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Synthesis of Oligonucleotides Labelled with a Novel Type of Chemically Stable Acridine Dye

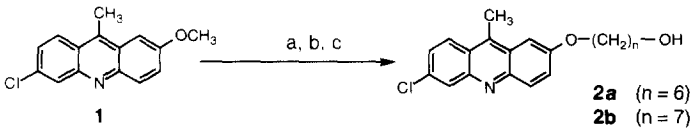
Karin Schütz, Markus Kurz and Michael W. Göbel*
Département de Chimie Organique; Université de Genève; 30, quai Ernest-Ansermet
CH-1211 Genève 4, Switzerland

Abstract: Alkoxy derivatives of 6-chloro-2-methoxy-9-methylacridine **1** have been attached to oligonucleotides **6** - **10**. The coupling step may be performed either with phosphoramidite-, *H*-phosphonate or phosphodiester reagents prepared from **2**. This dye, in contrast to 9-aminoacridines, is completely stable under the conditions of oligonucleotide deprotection (NH_3 or CH_3NH_2). The dye conjugates tightly bind to complementary strands and are useful tools in the study of nonenzymatic oligomerizations of ribonucleotides according to L. Orgel.

In our continuing search for the catalysis of transphosphorylation reactions [1] we are interested in the template directed but nonenzymatic oligomerization of ribonucleotides [2]. In order to monitor the proceeding of chain elongation we planned to apply short oligonucleotide primers labelled with a fluorescent dye. Especially, intercalating dyes should be advantageous in stabilizing the complexes between primers and templates [3,4]. Derivatives of 9-aminoacridines are well established for this purpose [3c-e]. However, their C9-N-bond is prone to hydrolysis which complicates preparation and use of such oligonucleotide conjugates. Here we present a novel type of chemically stable acridine dye [4] attached to oligonucleotides via side chains of variable length.

Starting from 6-chloro-2-methoxy-9-methylacridine **1** [5] the methyl ether was cleaved by use of hydrobromic acid. The crude phenole was then alkylated with the THP protected side chain building blocks [6] (91 - 98% overall yield). After acidolysis the acridine alcohols **2a** and **b** were obtained as readily crystallizing yellow compounds (93 - 96%).

Scheme 1: Attachment of side chains to 6-chloro-2-methoxy-9-methylacridine



1

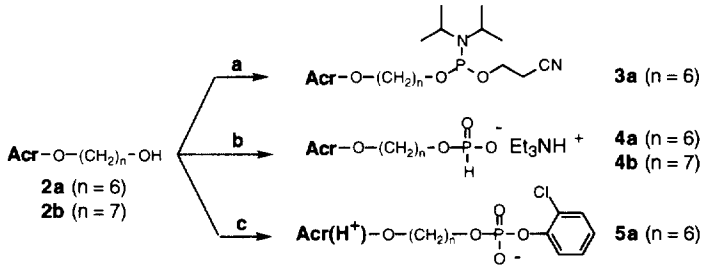
2a (n = 6)
2b (n = 7)

Reagents and conditions: **a**, HBr, glacial acetic acid, reflux, 7 h; **b**, 1.5 eq. Br-$(CH_2)_n$-OTHP, 5 eq. Cs_2CO_3, DMF, 110 °C, 10 h, 91 - 98%; **c**, dil. HCl, MeOH, reflux, 6 h, 93 - 96%.

2a and **b** could be transformed to all major types of phosphorylated derivatives used in oligonucleotide synthesis. Treatment of **2a** with bis(diisopropylamino)cyanoethoxyphosphine gave phosphoramidite **3a** in 69% yield [7]. The *H*-phosphonates **4a** and **b** were obtained by reaction of **2a** and **b** with phosphorus trichloride, 1*H*-1,2,4-triazole and *N*-methylmorpholine in 65% yield [8]. The phosphodiester **5a** was pre-

pared from **2a** and 2-chlorophenyldichlorophosphate for application in the phosphotriester technique [9]. Alternatively conjugation of **2a** with oligonucleotides was possible in one step according to van Boom's in situ phosphorylation/coupling method [10].

Scheme 2: Preparation of phosphorylated acridines **3** - **5** [16]



Reagents and conditions: **a**, 2.2 eq. bis(diisopropylamino)cyanoethoxyphosphine, 1.1 eq. diisopropylammonium-tetrazolide, pyridine, rt, 5 h, 69%; **b**, 40 eq. 1*H*-1,2,4-triazole, 50 eq. *N*-methylmorpholine, 5 eq. PCl_3, THF, pyridine, rt, 1.5 h, 65%; **c**, 1) 2 eq. 2-chlorophenyldichlorophosphate, pyridine, -10 °C, 10 min, 2) H_2O, 65%.

In order to obtain large amounts of labelled oligonucleotides (20 - 100 mg) we used both solution chemistry and manual solid phase preparation with highly charged supports [11]. Our acridine dye proved to be stable under the conditions of oligonucleotide synthesis, especially during base deprotection with ammonia or methylamine [12]. Results are summarized in table 1.

Table 1: Preparation of Acr-O-$(CH_2)_n$-O-PO_2-O-(oligonucleotides) **6** - **10** [15]

acridine building block	nucleotide moiety	product	method
H-phosphonate **4a** (n = 6)	- - - dG-dC-dA-dC-G - - - dG-dG-G - - - dA-dA-dA-dA-dA	**6** **7** **8**	a) H-phosphonate, solid phase
phosphoramidite **3a** (n = 6)	- - - dG-dG-G	**7**	b) phosphoramidite, solid phase
acridine alcohol **2b** (n = 7)	- - - dA-dA-A	**9**	c) phosphotriester, solution (v. Boom)
phosphodiester **5a** (n = 6)	- - - dT-dT-U	**10**	d) phosphotriester, solution

Reagents and conditions: **a**, 1) 5 eq. **4a**, 15 eq. pivaloyl chloride, oligonucleotide attached to Tentagel® (ca. 0.2 mmol/g), acetonitrile/pyridine 1 : 1, rt, 2 min; 2) 0.1 M I_2 in pyridine/*N*-methylmorpholine/H_2O/THF 5 : 1 : 5 : 90 followed by 0.1 M I_2 in NEt_3/H_2O/THF 5 : 5 : 90; 3) methylamine/ammonia 1 : 1, rt, 90 min or ammonia, 55 °C, 20 h; **b**, 1) 30 eq. **3a**, 60 eq. tetrazole, oligonucleotide attached to CPG-support (1 µmol), acetonitrile, rt, 2 h; 2) 0.1 M I_2 in THF/H_2O/lutidine 2 : 2 : 1; 3) methylamine/ammonia 1 : 1, rt, 4 h; **c**, 1) 1.1 eq. 2-chlorophenyldichlorophosphate, 2.2 eq. CF_3HOBt [10c], 2.2 eq. pyridine; 2) 1 eq. N_6,2',3'-benzoyl-protected dAdArA; 3) 4 eq. **2b**, THF, rt, 6h; 4) 25 eq. pyridine-2-carbaldoxime, 25 eq. tetramethylguanidine, THF, rt, 23 h; 5) ammonia, 55 °C, 72 h; **d**, 1) 1 eq. 2',3'-isopropylidene-protected dTdTU, 1.2 eq. **5a**, 11 eq. MSNT, pyridine, rt, 6 h; 2) 25 eq. pyridine-2-carbaldoxime, 25 eq. tetramethylguanidine, THF, rt, 12 h; 3) 80% acetic acid, reflux, 40 min.

The interactions of **6** - **10** with complementary oligonucleotides were investigated by CD- and UV-measurements. A strong induced Cotton effect in the visible band of the dye was observed in the CD spectrum of **9** when mixed with poly-U [13] (figure 1a). Together with a pronounced hypochromicity at 354 nm this observation proves a strong interaction between the dye and the bases which can be explained most likely by intercalation. No effect was visible either with **9** alone or in the presence of the noncomplementary poly-A. To quantify the stability of the aggregate, the melting point was determined to 42°C as shown in figure 1b [14]. However, the exact nature of these aggregates (duplex versus triplex) is still unknown. An unequivocal case of duplex formation (T_M = 49°C) was observed with the labelled oligonucleotide **6** Acr-d(GCAC)rG and d(CCCC**CGTGC**G), also proven by UV- and CD-measurements.

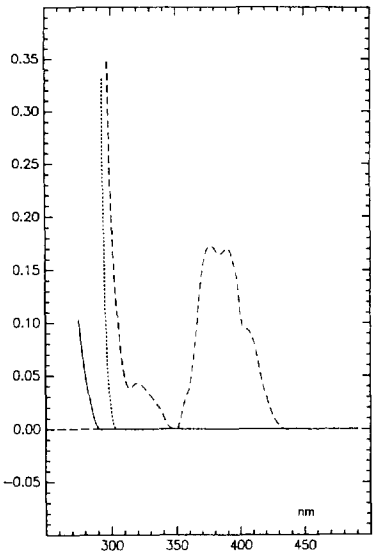


Figure 1a: CD spectrum of **9** [14]
(— **9** / - - - **9** + poly-U / ··· **9** + poly-A)

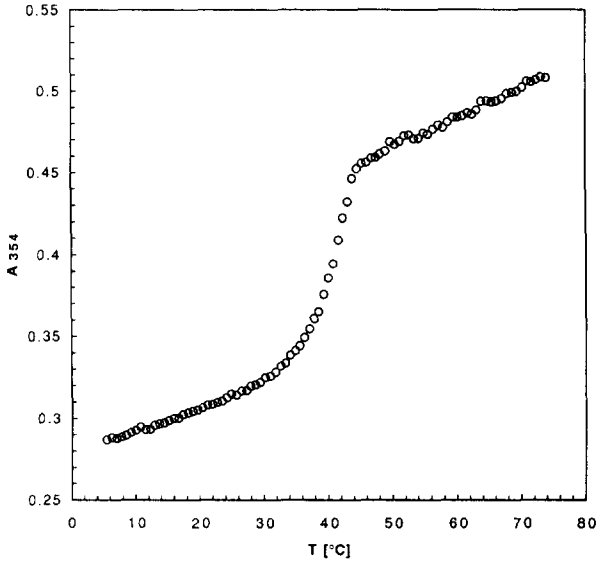


Figure 1b: Thermal dissociation profile of **9** and poly-U [14]
(y-axis: absorption of the acridine at 354 nm)

Template assisted elongation experiments of **6** and **9** according to Orgel [2a] were carried out with guanosine-5'-phosphoro(2-methylimidazolide) and the adenosine derivative, respectively. The reactions could be successfully monitored by HPLC, due to the colour, fluorescence and lipophilic properties of the acridine moiety. So, the new intercalating agent is well suited for our purposes. Furthermore some applications in antisense strategy may be envisaged.

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14. CD: 0.18 mM **9**, 2 mM poly-U or poly-A (calculated as triplets), H_2O, 10 mM Tris-buffer pH 8.3, 1 M NaCl; the effects changed only slightly between pH 5.6 and pH 10.3 and disappeared at pH 10.9; UV: 0.1 mM **9**, 0.5 mM poly-U, H_2O, 100 mM Tris-buffer pH 5.2, 1 M NaCl; the same value of T_M was observed at pH 7.0.
15. Satisfactory ^1H NMR and ESI MS data have been obtained.
16. Selected physical data: **2a**: mp. 162°C (ethyl acetate); UV/VIS: (ethanol); λ_{max} (ε) = 263 (142500), 319 (2670), 335 (5000), 352 (7360), 382 (6500), 401 nm (6190); **2b**: mp. 154 - 155°C (ethyl acetate); UV/VIS: like spectrum of 2a; ^1H NMR: (270 MHz, [D_6]DMSO): δ = 1.33 - 1.53 (m, 8 H, methylene-H), 1.82 (m, 2 H, methylene-H), 3.02 (s, 3 H, CH_3), 3.39 (m, 2 H, methylene-H), 4.18 (t, J = 6.5 Hz, 2 H, methylene-H), 4.34 (t, J = 5.1 Hz, 1 H, OH), 7.44 (d, J = 2.6 Hz, 1 H, 1-H), 7.52 (dd, J = 9.4 and 2.5 Hz, 1 H, 3-H), 7.56 (dd, J = 9.2 and 2.1 Hz, 1 H, 7-H), 7.99 (d, J = 9.4 Hz, 1 H, 4-H), 8.09 (d, J = 2.1 Hz, 1 H, 5-H), 8.36 (d, J = 9.4 Hz, 1 H, 8-H); **3a**: ^{31}P NMR: (162 MHz, C_6D_6): δ = 148.5 (m); **4a** / **4b**: ^{31}P NMR: (162 MHz, [D_6]-DMSO): δ = 2.55 (dt, J_d = 589 Hz, J_t = 8.1 Hz); **5a**: ^{31}P NMR: (162 MHz, [D_6]DMSO): δ = - 5.50 (t, J = 7.1 Hz).

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