATES

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SYNTHESIS OF FLUORESCEIN PHOSPHATES AND SULFATES

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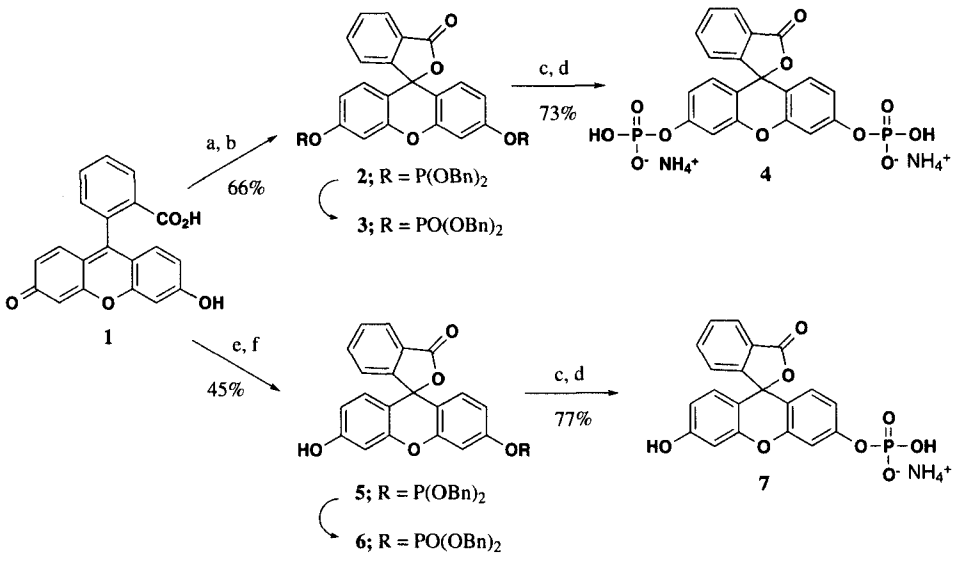
The sensitivity of fluorescence techniques makes them attractive for various enzyme assays and the phosphate derivatives of fluorescein are ideally suited in these assays due to their high extinction coefficients and fluorescence quantum yields[1]. Reported preparations of fluorescein diphosphate (FDP) by phosphorylation of fluorescein with phosphorus oxychloride in pyridine were found to be very inefficient and led to low yields of uncharacterized FDP.[2,3] The tetrazole promoted phosphitilation and subsequent oxidation method developed by Johns[4,5] for phosphorylation of alcohols was successfully applied to fluorescein. Mild acidolytic cleavage of *tert*-butyl groups[6,7] prompted a synthesis of FDP using di-*tert*-butyl N,N-diethylphosphoramidite[5] as the phosphitilating reagent. However, hydrolysis of the FDP precursor, fluorescein *bis*(di-*tert*-butylphosphate) generated about 20% fluorescein monophosphate (FMP) contamination and other fluorescein related impurities which were extremely difficult to remove. Tedious chromatographies on cellulose with small quantities of mixture gave FDP of unacceptable quality for our purposes. We now report a method for preparing gram quantities of FDP (**4**) and FMP (**7**) of sufficient quality for enzymatic and kinetic studies. We also describe the synthesis of the corresponding sulfate analogs of fluorescein, FMS (**10**) and FDS (**11**) and the mixed sulfate-phosphate derivative FMSP (**13**).

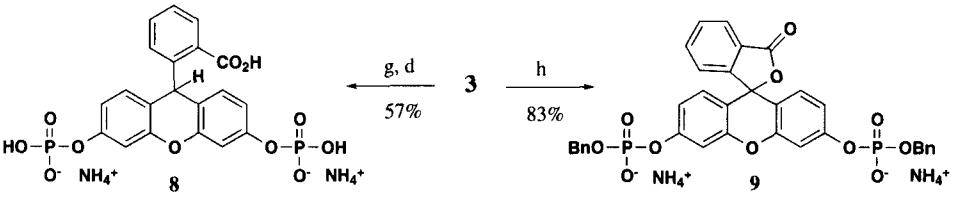
Instead of a hydrolysis method for the deprotection step, the FDP precursor **3** was synthesized with benzyl groups which could be removed under mild reduction conditions. Dibenzyl diisopropylphosphoramidite[8,9] was used to phosphitilate fluorescein. The resulting fluorescein bisphosphite intermediate **2** is not stable on silica gel and must be oxidized and purified as its bisphosphate **3**. Any **2** or partially oxidized **2** in the mixture will degrade during chromatography to produce fluorescein dibenzylphosphate **6** and fluorescein **1**. Fluorescein *bis*(dibenzylphosphate) (**3**) was then hydrogenolyzed to the fluorescein *bis*(dihydrogenphosphate) (**4**) which is very sensitive to acids, bases, heat and water and thus was converted to its more stable diammonium salt. The salt was then dissolved in water, filtered through Celite to remove the catalyst, lyophilized and was recrystallized (78%); it was shown to be greater than 98% pure by NMR and enzyme assays. Residual amounts of ammonium acetate could be sublimed out by prolonged pumping on the lyophilizer. While the reductive cleavage failed below -25°, at temperatures higher than 5°, the spiro lactone ring of **3** was



a) $(BnO)_2PN(iPr)_2$, (2.2 eq), Et_3N, Tetrazole, THF, 65°, 2.5 h b) MCPBA (3 eq), 0° to r.t., 30 min.
c) H_2, Pd / C, EtOH, -5°, 2 h d) NH_4OAc / EtOH e) $(BnO)_2PN(iPr)_2$, (1.1 eq), Et_3N, Tetrazole, THF, 65°, 2.5 h
f) MCPBA (1.6 eq), 0° to r.t., 30 min.

Scheme 1

reduced to 2-[3,6-*bis*(phosphonooxy)-9H-xanthen-9-yl]benzoic acid (**8**) (Scheme 2). This ring opened product **8** was identified by the presence of a singlet for the methine proton at δ 6.15. It is also interesting to note that when the reduction was carried out in the presence of ammonium acetate, only one benzyl from each phosphate was cleaved to give 3',6'-*bis*(benzyloxyhydroxyphosphoryloxy)spiro[isobenzofuran-1(3H),9'-[9H]xanthen]-3-one diammonium salt (**9**) even after a prolonged reduction period with addition of extra catalyst and higher temperature.



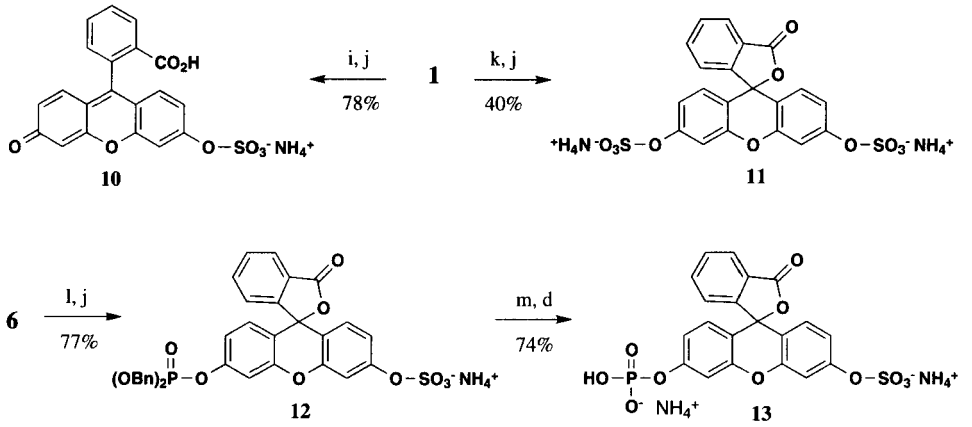
g) H_2, Pd / C, EtOH, 20°, 2h h) H_2, Pd / C, EtOH, NH_4OAc, -5°, 2 h

Scheme 2

By using the same conditions as for the synthesis of **3** but with 1.1 equivalents of dibenzyl diisopropylphosphoramidite, fluorescein dibenzylphosphate **6** was obtained in 45% yield after oxidation of the intermediate fluorescein monophosphite derivative **5** and purification from any bisproduct **3**. The isolated product **6** exists in the phenol-lactone form with a characteristic orange color and long wavelength visibility due to the conjugation involved. Reductive cleavage of the benzyl groups was carried

out as for FDP but the FMP ammonium salt failed to crystallize out. The ethanol was first pumped off and then the FMP was dissolved in water, filtered, and lyophilized. The FMP was then recrystallized from 10% ethanol in 2-propanol to afford a 77% yield for the reduction step. The large solubility difference between FDP and FMP in ethanol allowed for isolation of FDP, free from FMP contamination.

Fluorescein monosulfate (**10**) was synthesized using chlorosulfonic acid and a base such as N,N-dimethylaniline[10] which did not promote ring closure to the spiro lactone (Scheme 3). Without ring closure, there is only one phenolic group available for sulfation. The fluorescein sulfates are much more stable to silica gel than the fluorescein phosphates and could be chromatographed. The 4-(dimethylamino)benzenesulfonic acid which was also formed, was readily eluted as its ammonium



i) $C_6H_5N(CH_3)_2$, $ClSO_3H$, $CHCl_3$, 65°, 18 h j) NH_4OH k) Et_3N, $ClSO_3H$, $CHCl_3$, 65°, 18 h
l) Et_3N, $ClSO_3H$, $CHCl_3$, 65°, 2 h m) H_2, Pd / C, EtOH, $CHCl_3$, -15°, 1 h

Scheme 3

salt under the chromatography conditions. The resulting fluorescein monosulfate aniline salt, when chromatographed with ammonium hydroxide:methanol:chloroform (1:4:8), gave the FMS ammonium salt in 78% yield and had the characteristic ^1H NMR spectrum of a monosubstituted fluorescein molecule. When the identical reaction was carried out with triethylamine as base, fluorescein disulfate diammonium salt (**11**) was obtained in 40% yield.

Fluorescein monosulfate phosphate diammonium salt (**13**) was synthesized from the intermediate fluorescein dibenzylphosphate **6** (Scheme 3). **6** was reacted with chlorosulfonic acid and excess triethylamine maintaining closure of the lactone ring and the phenol was sulfated. Isolation, chromatography and lyophilization gave fluorescein dibenzylphosphate monosulfate ammonium salt (**12**) in 77% yield. Reductive removal of the benzyls required a 1:1 chloroform:ethanol mixture to solubilize the salt but the presence of chloroform seemed to promote reductive opening of the spiro lactone at -5°. At -15° this did not occur. It is also interesting to note here that the reduction was carried out on an ammonium salt. In the presence of ammonium acetate, only one benzyl was reduced off each phosphate of **3** as was observed for the reduction of **3** to **9**. **12** was lyophilized before reduc-

tion and so no excess ammonia was present. The stronger acidity of the sulfate group may have prevented an equilibration of its ammonia counterion with the phosphate group during reduction. Both benzyls were hydrogenolyzed and after workup with ammonium acetate, the lyophilized yield of FMSP was 74% for the reduction step.

EXPERIMENTAL SECTION

Melting points were measured by use of a Buchi 510 apparatus in open capillary tubes and are uncorrected. NMR spectra were obtained on Bruker AMX500, Bruker ARX400 and Varian Gemini 200 spectrometers and proton chemical shifts are relative to TMS as an internal standard. NMR assignments were obtained by heteronuclear multiple quantum correlation (HMQC) and heteronuclear multiple bond correlation (HMBC) experiments.. 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 by Guelph Chemical Laboratories, Guelph, Ontario. Mass spectral analyses were provided by Dr. J. Yergey and Chun Li of these laboratories and by the Biomedical Mass Spectrometry Unit at McGill University, Montreal, Quebec. Dibenzyl diisopropylphosphoramidite and fluorescein (95%) were purchased from Aldrich Chemical Co. and used without further purification.

3',6'-*bis*[*bis*(Benzyloxy)phosphoryloxy]spiro[isobenzofuran-1(3H),9'-[9H]xanthen]-3-one (3).- To a suspension of fluorescein (7.5 g, 22.5 mmol) in THF (150 mL) was added dibenzyl diisopropylphosphoramidite (17 g, 49.5 mmol), triethylamine (3 g, 30 mmol) and 1H-tetrazole (4.0 g, 58.5 mmol). The mixture was stirred in an oil bath at 65° for 2.5 h. An analytical sample of the solution was diluted with chloroform and treated with excess m-chloroperoxybezoic acid (MCPBA). TLC eluting with 3% methanol in dichloromethane showed the non highly fluorescent fluorescein *bis*(dibenzylphosphate) **3** at R_f 0.7 under short wavelength. The mixture was cooled, filtered and the filtrate concentrated. The crude intermediate 3',6'-*bis*[*bis*(benzyloxy)phosphanyloxy]spiro[isobenzofuran-1(3H),9'-[9H] xanthen]-3-one (**2**) mixture was taken up in chloroform (200 mL) and cooled to 0°. MCPBA, 55% quality (21 g, 67.5 mmol) was added in portions and then the mixture stirred for 30 min. at 20°. The mixture was diluted with more chloroform (200 mL) and washed with 1.5 M Na_2SO_3 (2 x 125 mL), saturated $NaHCO_3$ (100 mL), dried (Na_2SO_4), filtered and the filtrate concentrated. Purification on silica gel required two chromotographies. Elution with 10% ethyl acetate in dichloromethane separated fluorescein *bis*(dibenzylphosphate) **3** from the more polar fluorescein dibenzylphosphate **6** by-product. A subsequent chromatography eluting with 50% ethyl acetate in hexane R_f 0.5 yielded 12.7 g (66%) of the title compound **3** as a colorless syrup. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.06 (d, 1H), 7.83 (t, 1H), 7.77 (t, 1H), 7.29 - 7.43 (m, 21H), 7.20 (d, 1H), 6.97 (dd, 2H), 6.86 (d, 2H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 168.3, 152.0, 151.47, 151.42, 150.8 (2), 135.9, 135.8, 135.4, 135.3, 130.5, 129.6 - 127.3 phenyls, 125.3, 124.9, 123.9, 116.66, 116.62, 115.6 (2), 108.27, 108.23, 80.6, 69.67 ($^2J_{cp}$ = 5.7 Hz). LRMS (APCI): m/z = 853 $(M + 1)^+$.

Anal. Calcd. for $C_{48}H_{38}O_{11}P_2$: C, 67.60; H, 4.49; P, 7.26. Found: C, 67.56; H, 4.44; P, 7.08

3',6'-*bis*(Phosphonooxy)spiro[isobenzofuran-1(3H),9'-[9H]xanthen]-3-one Diammonium Salt

(FDP) (4).- Fluorescein *bis*(dibenzylphosphate) **3** (5.2 g, 6.1 mmol) was dissolved in ethanol (240 mL) and cooled to -5° internal temperature using an ice-ethanol bath. Catalyst, 5% Pd/C (500 mg) was added and the mixture stirred for 1.5 h under a slight positive pressure of H_2 gas using a balloon. The reduction was followed by TLC using 60% ethyl acetate in hexane. When no more starting material was evident and only the product spot observed at the baseline, 8 equivalents of ammonium acetate (3.75 g, 50 mmol) dissolved in ethanol (20 mL) were added at such a rate as not to cause a surge in temperature. The solution was stirred for 1h at -5°. The precipitate was filtered off on a filter paper and washed with a little cold ethanol. The black solid was dissolved in water (50 mL) and passed through a Celite plug to remove the palladium catalyst. The solution was then pumped on at room temperature for a short while to diminish the quantity of remaining ethanol and then lyophilized to obtain 3.0 g of a yellow colored solid. The crude FDP solid was prepurified by stirring at r.t. for 30 min. with enough ethanol to mobilize all the solid, filtering, washing with some ethanol and twice with Et_2O. The solid was transferred as soon as possible to a flask before it could pick up too much moisture and pumped on to obtain 2.8 g of partially purified FDP. The solid was crystallized using a ratio of 7.5 mL of methanol per gram of FDP and using the following method. Methanol (21 mL) was added to the 2.8 g of FDP and the mixture stirred in a closed flask at r.t. for 2h. The material forms a gum initially but gradually becomes crystalline. The mixture was then diluted slowly with ethanol (84 mL), cooled to 0° and stirred for 30 min. The temperature was lowered to -10°, filtered, washed with precooled ethanol, diethyl ether and then pumped on by high vacuum to yield 2.35 g (73%) of the title compound: m.p. 230-240°. ^{1}H NMR (500 MHz, D_2O/DMSO-d_6 1:1): δ 7.98 (d, 1H, J = 7.6 Hz, H - 4), 7.78 (td, 1H, J = 7.5, 1.0 Hz, H - 6), 7.70 (td, 1H, J = 7.4, 0.77 Hz, H - 5), 7.22 (d, 1H, J = 7.7 Hz, H - 7), 7.16 (d, 2H, J = 2.3 Hz, H - 4', H - 5'), 6.83 (dd, 2H, J = 2.3, 8.8 Hz, H - 2', 7'), 6.56 (d, 2H, J = 8.8 Hz, H - 1', 8'). ^{13}C NMR (125 MHz, D_2O/DMSO-d_6 1:1): δ 171.4 (C-3), 156.3 (C-3', 6'), 153.4 (C-7a), 152.5 (C-4'a, 10'a), 137.5 (C-6), 131.8 (C-5), 129.6 (C-1', 8'), 126.6 (C-3a), 126.2 (C-4), 125.1 (C-7), 118.0 (C-2', 7'), 113.7 (C-8'a, 9'a), 109.0 (C-4', 5'), 84.6 (C-1). MS (FAB$^+$): m/z = 493 $(M+H)^+$, 413 $(M+H\text{-}PO_3)^+$.

Anal. Calcd. for $C_{20}H_{20}N_2O_{11}\bullet 2H_2O$: C, 42.72; H, 4.30; N, 4.98; P, 11.02

Found: C, 43.22; H, 4.10; N, 5.12; P, 10.82

6'-Hydroxy-3'-*bis*(benzyloxy)phosphoryloxyspiro[isobenzofuran-1(3H),9'-[9H]xanthen]-3-one (6).- A mixture of fluorescein (10.0 g, 30 mmol), THF (150 mL), dibenzyl diisopropylphosphoramidite (11.3 g, 33 mmol), triethylamine (1.5 g, 15 mmol) and 1H- tetrazole (2.7 g, 39 mmol) was reacted and then oxidized as described for the preparation of **3**. Purification by column chromatography eluting with 3% methanol in dichloromethane (R_f 0.4) gave 8.0 g (45%) of the title compound **6** as a yellow foam. ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.02 (dd, 1H), 7.70 - 7.58 (m, 2H), 7.55 (s, 1H, OH), 7.35 (s, 10H, Ph), 7.10 (s, 1H), 7.05 - 6.95 (m, 1H), 6.80 - 6.50 (m, 5H), 5.20 (s, 2H), 5.15 (s, 2H). ^{13}C NMR (125 MHz, D_2O/DMSO-d_6 1:4): δ 170.7, 160.7, 153.4, 153.2, 152.9, 152.7, 137.5, 136.49, 136.44, 132.0, 130.8 -129.6 phenyls, 126.9, 126.4, 125.1, 117.6, 117.5, 114.6, 110.6, 109.7, 103.8, 83.7, 71.62 ($^2J_{cp}$ = 5.9 Hz). LRMS (APCI): m/z = 593 $(M+1)^+$.

Anal. Calcd. for $C_{34}H_{25}O_8P$: C, 68.92; H, 4.25; P, 5.23. Found: C, 68.66; H, 4.18; P, 4.78

6'-Hydroxy-3'-phosphonooxyspiro[isobenzofuran-1(3H),9'-[9H]xanthen]-3-one Ammonium Salt (FMP) (7).- Fluorescein dibenzylphosphate **6** (1.0 g, 1.7 mmol) was reduced as described for **4** and then neutrilized with 6 equivalents of ammonium acetate in ethanol (10 mL). The resultant mixture was filtered through a Celite plug to remove the catalyst, the ethanol was then gently pumped off (<20°) and the residue dissolved in water and lyophilized to obtain 724 mg of a yellow colored solid. The crude FMP ammonium salt was stirred at r.t. for 20 h with 5% ethanol in 2-propanol (21 mL), filtered, washed with some solvent mixture, ether and then dried at a reduced pressure provided by a high vacuum pump to yield 565 mg (77%) of the title compound: m.p. >185° (gradual). ^{1}H NMR (500 MHz, D_2O/DMSO-d_6 1:1): δ 7.96 (d, 1H, J = 7.5 Hz, H-4), 7.76 (t, 1H, J = 7.5 Hz, H-6), 7.68 (t, 1H, J = 7.5 Hz, H-5), 7.18 (d, 1H, J = 8.1 Hz, H-7), 7.17 (d, 1H, J = 2.2 Hz, H-4'), 6.82 (dd, 1H, J = 2.1, 8.7 Hz, H-2'), 6.70 (s, 1H, H-5'), 6.55 (d, 1H, 8.7 Hz, H-1'), 6.52 (s, 2H, H-7', 8'). ^{13}C NMR (125 MHz, D_2O/DMSO-d_6 1:1): δ 169.9 (C-3), 160.7 (C-6'), 156.5 (C-3'), 152.7 (C-10'a), 152.4 (C-7a), 151.9 (C-4'a), 136.3 (C-6), 130.9 (C-5), 129.7 (C-8'), 128.8 (C-1'), 126.9 (C-3a), 125.5 (C-4), 124.7 (C-7), 117.3 (C-2'), 113.8 (C-7'), 112.7 (C-9'a), 110.2 (C-8'a), 107.9 (C-4'), 103.0 (C-5'), 86.1 (C-1). MS(FAB$^+$): m/z = 413 $(M+H)^+$, 333 $(M+H-PO_3)^+$.

2-(3,6-Diphosphonooxy-9H-xanthen-9-yl)benzoic Acid Diammonium Salt (8).- Fluorescein *bis*(dibenzylphosphate) **3** (650 mg, 0.75 mmol) was reduced as described for **4** but at r.t. 320 mg (57%) of the title compound was obtained as a yellow foam. ^{1}H NMR (400 MHz, D_2O/DMSO-d_6 1:1): δ 7.69 (dd, 1H), 7.32 (td, 1H), 7.18 (td, 1H), 6.95 (d, 2H), 6.89 (d, 1H), 6.83 (d, 2H), 6.70 (dd, 2H), 6.15 (s, 1H). ^{13}C NMR (125 MHz, D_2O/DMSO-d_6) 1:1): δ 171.8, 157.0, 153.9, 153.8, 152.8, 151.8, 145.4, 140.9, 137.6, 131.6, 129.6 (2) 126.8, 120.7 (2), 113.2 (2) 108.8 (2), 85.3. MS (FAB$^-$): m/z = 493 $(M-H)^-$, 413 $(M-H-PO_3)^-$.

3',6'-*bis*(Benzyloxyhydroxyphosphoryloxy)spiro[isobenzofuran-1(3H),9'-[9H]xanthen]-3-one Diammonium Salt (9).- Fluorescein *bis*(dibenzylphosphate) **3** (1.4 g, 1.7 mmol) was reduced as described for **4** but in the presence of ammonium acetate (390 mg, 5.0 mmol). The mixture was filtered through Celite to remove the catalyst and then concentrated on a rotavap (30° - 35°) with further pumping by high vacuum. The sticky foam was dissolved in water (15 mL), refiltered through Celite to obtain a clear solution and then lyophilized to obtain 1.0 g (83%) of the title compound as a light yellow foam. ^{1}H NMR (400 MHz, D_2O/DMSO-d_6 1:1): δ 7.95 (d, 1H), 7.76 (t, 1H), 7.66 (t, 1H), 7.23 - 7.07 (m, 11H), 7.0 (d, 2H), 6.79 (dd, 2H), 6.57 (d, 2H), 4.78 (s, 2H), 4.75 (s, 2H). ^{13}C NMR (125 MHz, D_2O/DMSO-d_6 1:1): δ 171.5, 155.85, 155.80, 153.4, 152.5 (2), 138.68, 138.62, 137.6, 132.0, 129.8 - 128.9 phenyls, 126.7, 126.4, 125.0, 118.0 (2), 114.3 (2), 109.1 (2), 84.4, 68.67 ($^2J_{cp}$ = 5.5 Hz). MS (FAB$^-$): m/z = 493 $(M-H)^-$, 413 $(M-H-PO_3)^-$.

6'-Hydroxy-3'-sulfooxyspiro[isobenzofuran-1(3H),9-[9H]xanthen]-3-one Ammonium Salt (FMS) (10).- A solution of chlorosulfonic acid (1 g, 8.6 mmol) in chloroform (20 mL) was added dropwise to a precooled solution (-78°) of N,N-dimethylaniline (2.5 g, 20 mmol) in chloroform (20 mL). The solution was stirred and allowed to warm to r.t. Fluorescein (0.9 g, 2.7 mmol) dissolved in chloroform (20 mL) was added dropwise and the resultant mixture refluxed at 65° for 18 h. TLC

eluting with NH_4OH:MeOH:$CHCl_3$ 1:4:8 showed no fluorescein left at R_f 0.4 but a new highly fluorescent spot at R_f 0.45 under long wavelength. The mixture was concentrated and chromatographed on silica gel using NH_4OH:MeOH:$CHCl_3$ 1:4:8 as eluent. By-product 4-(dimethylamino)-benzenesulfonic acid ammonium salt was eluted first followed by the title compound. The separated FMS ammonium salt 0.9 g (78%) was dissolved in water and lyophilized. ^1H NMR (200 MHz, D_2O/DMSO-d_6 1:1): δ 8.01 (d, 1H), 7.90 - 7.65 (m, 2H), 7.35 - 7.15 (m, 2H), 6.98 - 6.81 (m, 2H), 6.80 - 6.62 (m, 2H), 6.55 (d, 1H). ^{13}C NMR (125 MHz, D_2O/DMSO-d_6 1:1): δ 171.8, 171.5, 155.4, 155.0, 152.8, 152.4, 137.7, 136.6, 130.9, 130.2, 130.1, 127.0, 126.5, 118.7, 116.6, 115.9, 111.9, 110.2, 104.1, 83.9. MS(FAB$^-$): m/z = 411 $(M-H)^-$, 331 $(M-H-SO_3)^-$.

3',6'-*bis*(Sulfooxy)spiro[isobenzofuran-1(3H),9'-[9H]xanthen]-3-one Diammonium Salt (FDS) (11).- A solution of chlorosulfonic acid (5 g, 77 mmol) in chloroform (20 mL) was added dropwise to a precooled solution (-78°) of triethylamine (13 g, 130 mmol) in chloroform (20 mL). The solution was stirred and allowed to warm to r.t. Fluorescein (1 g, 3 mmol) dissolved in chloroform (20 mL) was added dropwise and the resultant mixture refluxed at 65° for 18 h. TLC eluting with NH_4OH:MeOH:$CHCl_3$ 1:4:8 showed no fluorescein left at R_f 0.4 but a new non highly fluorescent spot at R_f 0.5 under short wavelength. The solution was concentrated and the residue chromatographed on silica gel eluting with $CHCl_3$, then with NH_4OH:MeOH:$CHCl_3$ 1:10:75 (removes triethylamine salts) followed by NH_4OH:MeOH:$CHCl_3$ 1:4:8 to yield 0.6 g (40%) of fluorescein disulfate diammonium salt. The compound was redissolved in water, filtered, frozen and lyophilized. ^1H NMR (200 MHz, D_2O/DMSO-d_6 1:1): δ 8.03 (d, 1H), 7.82 (td, 1H), 7.72 (td, 1H), 7.28 (d, 1H), 7.25 (d, 2H), 6.91 (dd, 2H), 6.7 (d, 2H). ^{13}C NMR (100 MHz, D_2O/DMSO-d_6 1:1): δ 171.3 (C-3), 155.1 (C-3', 6'), 153.3 (C-7a), 152.3 (C-4'a, 10'a), 137.4 (C-6), 131.9 (C-5), 129.9 (C-1', 8'), 126.4 (C-4), 126.2 (C-3a), 125.0 (C-7), 118.5 (C-2', 7'), 115.6 (C-8'a, 9'a), 109.9 (C-4', 5'), 83.7 (C-1). MS(FAB$^-$): m/z = 491 $(M-H)^-$, 411 $(M-H-SO_3)^-$, 331 $(M-H-2SO_3)^-$.

Anal. Calcd. for $C_{20}H_{18}N_2S_2O_{11}\cdot 2H_2O$: C, 42.70; H, 3.94; N, 4.98; S, 11.40

Found: C, 42.88; H, 3.89; N, 5.02; S, 11.53

3'-Sulfooxy-6'-*bis*(benzyloxy)phosphoryloxy-spiro[isobenzofuran-1(3H),9-[9H]xanthen]-3-one Ammonium Salt (12).-Fluorescein dibenzylphosphate **6** (2.0 g, 3.4 mmol) dissolved in chloroform (20 mL) was added dropwise to a solution of chlorosulfonic acid triethylamine salt (21 mmol) in chloroform (100 mL) prepared as described for FDS. The resultant mixture was refluxed at 65° for 2h. TLC eluting with NH_4OH:MeOH:$CHCl_3$ 1:10:75 showed the product spot at R_f 0.5 under short wavelength. Heating the TLC plate with a molybdate solution shows the fluorescein derivative as a dark green spot and heating with palladium chloride reveals the amine salts. The reaction mixture was cooled and washed with brine (100 mL), concentrated and then chromatographed on silica gel eluting first with chloroform and then with NH_4OH:MeOH:$CHCl_3$ 1:10:75. The separated product was redissolved in water and lyophilized to yield 1.8 g (77%) of **12** as an amorphous solid that was stored in a freezer due to gradual decomposition (3 days) at ambient temperatures. ^1H NMR (400 MHz, D_2O/DMSO-d_6 1:4): δ 8.0 (d, 1H), 7.80 (td, 1H), 7.72 (td, 1H), 7.35 - 7.25 (m, 10H), 7.22 (d, 1H),

7.18 (d, 1H), 7.03 (d, 1H), 6.90 (dd, 1H), 6.85 (dd, 1H), 6.72 (d, 1H), 6.70 (d, 1H), 5.12 (s, 2H), 5.09 (s, 2H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 168.5, 155.5, 152.3, 151.4, 151.2, 150.6, 135.8, 135.49, 135.44, 130.4, 129.5 - 127.8 phenyls, 125.5, 124.8, 124.0, 116.9, 116.2, 115.8, 112.7, 108.3, 107.4, 81.4, 69.71 ($^2J_{cp}$ = 5.7 Hz). MS (FAB$^+$): m/z = 673 $(M+H)^+$, 593 $(M+H-Bn)^+$, 503 $(M+H-2Bn)^+$.

3'-Sulfooxy-6'-phosphonooxyspiro[isobenzofuran-1(3H),9'-[9H]xanthen]-3- one Diammonium Salt (FMSP) (13).- Fluorescein dibenzylphosphate monosulfate ammonium salt **12** (0.9 g, 1.3 mmol) was reduced in a 1:1 mixture of ethanol and chloroform (20 mL) at -20° using 5% Pd/C (100 mg) for 1.5 h under a slight positive pressure of H_2 gas using a balloon. The reduction was followed by TLC using NH_4OH:MeOH:$CHCl_3$ 1:10:75. When no more starting material was evident and only the product spot observed at the baseline, ammonium acetate (0.5 g, 6.5 mmol) dissolved in ethanol (20 mL) was added dropwise and the mixture stirred for 0.5 h. The precipitate was collected and dissolved in water (25 mL) and then passed through a Celite plug to remove the palladium catalyst. The solution was pumped on for a short while to diminish the quantity of remaining ethanol and then lyophilized to obtain 510 mg (74%) of the title compound as an amorphous solid. ^{1}H NMR (200 MHz, D_2O/DMSO-d_6 1:1): δ 8.02 (d, 1H), 7.80 (t, 1H), 7.75 (t, 1H), 7.35 - 7.05 (m, 3H), 7.00 - 6.78 (m, 2H), 6.65 (d, 1H), 6.60 (d, 1H). ^{13}C NMR (125 MHz, D_2O/DMSO-d_6 1:): δ 172.2, 157.0, 154.9, 153.6, 152.8, 152.7, 137.8, 132.0, 130.2, 129.8, 126.7, 126.5, 125.2, 118.5, 118.4, 116.4, 113.3, 110.5, 109.1, 85.0 MS (FAB$^-$): m/z = 491 $(M-H)^-$, 411 $(M-H-SO_3)^-$.

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