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Tetrahedron 60 (2004) 1903–1911
Tetrahedron

New fluorescent probes for testing combinatorial catalysts with phosphodiesterase and esterase activities

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Received 17 April 2003; revised 11 December 2003; accepted 11 December 2003

Abstract—Combinatorial development of new catalysts with phosphodiesterase and esterase activities requires specific fluorescent probes for rapid visual detection of hydrolytic activity. Such fluorescent probes have been synthesized with special attention to solubility in water and stability towards spontaneous hydrolysis at a given pH. The probes reported here include compound **5** based on a fluorescein fluorophore, compound **12** for FRET-detection of phosphodiester hydrolysis and compound **25** based on a quinolinium fluorophore.

1. Introduction

Combinatorial chemistry[1] has a major impact on catalyst discovery and optimization.[2] However, the application of combinatorial techniques to this subject requires new high throughput screening methods to monitor a large number of reactions in a quick and simple way.[2a–g,3] While pursuing combinatorial approaches to the development of molecular catalysts for the hydrolysis of phosphodiesters and carboxylic esters, we became interested in the preparation and characterization of novel substrate probes that would allow to monitor hydrolysis reactions, by means of appearance of fluorescence. Our goal was to provide substrates that allow rapid qualitative visual screening of compound libraries for catalytic activity in phosphodiester and carboxylic ester hydrolyses. We were certainly led by known fluorescent probes for enzyme assays, but we did not intend to use these substrates for that purpose.

To design these substrates, we focused on the excellent fluorescent properties of fluorescein,[4] which possesses a relatively high absorptivity and excellent quantum yield. Fluorescein esters have been used previously as fluorescent probes to determine esterase activities. Guilbaut and Kramer[5] reported a study of the hydrolysis of various fluorescein esters by lipases. Also, *p*-guanidinobenzoic acid esters of fluorescein have been used as active site titrants of serine proteases[6] and water insoluble fluorescein monoesters, for example, 3′- or 6′-laureates or myristates, have been used for medicinal applications, determining the activity of pancreas enzymes, lipases or of chymotrypsin in blood, duodenal fluid, or urine.[7] Moreover, the use of fluorescein in the preparation of fluorogenic substrates to continuously monitor the activity of the enzyme phosphatidylinositide-specific phospholipase C has been previously described[8] and Scheigetz and co-workers[9] have prepared 3′,6′-fluorescein diphosphate and different fluorescein monophosphates for highly sensitive and continuous protein tyrosine phosphatase assays.

We therefore wanted to synthesize non-fluorescent esters of fluorescein, which upon hydrolysis liberate the fluorescent fluorescein molecule. Fluorescent probes of that nature could be used in concentrations of up to 10^{-3} M, which is necessary to ensure a reasonable reaction rate on reaction with a catalyst which is present in even lower concentration. If one aims at fluorescent probes which could be used at much lower concentration, one can turn to FRET-based systems,[10] see for instance the system described by Berkessel and co-workers.[11] This methodology requires the presence of a fluorophore and a quencher group linked through an ester or phosphodiester bond. Upon irradiation, a rapid energy transfer occurs between these two parts and the phosphodiester does not fluoresce. When hydrolysis occurs, the fluorophore and the quencher are separated and the internal quenching is disrupted with the consequence that fluorescence can be observed. In the design of our catalytic systems, we tried to emulate nature using aqueous media. Therefore, the fluorescent probes of interest to us should be soluble in water over a broad concentration range and should be stable towards spontaneous hydrolysis at a given pH.

Keywords: Combinatorial catalysis; Fluorescence; Ester hydrolysis; Phosphodiester hydrolysis.
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doi:10.1016/j.tet.2003.12.032

2. Results and discussion

2.1. Phosphodiester probes

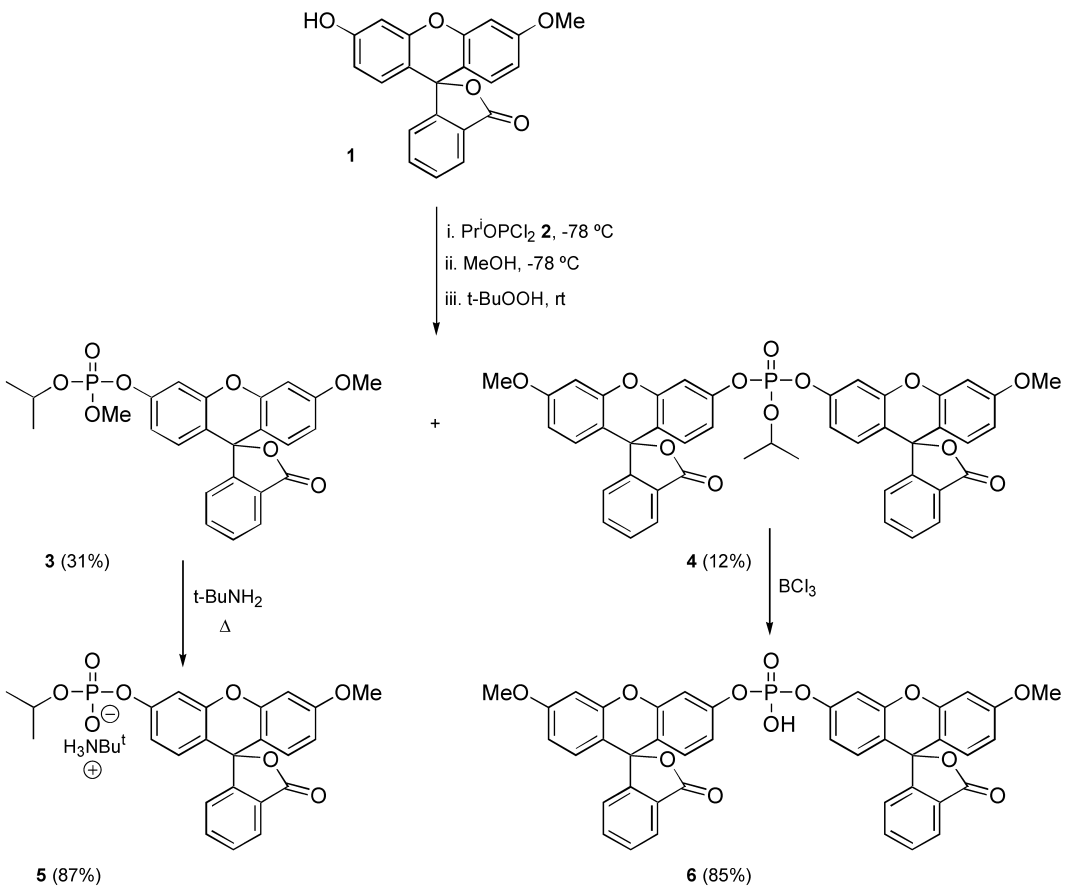
Taking into consideration the wide importance of the phosphodiester linker in nature, we wanted to prepare fluorogenic substrates to continuously monitor phosphodiesterase activity of artificial catalysts. We therefore targeted phosphodiesters of fluorescein. For their preparation, we used the dichloridite procedure previously developed for the synthesis of oligonucleotides.[12] In this vein, dichloridite **2** was reacted sequentially at −78 °C with methyl-fluorescein **1** and MeOH (Scheme 1). In situ oxidation at rt with *t*-BuOOH gave a mixture of two products that could be separated by column chromatography: the phosphate **3** in 31% yield, and the product **4** resulting from the coupling of two molecules of methyl-fluorescein **1** to dichloridite **2** in 12% yield (Scheme 1). Product **3** was subsequently demethylated by treatment with *t*-$BuNH_2$ under reflux, affording phosphodiester **5** in 87% yield after crystallization. The isopropyl group present in phosphate **4** was cleaved by treatment with BCl_3 to give the corresponding phosphodiester **6** in 85% yield (Scheme 1).

For the preparation of a FRET-based system, we aimed at one which would quickly detect phosphodiesterase activity of potential catalysts by using a fluorophore, which could be excited by a simple UV-lamp ($\lambda_{abs}\approx360$ nm) and which would produce a visible fluorescence ($\lambda_{em}>500$ nm) upon hydrolysis of the phosphodiester bond. We therefore modified the Berkessel system and focused on compound **12** shown in Scheme 2. We envisaged that compound **12** would be a suitable substrate, which allows to detect hydrolysis by simple visual observation of fluorescence avoiding the use of spectrophotometers. The synthesis of **12** started with the separate preparation of both the fluorophore **7** and the quencher **10** following procedures described in the literature (Scheme 2).[13,14] For the combination of these two parts, the phosphoramidite coupling method developed by Beaucage and Caruthers[15] for the synthesis of oligonucleotides was used. Thus, fluorophore **7** was reacted with phosphoramidite **8** to afford compound **9** in 61% yield (Scheme 2). Afterwards, compound **9** could be coupled with the azo-derivative **10** in the presence of tetrazole, and the crude product was oxidized in situ with *t*-BuOOH to give the phosphate **11** in 64% yield. The last step was the cleavage of the methyl ester group in **11** using Me_3SiBr[16] to form the desired phosphodiester **12** (Scheme 2).

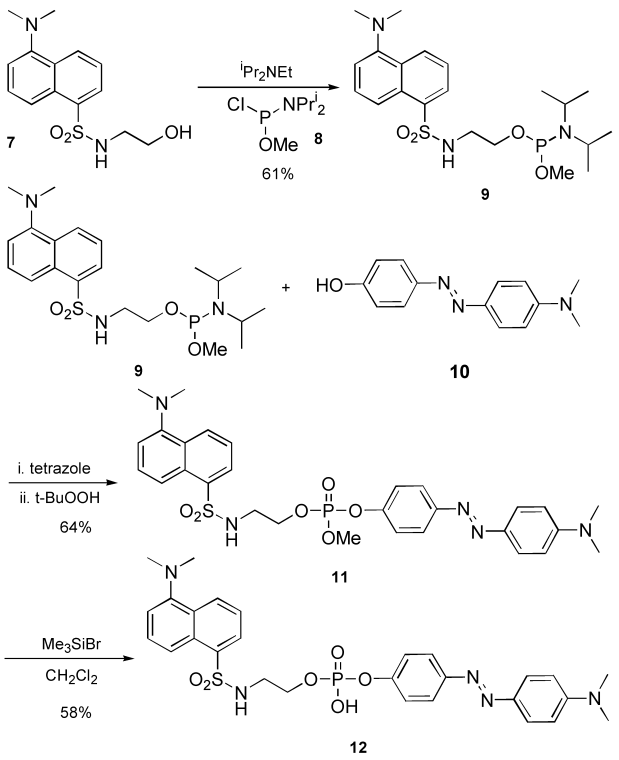
2.2. Carboxylic ester probes

Aiming at water soluble fluorescent probes capable of indicating hydrolysis of carboxylic esters, the attachment of polyol units to fluorescein derivatives was envisioned as a method to increase solubility in water. This could be done either through a polyol moiety bound via the phenolic hydroxyl group to fluorescein or by a polyol group bound via a carboxyl link to the fluorescein core.

Regarding the first approach, our synthetic targets were

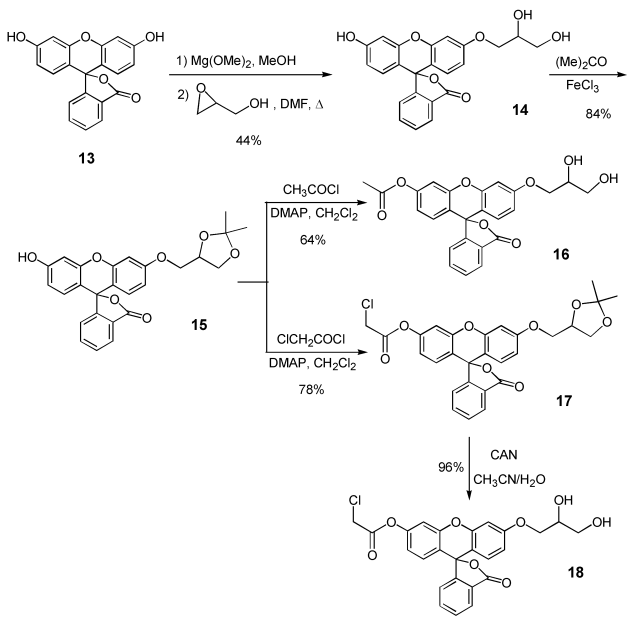


Scheme 1.



Scheme 2.

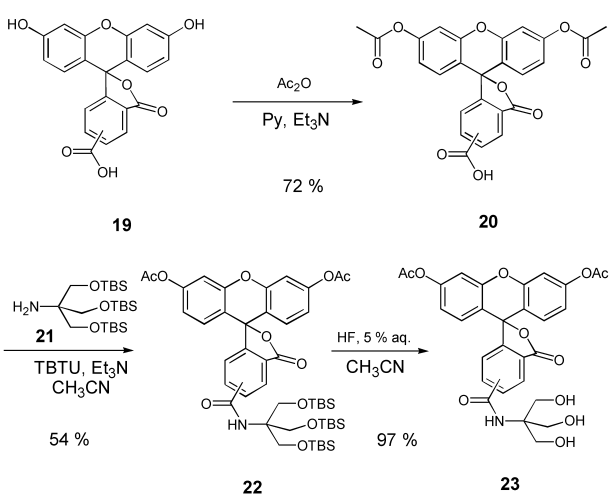
compounds **16** and **18**, having a diol moiety to increase solubility in water (Scheme 3). These compounds were prepared starting from commercially available fluorescein **13** in the following way: the magnesium salt of fluorescein was reacted with glycidol to afford diol **14** in 44% yield. After protection of the diol function as a ketal, the phenolic hydroxyl group of **15** was reacted with acetyl chloride, affording ester **16** directly in 64% yield (Scheme 3), as ketal deprotection took place during the work-up. Likewise compound **15** was treated with chloroacetyl chloride to give compound **17** in 78% yield. After purification by flash chromatography ketal deprotection was achieved under essentially neutral conditions with cerium ammonium nitrate[17] in 96% yield (Scheme 3).



Scheme 3.

Unfortunately, despite the presence of a diol group, did the solubility in water of neither **16** nor **18** exceed 10^{-5} M.

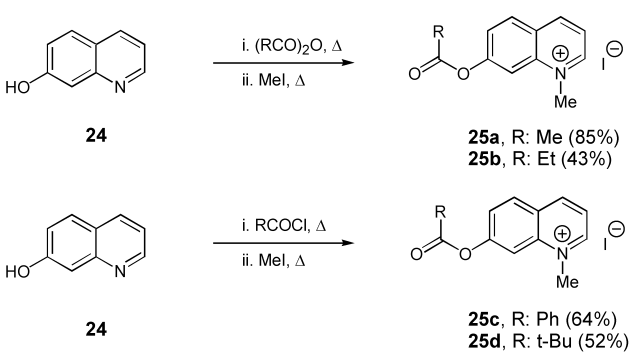
In order to test other possibilities, we turned to carboxyfluorescein **19** to introduce a polyol unit attached via the pendant carboxyl group. Thus, acetylcarboxyfluorescein **20** was coupled to the triethanolamine derivative **21** using *O*-benzotriazolyl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate in 54% yield and the *tert*-butyldimethylsilyl groups of intermediate **22** were removed by treatment with 5% aq. HF, yielding compound **23** in 97% yield (Scheme 4).



Scheme 4.

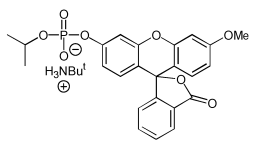
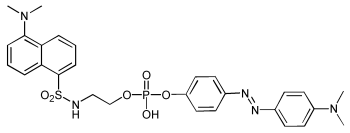
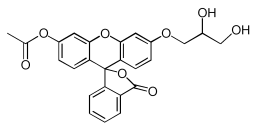
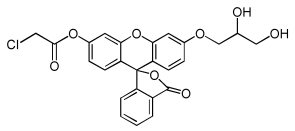
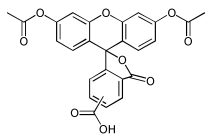
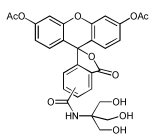
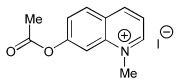
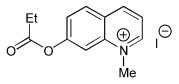
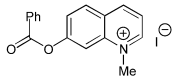
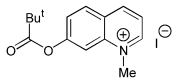
However, again the solubility of compound **23** in water did not exceed 10^{-5} M. Likewise, the solubility of diacetylcarboxyfluorescein **20**, a potential candidate, was also around 10^{-5} M.

Due to the difficulties to increase the water solubility of fluorescein derivatives, we were intrigued by a study of Menger and co-workers[18] who tested the activity of some esterases like acetylcholinesterase and chymotrypsin, using carboxylic esters derived from 7-hydroxyquinoline **24**. The synthesis of compounds **25** was achieved by acylating 7-hydroxyquinoline **24** with an anhydride or an acid chloride and treating the resulting esters with MeI (Scheme 5).



Scheme 5.

Table 1. Qualitative characterization of fluorescent probes

	Fluorescent probes	Solubility	Stability	Enzymatic assay[a]	Outcome[b]
5		$>10^{-3}$ M, pH=8.8[c]	1 d, 10^{-3} M, pH=8.8[c]	PDI[d] 10 min, 10^{-3} M, pH=8.8[c]	Green fluorescence
12		10^{-5} M, pH=8.8[c]	6 h, 10^{-5} M, pH=8.8[c]	PDI,[d] 10 min, 10^{-6} M, pH=8.8[c]	Green fluorescence
16		10^{-5} M, pH=7.0[e]	6 h, 10^{-5} M, pH=7.0[e]	PPL,[f] 10 min, 10^{-5} M, pH=7.0[e]	Green fluorescence
18		10^{-5} M, pH=7.0[e]	2 h, 10^{-5} M, pH=7.0[e]	PPL,[f] 10 min, 10^{-5} M, pH=7.0[e]	Green fluorescence
20		10^{-5} M, pH=8.8[c]	2 d, 10^{-5} M, pH=8.8[c]	PPL,[f] 10 min, 10^{-5} M, pH=8.8[c]	Green fluorescence
23		10^{-5} M, pH=8.8[c]	1 d, 10^{-5} M, pH=8.8[c]	—[g]	—[g]
25a[h]		$>10^{-2}$ M, pH=7.0[e]	2 h, 10^{-3} M, pH=7.0[e]	—[g]	—[g]
25b[h]		$>10^{-2}$ M, pH=7.0[e]	3 h, 10^{-3} M, pH=7.0[e]	PPL,[f] 20 min, 10^{-3} M, pH=7.0[e]	Green fluorescence
25c[i]		$>10^{-2}$ M, pH=7.0[e]	24 h, 10^{-3} M, pH=7.0[e]	PPL,[f] 120 min, 10^{-3} M, pH=7.0[e]	Green fluorescence
25d[i]		$>10^{-2}$ M, pH=7.0[e]	30 h, 10^{-3} M, pH=7.0[e]	PPL,[f] 240 min, 10^{-3} M, pH=7.0[e]	Green fluorescence

[a] All enzymatic assays were done taking 1 mL of a stock solution of the corresponding fluorescent probe in the buffer and concentration quoted and adding the enzyme as solid.
[b] The estimated visual detection threshold of all the probes is below 10^{-6} M.
[c] 0.1 M AMPSO buffer {3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid}.
[d] Phosphodiesterase I (EC 3.1.4.1, type IV from crotalus atrox crude dried venom): 10 mg, 0.1 units.
[e] 0.1 M HEPES buffer [*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid)].
[f] Porcine pancreas lipase (EC 3.1.1.3. type II, crude): 4 mg, 50 units.
[g] Compound not tested because of a lack of enough material.
[h] A 10^{-3} M solution of this compound in water shows a blue color.
[i] A 10^{-3} M solution of this compound in water shows a violet color.

The water solubility of this class of fluorescent probes was in all cases, larger than 10^{-2} M providing a set of compounds which should allow easy visual detection of ester hydrolysis when exposed to potential catalysts.

3. Testing

In order to characterize the new fluorescent probes in a qualitative manner, these were first subjected to stability

tests at pH 7 or 8.8. Lacking active synthetic catalysts at this stage of our work we tested the viability of our probes by subjecting them to cleavage by phosphodiesterase I or porcine pancreas lipase under buffered conditions. The results reported in Table 1 reflect the appearance of color as determined by the naked eye.

As we can see from the table, the fluorescein derived phosphodiester **5** showed good solubility in water ($>10^{-3}$ M) and is stable in aqueous pH 8.8 buffer safely over one day. Phosphodiester **6** was not tested because of a lack of enough material. In the case of the phosphodiester **12**, its solubility in water was only around 10^{-5} M, but higher concentrations are anyhow not tolerated if the FRET technique is to be applied to monitor ester hydrolysis.

Turning to the carboxylic esters **16** and **18**, they showed a low solubility in water (10^{-5} M as maximum) and also low stability towards spontaneous hydrolysis.

The problem of insufficient stability was overcome with compounds **20** and **23** derived from carboxyfluorescein **19**, stability in the buffer system extended to around 2 d for compound **20**. The solubility of compounds **20** and **23** in water is still low (10^{-5} M). The triol moiety therefore did not contribute too much to increase the solubility in water. Nevertheless the strong green fluorescence emitted by the fluorophore can easily be detected, once the carboxylic ester is hydrolyzed.

In the case of the quinolinium derivatives shown in Table 1, the solubility was not a problem, being higher than 10^{-2} M. The stability to spontaneous hydrolysis ranges from 2 h, for compound **25a**, to 30 h in the case of compound **25d**. Therefore, compounds **25a** and **25b** are too labile to be considered useful fluorescent probes.

In summary, the best fluorescent probe for monitoring the cleavage of phosphodiesters is compound **5**. Regarding fluorescent probes for cleavage of carboxylic esters, the best candidates are compound **20**, derived from carboxyfluorescein **19** and the quinolinium derivatives **25c** and **25d**, showing good solubility in water and a useful stability against spontaneous hydrolysis.

4. Experimental

4.1. General

All temperatures quoted are not corrected. Reactions were carried out under dry nitrogen or argon. ^{1}H and ^{13}C NMR spectra were recorded on Bruker ARX-200 and AC-300 spectrometers. Spectra were recorded for ca. 0.2 mM solutions in $CDCl_3$ (99% d), which was also used as an internal standard. Coupling constants are quoted in Hz. Flash chromatography was run using silica gel Si 60 (40–63 μm, E. Merck AG, Darmstadt). Electron impact (EI, 70 eV) mass spectra were recorded on a Varian CH 7A instrument.

4.2. 3′-(Isopropoxy(methoxy)phosphoryl(-6′-methoxy-spiro(isobenzo-furan-1(3*H*)-9′(9′*H*)-xanthen(-3-one (3) and isopropylbis{6′-methoxy-spiro[isobenzofuran-1(3*H*)-9′(9′*H*)-xanthen]-3-one-3′-yl}phosphate (4)

Pyridine (1 mmol, 81 μL) was added via syringe to a solution of Pr^iOPCl_2 **2**[19] (0.265 mmol, 43 mg) in dry THF (0.35 mL) maintained at −78 °C in a small flask equipped with a septum cover. To the solution was added methylfluorescein (0.24 mmol, 83 mg) dissolved in 0.65 mL of dry THF. After a total of 10 min, methanol (0.19 mmol) was added (via syringe). The solution was maintained for 15 min at −78 °C. The reaction mixture was warmed to rt and to it was added a solution of *t*-BuOOH in CH_2Cl_2 (7 M, 125 μL). After 2 h at rt, H_2O was added and the aqueous phase was extracted with $CHCl_3$ (3×15 mL). The organic layer was dried ($MgSO_4$) and concentrated to afford a residue which was purified by flash chromatography (eluent: CH_2Cl_2/EtOAc: 93/7), yielding phosphates **3** and **4** with the yield mentioned in the text. Compound **3**. R_f=0.36 ($CHCl_3$/EtOAc: 9/1). ^{1}H NMR (200 MHz, $CDCl_3$): δ=1.38 (m, 6H), 3.84 (s, 3H), 3.86 (d, *J*=14.0 Hz, 3H), 4.79 (m, 1H), 6.64–6.79 (m, 5H), 7.19 (m, 2H), 7.66 (m, 2H), and 8.03 (d, *J*=7.0 Hz, 1H). ^{13}C NMR (50 MHz, $CDCl_3$): δ=23.6, 54.7, 54.9, 55.6, 74.3, 74.4, 82.5, 100.9, 108.5, 111.0, 112.0, 115.8, 116.0, 124.0, 125.1, 126.6, 129.0, 129.4, 129.9, 135.1, 152.1, 152.3, 153.1, 161.5, and 169.3. ^{31}P NMR (81 MHz, $CDCl_3$): δ=−5.98. HR-MS: $C_{24}H_{20}PO_8-CH_3$ requires 467.0896; found 467.0903. Compound **4**. R_f=0.57 ($CHCl_3$/EtOAc: 9/1). Mp 97–99 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ=1.41 (m, 6H), 3.83 (s, 6H), 4.94 (m, 1H), 6.64–7.00 (m, 10H), 7.17 (m, 4H), 7.65 (m, 4H), and 8.03 (d, *J*=7.0 Hz, 2H). ^{13}C NMR (50 MHz, $CDCl_3$): δ=23.5, 25.8, 55.6, 75.8, 82.3, 100.9, 108.7, 110.9, 112.1, 115.8, 116.5, 123.9, 125.1, 126.5, 129.0, 129.6, 129.9, 135.2, 151.6, 152.1, 152.2, 153.0, 161.5, and 169.2. ^{31}P NMR (81 MHz, $CDCl_3$): δ=−13.10. *m/z* 360 (M^+−436, 8%). HR-MS: $C_{20}H_{10}PO_5$ requires 360.0188; found 360.0981.

4.3. *tert*-Butylammonium isopropyl-{6′-methoxy-spiro-(isobenzofuran-1(3*H*)-9′(9′*H*)-xanthen(-3-one-3′-yl}phosphate (5)

A solution of phosphate **3** (230 mg) in *t*-$BuNH_2$ (140 mL) was heated at reflux for 8 h. The solvent was removed at reduced pressure affording a solid which was recrystallized from EtOAc/hexane to afford 200 mg of pure phosphodiester **5** (87%). R_f=0.27 ($CHCl_3$/MeOH: 9/1). Mp 205–206 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ=1.20 (d, *J*= 6.2 Hz, 6H), 1.31 (s, 9H), 3.83 (s, 3H), 4.50 (m, 1H), 6.58–7.24 (m, 7H), 7.63 (m, 2H), and 8.01 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$): δ=23.9, 27.7, 51.4, 55.6, 70.5, 83.0, 100.9, 107.7, 107.8, 111.1, 111.7, 113.6, 116.0, 116.1, 123.8, 125.0, 126.8, 128.7, 129.0, 129.7, 135.0, 151.9, 152.5, 153.2, 154.7, 154.8, 161.4, and 169.4. ^{31}P NMR (81 MHz, $CDCl_3$): δ=−6.23. *m/z* 466 ($M^+-H_3N^+Bu^t$-1, 2%). HR-MS: $C_{24}H_{20}O_8P$ requires 467.0894; found 467.1537.

4.4. Bis{6′-methoxy-spiro[isobenzofuran-1(3*H*)-9′(9′*H*)-xanthen]-3-one-3′-yl}phosphate (6)

To a solution of phosphate **4** (48 mg, 0.06 mmol) in dry CH_2Cl_2 (4 mL) was added with stirring a 1 M solution of

BCl_3 in heptane at −10 °C. The stirring was continued under N_2 for 45 min at the same temperature, and then 1 M HCl was added. The resulting mixture was extracted with EtOAc (3×15 mL). The organic phase was washed with H_2O, dried ($MgSO_4$) and concentrated to afford 38 mg (85% yield) of phosphodiester **6**, essentially pure. R_f=0.24 ($CHCl_3$/MeOH: 85/15). Mp 198–200 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ=3.69 (s, 6H), 6.50–7.11 (m, 14H), 7.51 (m, 4H), 7.90 (m, 2H), and 8.60 (br s, 1H). ^{13}C NMR (50 MHz, $CDCl_3$): δ=55.7, 100.7, 108.6, 111.2, 111.2, 112.6, 116.2, 116.5, 124.3, 125.4, 126.6, 129.0, 129.4, 129.9, 135.0, 152.2, 152.6, 162.1, and 169.1. ^{31}P NMR (81 MHz, $CDCl_3$): δ=−12.20. *m/z* 360 (M^+−395, 66%). HR-MS: $C_{20}H_{10}PO_5$ requires 360.0188; found 360.0981.

4.5. *N*-[2-(Diisopropylamino-methoxyphosphoxy)ethyl]-5-dimethylamino-1-naphthalenesulfonamide (9)

Chloro(diisopropylamino)methoxyphosphine **8** (268 mg, 1.35 mmol) was added to a solution of compound **7** (200 mg, 0.679 mmol) in CH_2Cl_2 (3 mL), containing diisopropylethylamine (473 μL, 2.7 mmol). The mixture was stirred for 35 min at rt. The resulting solution was diluted with CH_2Cl_2 (50 mL) and washed with 5% aq. $NaHCO_3$ (2×25 mL), brine (820 mL), dried ($MgSO_4$), filtered and concentrated. The crude phosphoramidite was purified by chromatography (5 g of silica gel, prewashed with a mixture of pentane/EtOAc/Et_3N: 50/50/1). The product was eluted with the same mixture and evaporation of appropriate fractions gave the phosphoramidite as a yellow oil (71% yield). R_f=0.53 (pentane/EtOAc/Et_3N: 50/50/1). ^{1}H NMR (300 MHz, $CDCl_3$): δ=1.00 (d, *J*=6.8 Hz, 6H), 2.78 (s, 6H), 2.99 (t, *J*=5.83 Hz, 2H), 3.16 (d, *J*=12.8 Hz, 3H), 3.39–3.48 (m, 4H), 6.56 (t, *J*=9.8 Hz, 1H), 7.16 (m, 1H), 7.47–7.55 (m, 2H), and 8.11–8.48 (m, 2H). ^{31}P NMR (81 MHz, CDCl3) δ=149.5. ^{13}C NMR (50 MHz, $CDCl_3$): δ=25.0, 43.2, 45.3 (d, *J*=13.5 Hz), 50.5 (d, *J*=10.06 Hz), 62.5 (d, *J*=17.1 Hz), 115.4, 119.2, 123.5, 128.6, 129.7, 130.0, 130.3, 130.4, 135.1, 137.0.

4.6. 2-(5-Dimethylamino-1-naphthalenesulfonyl-amide)ethoxy-4-(4-dimethyl-aminophenylazo)-phenyl-methylphosphate (11)

A solution of phosphoramidite **9** (196 mg, 0.43 mmol) in dry and acid free CH_2Cl_2 (2 mL) was added to a solution of the azo-compound **10** (103 mg, 0.43 mmol) in CH_2Cl_2 (1 mL) containing tetrazole (1.9 mL, 0.45 M in CH_3CN). The mixture was stirred for 2 h and then *t*-BuOOH (3 M solution in isooctane, 430 μL) was added and this mixture was stirred for an additional hour. The resulting solution was diluted with CH_2Cl_2 (30 mL) and washed with 5% aq. $NaHCO_3$ (2×15 mL), brine (20 mL), and dried ($MgSO_4$). The solution was filtered and evaporated under vacuum and the residue was chromatographed (silica flash, 5 g, CH_2Cl_2/EtOAc: 10/1 and 7/3). Evaporation of appropriate fractions gave the phosphotriester **11** as an oil (64% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ=2.74 (s, 6H), 2.97 (s, 6H), 3.36 (dd, *J*=5.5, 5.4 Hz, 2H), 3.65 (d, *J*=11.4 Hz, 3H), 4.03 (ddd, *J*=9.2, 5.6 Hz, 2H), 6.73 (d, *J*=9.2 Hz, 1H), 7.16 (m, 4H), 7.43–7.47 (m, 2H), 7.69 (t *J*=9.2 Hz, 4H), 8.12 (dd, *J*=7.3, 1.3 Hz, 1H), 8.26 (d, *J*=8.3 Hz, 1H), 8.44 (d, *J*=8.6 Hz, 1H). ^{31}P NMR (81 MHz, CDCl3) δ=−4.36. ^{13}C NMR (50 MHz, $CDCl_3$): δ=40.7, 43.8, 55.6 (d, *J*= 6.0 Hz), 67.7 (d, *J*=6.0 Hz), 111.8, 115.7, 119.1, 120.6, 120.7, 123.5, 124.0, 125.4, 128.9, 129.8, 129.9, 130.3, 131.0, 135.0, 143.8, 151.2 (d, *J*=7.0 Hz), and 152.9.

4.7. 2-(5-Dimethylamino-1-naphthalenesulfonyl-amide)ethoxy-4-(4-dimethylaminophenylazo)-phenyl-phosphate (12)

To a solution of phosphate **11** (84 mg, 0.14 mmol) in CH_2Cl_2 (4 mL), was added Me_3SiBr (38.2 μL, 0.28 mmol). The solution was stirred for 3 h at rt and then evaporated under vacuum (30 °C). The residue was dissolved in acetone (6.1 mL) followed by addition of water (1.15 mL). The solution was stirred for 30 min at rt and then evaporated in vacuo to give a residue that was dissolved in $CHCl_3$ (50 mL), washed with water (20 mL), brine (20 mL), dried ($MgSO_4$) and concentrated to yield a crude phosphodiester which was purified by chromatography (silica flash, 5 g, Et_2O, $CHCl_3$/MeOH: 10/1). Concentration of the appropriate fractions gave phosphodiester **12** as a red solid (34% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ=2.76 (s, 6H), 2.87 (s, 6H), 2.87 (m, 2H), 4.02 (ddd, *J*=9.2, 5.6 Hz, 2H), 6.51 (m, 2H), 6.75 (m, 1H), 6.80 (d, *J*=9.2 Hz, 1H), 7.36 (t, *J*=9.0 Hz, 2H), 7.74 (m, 5H), 8.20 (d, *J*=7.3 Hz, 1H), 8.37 (d, *J*=7.4 Hz, 1H), 8.40 (d, *J*=8.6 Hz, 1H). ^{31}P NMR (81 MHz, $CDCl_3$) δ=−3.68. ^{13}C NMR (50 MHz, $CDCl_3$): δ=40.1, 40.4, 42.3, 62.4 (d, *J*=9.4 Hz), 118.8, 120.0, 121.8, 122.5, 122.8, 123.2, 123.8, 124.7, 125.4, 126.0, 135.6, 136.6, 140.1, 142.2, 148.1, 153.7 (d, *J*=10.1 Hz), and 154.1.

4.8. 3′-Hydroxy-6′-(2,3-dihydroxypropoxy)spiro-(isobenzofuran-1(3*H*)-9′(9′*H*)-xanthen(-3-one (14)

Fluorescein **13** (332 mg, 1 mmol) was added in small portions under stirring at rt to a methanol solution of $Mg(OCH_3)_2$ (0.25 M, 8 mL). After stirring for 40 min the solvents were evaporated to dryness in vacuum. The resulting solid was powdered and added to a solution of glycidol (271 μL, 4 mmol) in DMF (8 mL). The mixture was stirred at 120 °C for 16 h. The resulting solution was diluted with 1 M HCl to pH=2 and extracted by continuous extraction with CH_2Cl_2 during 1 d. The organic layer was dried ($MgSO_4$) and evaporated to give a residue which was purified by flash chromatography ($CHCl_3$/MeOH: 85/15) on silica gel, affording 179 mg (44%) of compound **14** as a yellow solid (mixture of diastereomers). R_f=0.25 ($CHCl_3$/CH_3OH: 85/15). ^{1}H NMR (300 MHz, CD_3OD): δ=3.52–3.74 (m, 2H), 3.89–4.02 (m, 3H), 4.78 (broad s, 2H), 6.39–6.25 (m, 5H), 6.74 (d, *J*=2.2 Hz, 1H), 7.04 (m, 1H, ArH), 7.59 (m, 2H), and 7.86 (m, 1H). ^{13}C NMR (75 MHz, CD_4OD): δ=61.1, 63.3, 69.9, 70.8, 78.6, 80.2, 85.5, 101.9, 102.9, 110.4, 110.7, 112.2, 112.9, 124.4, 124.6, 125.0, 125.1, 127.2, 127.5, 129.2, 129.3, 129.4, 130.3, 135.7, 135.8, 153.1, 153.3, 153.5, 160.3, 160.5, 161.4, 170.8, and 170.9. *m/z* 406 (M^+, 1%). HR-MS: $C_{23}H_{18}O_7$ requires 406.1053; found 406.1052.

4.9. 6′-Hydroxy-3′-(2,2-dimethyl-1,3-dioxolan-4-yl-methoxy)spiro(isobenzo-furan-1(3*H*),9′(9′*H*)-xanthen(-3-one (15)

Anhydrous $FeCl_3$ (33 mg, 0.20 mmol) was added at rt to a

solution of compound **14** (100 mg, 0.25 mmol) in dry acetone (13 mL), stirring the mixture at 36 °C. After 2 h, evaporation of solvent left a residue, which was purified by flash chromatography ($CHCl_3$/MeOH: 95/5) to afford the ketal **15** as a yellow solid (94 mg, 84%, mixture of diastereomers). R_f=0.20 ($CHCl_3$/CH_3OH: 95/5). ^1H NMR (300 MHz, $CDCl_3$): δ=1.41, 1.47 (2 s, 6H), 3.90 (dd, J=8.3, 5.8 Hz, 1H), 3.97 (dd, J=9.6, 5.7 Hz, 1H), 4.06 (dd, J=9.6, 5.4 Hz, 1H), 4.17 (m, 1H), 4.50 (m, 1H), 6.60–6.76 (m, 6H), 7.17 (d, J=7.2 Hz, 1H), 7.64 (m, 2H), and 8.02 (d, J=6.9 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ=25.3, 26.7, 66.6, 69.0, 73.8, 84.5, 101.6, 103.1, 110.0, 110.7, 111.5, 111.9, 112.5, 124.0, 125.0, 126.7, 129.1, 129.2, 129.7, 135.1, 152.4, 153.1, 158.3, 160.2, and 170.1. m/z 446 (M^+, 2%), and 431 (M^+-15, 12%). HR-MS: $C_{26}H_{22}O_7$ requires 446.1366; found 446.1372.

4.10. 6′-Acetoxy-3′-(2,3-dihydroxypropoxy)spiro-(isobenzo-furan-1(3*H*),9′(9′*H*)-xanthen(-3-one (16)

A solution of ketal **15** (118 mg, 0.26 mmol), acetyl chloride (21 μL, 0.29 mmol) and an excess of 4-dimethylamino-pyridine (DMAP) in dry CH_2Cl_2 (4 mL) was heated under reflux for 3 h. Then, one more portion of acetyl chloride (21 μL, 0.29 mmol) was added, and the mixture was refluxed for 4 h and kept overnight at rt H_2O was added, the phases were separated and the aqueous layer was extracted with $CHCl_3$ (3×15 mL). The combined organic layers were dried ($MgSO_4$) and evaporated in vacuum to give a residue which was purified by silica flash chroma-tography eluting with $CHCl_3$/MeOH: 95/5 to give the ester **16** (72 mg, 64%) as a mixture of diastereomers. R_f=0.20 ($CHCl_3$/CH_3OH: 95/5). ^1H NMR (300 MHz, $CDCl_3$): δ=1.67 (broad s, 1H), 2.31 (s, 3H), 2.67 (m, 1H), 4.06 (m, 2H), 4.14–4.34 (m, 3H), 6.58–6.83 (m, 5H), 7.08 (m, 1H), 7.17 (m, 1H), 7.66 (m, 2H), and 8.03 (m, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ=21.1, 65.2, 68.4, 69.1, 84.5, 101.7, 110.3, 112.2, 116.7, 117.5, 124.0, 125.1, 126.5, 129.0, 129.1, 129.9, 135.1, 151.8, 152.0, 152.2, 153.0, 160.1, 168.8, and 171.1. m/z 446 (M^+-2, 4%). $C_{25}H_{20}O_8$: requires C, 66.96; H, 4.50; found C, 66.85; H, 4.66.

4.11. 6′-(2-Chloroacetoxy)-3′-(2,2-dimethyl-1,3-dioxo-lan-4-yl-methoxy)spiro-(isobenzofuran-1(3*H*),9′(9′*H*)-xanthen(-3-one (17)

A solution of compound **15** (64 mg, 0.14 mmol), chloro-acetyl chloride (13 μL, 0.16 mmol) and an excess of 4-dimethylaminopyridine (DMAP) in dry CH_2Cl_2 (3 mL) was heated under reflux for 6 h. The volatile components were removed under reduced pressure and the residue was purified by flash chromatography (pentane/EtOAc: 1/1) on silica gel to give the ester **17** as almost colorless solid (57 mg, 78%, mixture of diastereomers). R_f=0.38 ($CHCl_3$/CH_3OH: 10/1). ^1H NMR (3×00 MHz, $CDCl_3$): δ=1.41, 1.47 (2s, 6H), 3.90 (dd, J=8.3, 6.0 Hz, 1H), 3.98 (dd, J=9.6, 5.8 Hz, 1H), 4.08 (dd, J=9.6, 5.5 Hz, 1H), 4.18 (dd, J=8.3, 6.7 Hz, 1H), 4.32 (s, 2H), 4.49 (q, J= 5.8 Hz, 1H), 6.61–6.84 (m, 5H), 7.10–7.20 (m, 2H), 7.67 (m, 2H), and 8.04 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$): δ=25.3, 26.7, 40.7, 66.6, 69.1, 73.7, 82.2, 101.6, 109.9, 110.0, 111.3, 112.3, 116.9, 117.3, 123.9, 125.2, 126.4, 129.1, 129.3, 129.9, 135.2, 151.3, 151.8, 152.0, 152.9, 160.4, 165.4, and 169.2. m/z 521 (M^+-1, 2%), and 523 (M^+, 2%). HR-MS: $C_{28}H_{22}ClO_8$ requires 521.1003; found 521.0989.

4.12. 6′-(2-Chloroacetoxy)-3′-(2,3-dihydroxypropoxy)-spiro-(isobenzofuran-1(3*H*),9′(9′*H*)-xanthen(-3-one (18)

A solution of CAN (123 mg, 0.22 mmol) in 6 mL of H_2O was added at 70 °C under inert atmosphere to a stirred solution of the protected ester **17** (45 mg, 0.09 mmol) in 3 mL of CH_3CN. The mixture was stirred at 70 °C during 5 min. Then, H_2O was added (15 mL) and this mixture was extracted with $CHCl_3$ (3×15 mL). The combined organic layers were dried over $MgSO_4$ and the solvent was evaporated under reduced pressure affording 40 mg of pure product **18** (96%) as mixture of diastereomers. R_f=0.32 (pentane/EtOAc: 1/4). ^1H NMR (500 MHz, $CDCl_3$): δ= 2.04, 2.61 (2 broad s, 2H), 3.75 (dd, J=11.4, 5.4 Hz, 1H), 3.85 (dd, J=11.4, 3.8 Hz, 1H), 4.05–4.16 (m, 3H, CH_2), 4.31 (s, 2H), 6.62–6.84 (m, 5H), 7.12–7.17 (m, 2H), 7.67 (m, 2H), and 8.03 (d, J=7.5 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ=40.8, 63.5, 69.4, 70.2, 82.1, 101.7, 110.0, 111.5, 112.3, 117.0, 117.4, 123.9, 125.2, 126.4, 129.2, 130.0, 135.2, 151.4, 151.8, 152.1, 152.9, 160.3, 165.4, and 169.2. m/z 439 (M^+-44, <1%).

4.13. 3′,6′-Diacetyl-5(6)-carboxyspiro-(isobenzofuran-1(3*H*),9′(9′*H*)-xanthen(-3-one (20)

Acetic anhydride (1.08 g, 10.58 mmol) was added dropwise to a solution of carboxyfluorescein (2.00 g, 5.31 mmol) in dry pyridine (25 mL) and dry Et_3N (14 mL). The yellow solution was stirred for 36 h and then $HCCl_3$ (30 mL) was added, washed with HCl 10% (4 x 20 mL) and dried (Na_2SO_4). The residue was crystallized in EtOAc–pentane to obtain the desired diacetyl carboxyfluorescein **20** (1.75 g, 71%) as a white solid.

The observed spectral data were in accord with those reported in literature.[20]

4.14. 3′,6′-Diacetyl-5(6)-{tris[(*tert*-butyldimethyl)-silyl-oxymethyl]methylcarbamoyl}spiro-(isobenzo-furan-1(3*H*),9′(9′*H*)-xanthen(-3-one (22)

To a solution of compound **20** (200 mg, 0.43 mmol), amine 24 (202 mg, 0.43 mmol), and triethylamine (0.13 mL, 0.93 mmol) in dry acetonitrile (10 mL) was added TBTU (167 mg, 0.52 mmol). After stirring at rt for 25 min, brine (20 mL) was added and the aqueous layer extracted with EtOAc (3×15 mL). Organic layers were washed succes-sively with HCl 10% (20 mL), water (20 mL), $NaHCO_3$ 5% (2×25 mL), and water (20 mL), and dried with Na_2SO_4. Solvent was removed to obtain a yellow foam which was purified by silica gel flash chromatography (Et_2O/pentane: 1/1) affording fluorescein derivative **22** as a white foam (210 mg, 54%). ^1H NMR (200 MHz, $CDCl_3$, mixture of two compounds) δ=−0.03 (s, $18H_B$), 0.07 (s, $18H_A$), 0.78 (s, $27H_B$), 0.89 (s, $27H_A$), 2.32 (s, $6H_A$+$6H_B$), 3.88 (s, $6H_B$), 3.98 (s, $6H_A$), 6.28 (s, $1H_B$), 6.77 (s, $4H_A$+$4H_B$), 7.07 (s, $2H_A$+$2H_B$), 7.20 (m, $1H_A$), 7.36 (s, $1H_B$), 7.95 (d, J= 4.2 Hz, $1H_B$), 8.05 (m, $1H_A$+$1H_B$), 8.29 (s, $1H_A$). m/z 890 (M^+-Me, 52%), 848 ($MH^+-Me-Ac$, 100%), 806 ($MH^+-Me-2Ac$, 4%).

4.15. 3′,6′-Diacetyl-5(6)-[tris[(hydroxymethyl)methylcarbamoyl]spiro-(isobenzofuran-1(3*H*),9′(9′*H*)-xanthen(-3-one (23)

To a solution of fluorescein derivative **22** (100 mg, 0.11 mmol) in acetonitrile (2 mL) was added 1 mL of a solution of HF 5% (acetonitrile/water). Ten minutes later, CH_2Cl_2 (10 mL) and water (10 mL) were added. The aqueous layer was extracted with CH_2Cl_2 (3×10 mL), and organic layers were washed with brine (2×10 mL), and dried with Na_2SO_4. Solvent was removed to obtain fluoresceintriol **23** as a white solid (60 mg, 97%). ^{1}H NMR (200 MHz, $CDCl_3$, mixture of two compounds) δ= 2.19 (s, $6H_A$+$6H_B$), 3.60 (s, $6H_B$), 3.68 (s, $6H_A$), 6.60 (m, $3H_A$+$4H_B$), 6.98 (m, $2H_A$+$2H_B$), 7.15 (d, J=4.0 Hz, $1H_A$), 7.24 (s, $1H_B$), 7.42 (s, $1H_A$), 7.56 (s, $1H_B$), 7.95 (m, $1H_A$), 8.08 (d, J=4.2 Hz, $1H_B$), 8.39 (s, $1H_A$). *m/z* 564 (MH^+, 8%).

4.16. 7-Acetoxy-*N*-methylquinolinium iodide (25a)

A solution of 7-hydroxyquinoline **24** (60 mg, 0.41 mmol) in acetic anhydride (3 mL, 31.8 mmol) was stirred for 40 h at 37 °C. Methyl iodide (1.50 mL, 24.09 mmol) was added and the mixture was stirred at 50 °C for 24 h. A solid was formed. Ethyl ether was added to ensure complete precipitation, and the solution was filtered. The crude solid was crystallized three times from methanol–ethyl ether to obtain 115 mg (85%) of yellow crystals. Mp 202–203 °C (decomp.). ^{1}H NMR (200 MHz, $CDCl_3$) δ=2.43 (s, 3H), 4.73 (s, 3H), 7.74 (dd, J=9.0, 2.0 Hz, 1H), 8.09 (dd, J=8.3, 5.8 Hz, 1H), 8.17 (d, J=1.5 Hz, 1H), 8.33 (d, J= 9.0 Hz, 1H), 9.18 (d, J=8.3 Hz, 1H), 9.94 (d, J=6.0 Hz, 1H). ^{13}C NMR (50 MHz, MeOH-d_4) δ=21.11, 46.59, 112.20, 122.63, 127.36, 129.19, 133.35, 141.39, 148.50, 151.57, 157.77, 170.06. m/z 187 (M^+−Me, 5%), 160 (MH^+−Ac, 7%), 145 (MH^+−Me−Ac, 100%), 142 (M^+−AcOH, 43%). HR-MS: $C_{11}H_9NO_2$ requires 187.0633; found 187.0637.

4.17. 7-Propionyloxy-*N*-methylquinolinium iodide (25b)

A mixture of 7-hydroxyquinoline **24** (48 mg, 0.33 mmol) in propionic anhydride (3 mL, 23.4 mmol) was stirred at 40 °C for 48 h. Methyl iodide (1.50 mL, 24.09 mmol) was added and the mixture was stirred at reflux for 24 h. Ethyl ether was added and the yellow precipitate was filtered. The crude solid was crystallized twice from methanol–ethyl ether to obtain 47 mg (43%) of dark yellow crystals. Mp 159–160 °C (decomp.). ^{1}H NMR (200 MHz, $CDCl_3$) δ=1.30 (t, J=7.6 Hz, 3H), 2.75 (q, J=7.5 Hz, 2H), 4.80 (s, 3H), 7.73 (dd, J=9.0, 2.0 Hz, 1H), 8.10 (dd, J=8.3, 5.7 Hz, 1H), 8.22 (d, J=1.5 Hz, 1H), 8.42 (d, J=9.0 Hz, 1H), 9.04 (d, J=8.3 Hz, 1H), 10.16 (d, J=5.7 Hz, 1H). ^{13}C NMR (50 MHz, MeOH-d_4) δ=9.12, 28.51, 46.65, 112.16, 122.61, 127.36, 129.16, 133.35, 141.41, 148.49, 151.55, 157.89, 173.59. *m/z* 201 (M^+−Me, 12%), 159 (M^+−EtCO, 11%), 145 (MH^+−Me−EtCO, 100%), 142 (M^+−$EtCO_2H$, 52%), 57 ($EtCO^+$, 30%), 29 (Et^+, 49%). HR-MS: $C_{12}H_{11}NO_2$ requires 201.0790; found. 201.0784.

4.18. *N*-Methyl-7-benzoyloxyquinolinium iodide (25c)

A mixture of 7-hydroxyquinoline **24** (50 mg, 0.34 mmol) and benzoyl chloride (0.24 mL, 2.06 mmol) in dry CH_2Cl_2 (2 mL) was heated at reflux for 29 h. Water (5 mL) was added, the aqueous layer extracted with CH_2Cl_2 (3×5 mL), and the combined organic layers were washed with sat. $NaHCO_3$ (1×15 mL) and dried (Na_2SO_4). The crude was solved in dry CH_2Cl_2 (5 mL) and methyl iodide (1.00 mL, 16.06 mmol) was added. The mixture was stirred at 50 °C for 24 h and a solid was formed. Ethyl ether was added to ensure complete precipitation, and the solution was filtered. The crude solid was crystallized twice from methanol–ethyl ether to obtain 85 mg (64%) of yellow crystals. Mp 213–214 °C (decomp.). ^{1}H NMR (200 MHz, $CDCl_3$) δ=4.87 (s, 3H), 7.59 (tt, J=7.5, 1.5 Hz, 2H), 7.74 (tt, J=7.5, 1.5 Hz, 1H), 7.93 (dd, J=9.0, 2.0 Hz, 1H), 8.16 (dd, J=8.3, 5.8 Hz, 1H), 8.25 (t, J=1.8 Hz, 2H), 8.28 (dd, J=2.0, 1.0 Hz, 1H), 8.40 (d, J=8.8 Hz, 1H), 9.06 (dm, J=8.0 Hz, 1H), 10.46 (dm, J=5.7 Hz, 1H). ^{13}C NMR (50 MHz, MeOH-d_4) δ= 46.55, 112.37, 122.73, 127.43, 129.37, 129.77, 130.14, 131.49, 133.49, 135.73, 141.49, 148.59, 151.64, 158.02, 165.71. *m/z* 249 (M^+−Me, 8%), 142 (M^+−$PhCO_2H$, 26%), 105 ($PhCO^+$, 100%), 77 (Ph^+, 34%). HR-MS: $C_{16}H_{11}NO_2$ requires 249.0790; found 249.0789.

4.19. 7-(*tert*-Butylcarbonyloxy)-*N*-methylquinolinium iodide (25d)

A mixture of 7-hydroxyquinoline **24** (50 mg, 0.34 mmol) and pivaloyl chloride (0.26 mL, 2.13 mmol) in dry CH_2Cl_2 (2 mL) was heated at reflux for 29 h. Water (5 mL) was added and the aqueous layer was extracted with CH_2Cl_2 (3×5 mL). The combined organic layers were washed with sat. $NaHCO_3$ (1×15 mL), dried (Na_2SO_4) and concentrated. The residue was dissolved in dry CH_2Cl_2 (5 mL) and methyl iodide (1.00 mL, 16.06 mmol) was added. Upon stirring the mixture at 50 °C for 24 h a solid formed. Ethyl ether was added to ensure complete precipitation, and the solution was filtered. The crude material was crystallized twice from methanol–ethyl ether to obtain 66 mg (52%) of yellow crystals. Mp 180–181 °C (Decomp.). ^{1}H NMR (200 MHz, $CDCl_3$) δ=1.44 (s, 9H), 4.84 (s, 3H), 7.72 (dd, J=9.0, 2.0 Hz, 1H), 8.10 (s, 1H), 8.14 (dd, J=8.3, 5.8 Hz, 1H), 8.35 (dd, J=9.0, 1.5 Hz, 1H), 9.08 (dm, J=8.0 Hz, 1H), 10.35 (dm, J=6.2 Hz, 1H). ^{13}C NMR (50 MHz, MeOH-d_4) δ=27.22, 40.32, 111.87, 122.45, 127.08, 129.04, 133.24, 141.27, 148.33, 151.37, 158.03, 177.34. *m/z* 244 (M^+, 0.5%), 229 (M^+−Me, 11%), 186 (M^+−Me−^{t}BuH, 1%), 159 (M^+−^{t}BuCO, 6%), 145 (MH^+−^{t}$BuCO_2$, 94%), 142 (M^+−^{t}$BuCO_2H$, 60%), 57 (^{t}Bu^+, 100%). HR-MS: $C_{14}H_{15}NO_2$ requires 229.1103; found 229.1105.

Acknowledgements

We thank Professor Dr. R. W. Hoffmann for his continuous support and advice during the realization of this work. This work has been supported by the European Commission through the TMR network ERB FMRX CT-960011.

References and notes

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