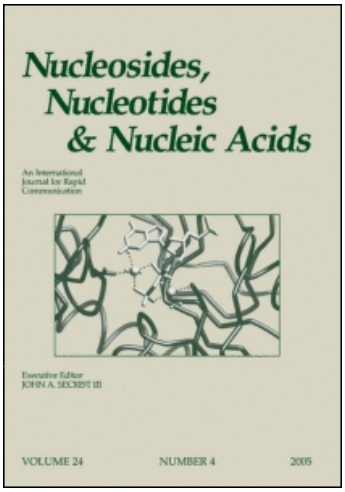

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SYNTHESIS OF NOVEL OLIGODEOXYNUCLEOTIDE CONJUGATES CONTAINING THE ANIONOPHORIC MOIETY OF PAMAMYCIN †

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ABSTRACT: A series of oligodeoxynucleotides bearing the alkaline hydrolysis product of pamamycin, an antibiotic from *Streptomyces aurantiacus* with penetration enhancement potency, were prepared by automated solid-phase synthesis. In order to allow conjugation of the anionophoric moiety to the 3'-terminus of oligodeoxynucleotides, an appropriately derivatized CPG support was synthesized.

The biological efficacy of antisense oligonucleotides is predominantly determined by their binding affinity to complementary target sequences, their stability against nucleases, and by their ability to penetrate cellular membranes [1, 2]. While there are many practicable solutions for stabilization of oligodeoxynucleotides against nuclease degradation and also for enhancing their binding affinity, the cellular uptake of oligonucleotides still requires optimization [3-5]. In order to improve the cellular uptake of oligodeoxynucleotides two major routes are described in the literature which are either the formulation of oligodeoxynucleotides with uptake enhancers, such as lipocations [6], or their conjugation to uptake-enhancing non-nucleotidic moieties [7], such as lipophilic derivatives of cholesterol and vitamin E [8, 9].

Recently, pamamycin **1** (FIG. 1) was isolated from *Streptomyces aurantiacus*. Pamamycin forms lipophilic ion pairs and therefore may be useful as a penetration enhancer for acidic pharmaceuticals [10, 11]. In preliminary experiments we found that pamamycin at concentrations of 2 to 10 mg/ml is able to convey an oligodeoxynucleotide from the aqueous layer into the toluene layer of a membrane lipophilicity model [12].

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† dedicated to late Professor Tsujiaki Hata

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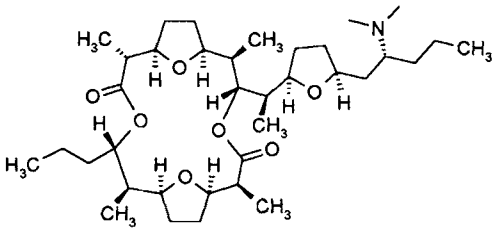

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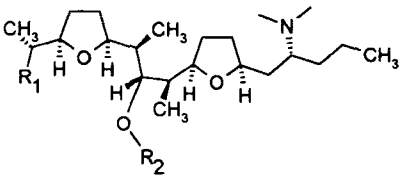


2 - 6

	R_1	R_2
2	COOH	H
3	CH_2OH	H
4	CH_2O-Dmt	H
5	CH_2O-Dmt	$C(O)CH_2CH_2COOH$
6	CH_2O-Dmt	$C(O)CH_2CH_2C(O)NH$--CPG

FIG. 1: Structure of pamamycin **1**, its hydrolysis product **2**, and DMAD derivatives **3 - 6**.

Furthermore, the hydrolysis product **2** of pamamycin shows much lower cytotoxicity as compared to pamamycin itself while its ability to form lipophilic ion pairs is retained [10]. This led us to the design and synthesis of novel oligodeoxynucleotide conjugates in which the anionophoric part of pamamycin, namely 2-[5-(2-dimethylamino-pentyl)-tetrahydro-furan-2-yl]-4-[5-(2-hydroxy-1-methyl-ethyl)-tetrahydro-furan-2-yl]-pentan-3-ol (DMAD) **3**, is covalently attached *via* a phosphodiester or a phosphorothioate linkage to the 3'-end of oligonucleotides.

RESULTS AND DISCUSSION

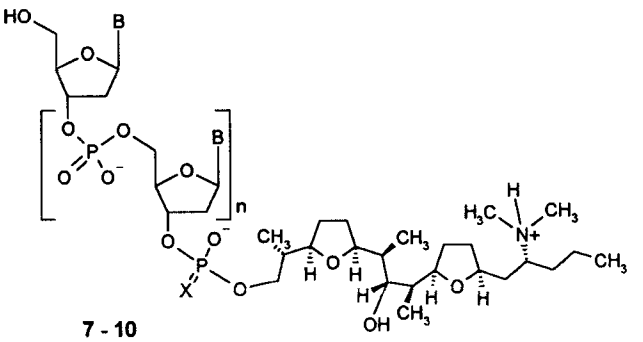
Pamamycin was hydrolyzed by alkaline treatment to carboxylic acid derivative **2** followed by reduction with lithium aluminium hydride to give DMAD **3** as described previously [10, 11]. DMAD **3** has both a primary and a secondary hydroxyl group allowing functionalization similarly to deoxynucleosides used in standard solid-phase synthesis of oligonucleotides. In a first step, the primary hydroxyl group of **3** could be selectively reacted with dimethoxytrityl (Dmt) chloride to give Dmt-DMAD derivative **4** which, without isolation, was succinylated by treatment with succinic anhydride in the presence of *N,N*-dimethylaminopyridine to give, after chromatographic purification, synthon **5** in 66 % overall yield. In the next step, the Dmt-DMAD succinate **5** was

anchored to controlled pore glass (CPG) by means of TBTU/*N*-ethylmorpholine activation to give the DMAD-derivatized CPG support **6** with a loading of 26 μmol/g which was then used for solid-phase oligonucleotide synthesis.

Four different oligodeoxynucleotides were synthesized (FIG. 2) having the DMAD moiety covalently bound to their 3'-terminus. In order to stabilize the oligodeoxynucleotides against nucleolytic degradation, they were modified either by end-capping (oligonucleotides **7** and **8**) or according to the 'minimal protection strategy' (oligonucleotides **9** and **10**) which is a combination of end-capping and additional protection at internal pyrimidine sites where endonuclease degradation is most serious [13]. Synthesis was performed using standard phosphoramidite chemistry on an automated DNA synthesizer with two necessary changes as compared to the standard protocol. Firstly, for efficient coupling of the first three bases the waiting time with phosphoramidite/tetrazole was increased to 240 seconds. Secondly, for cleavage of the DMAD-oligodeoxynucleotide conjugate from the CPG support the time of ammonia treatment was increased from 2 h (room temperature) to 16 h at 50°C resulting in complete cleavage from CPG and deprotection of the oligonucleotide. When the cleavage was performed under standard conditions (conc. ammonia for 2 h at room temperature), the yield was only about 35% of that of the overnight treatment.

The crude oligodeoxynucleotide conjugates were analyzed by ion exchange HPLC. The elution profile of crude DMAD-oligodeoxynucleotide conjugate **7** is depicted in Figure 3. The efficiency of synthesis using the DMAD-derivatized CPG support **6** almost equals the one with standard CPG supports, if the coupling time is extended for the first three bases as described above. The average coupling yields for all bases were estimated to be >98% by measurement of the dimethoxytrityl cation. The relatively strong impurity with a retention time of 26.4 min in the HPLC most likely results from incomplete sulfurization, since none of the oligodeoxynucleotides showed any (n-1) bands when analyzed by polyacrylamide gel electrophoresis. Furthermore, all new DMAD-oligodeoxynucleotide conjugates **7** - **10** were characterized by negative ion electrospray mass spectrometry which in all cases confirmed the expected molecular weight of the novel compounds (FIG. 2).

We have measured the melting temperatures (*Tm*) of the 3'-DMAD-modified oligonucleotides **8** and **9** against their complementary DNA (TABLE 1). The anionophoric moiety of pamamycin does not negatively influence binding affinity of these conjugates as can be seen by comparison with the *Tm*-values of the corresponding 3'-unmodified oligonucleotides **11** and **12**. There is even a tendency to stabilization of the duplexes containing DMAD moieties which may be explained by the positive charge of the dimethylaminopentyl residue.



7 - 10

	oligodeoxynucleotide sequence	X	M_c	M_f
7	5'-C*C*A G G G T A C A G G T G G C C G*G*C-DMAD	O	6710	6712
8	5'-G*C*G G G G C T C C A T G G G G G T*C*G-DMAD	O	6757	6758
9	5'-G*G*C* T G C*C A*T G G T*C*C*-DMAD	S	4846	4846
10	5'-G*G*G*A C C*A T*G G C A*G*C*-DMAD	S	4904	4903

FIG. 2: Sequence, phosphorothioate (*) pattern, calculated (M_c) and found (M_f) molecular weights, respectively, of oligodeoxynucleotides **7 - 10**.

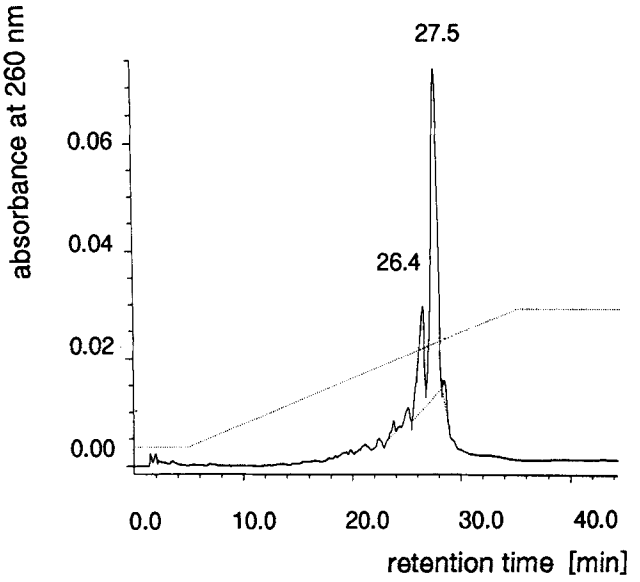


FIG. 3: HPLC-analysis of crude DMAD-oligodeoxynucleotide conjugate **7**.

TABLE 1: Binding affinities of DMAD-modified oligonucleotides **8** and **9** to complementary DNA as compared to the corresponding 3'-unmodified derivatives **11** and **12**, respectively.

	oligodeoxynucleotide	T_m (°C) [a]
8	5'-G*C*G G G G C T C C A T G G G G G T*C*G-**DMAD**	72.4 [b]
11	5'-G*C*G G G G C T C C A T G G G G G T*C*G-OH	71.3 [b]
9	5'-G*G*C* T G C*C A*T G G T*C*C*-**DMAD**	57.6 [c]
12	5'-G*G*C* T G C*C A*T G G T*C*C-OH	55.5 [c]

[a] Melting curves were measured against the complementary DNA at 1 μM in 140 mM NaCl, 10 mM HEPES, pH 7.5 at 15-85°C, heating rate 0.5°C/min on a Hewlett Packard 8452 Diode Array spectrophotometer. [b] measured against 3'-d(CGCCCCGAGGTACCCCCAGC)-5' [c] measured against 3'-d(CCGACGGTACCAGG-5').

Oligodeoxynucleotides **7** and **8**, directed against immediate early gene IE 110 of HSV-1 [14], and oligodeoxynucleotides **9** and **10**, directed against basic fibroblast growth factor, are now under investigation in cell-based assay systems with respect to their cellular uptake characteristics and biological activity in comparison to the 3'-underivatized analogues.

EXPERIMENTAL

1-O-Dimethoxytrityl-2-[5-{3-[5-(2-dimethylaminopentyl)tetrahydrofuran-2-yl]-2-hydroxy-1-methyl-butyl}tetrahydrofuran-2-yl]propanol (**4**). DMAD **3** (81 mg, 0.2 mmol) was prepared as reported previously [10, 11], dried by co-evaporation with pyridine and dissolved in anhydrous pyridine (0.6 mL). This solution was cooled with ice water, and a solution of dimethoxytrityl chloride (69 mg, 0.2 mmol) in pyridine (0.5 mL) was added dropwise within 30 min. The reaction mixture was stirred for additional 150 min at ambient temperature. Since the reaction was still incomplete after this time, three additional portions of dimethoxytrityl chloride (each 17 mg, 0.05 mmol, in 0.1 mL pyridine) were added at 2 hour time intervals. The reaction was quenched by the addition of water (5 μl) and concentrated in vacuum. The residue was dissolved in methylene chloride (4 mL) and the resulting solution was extracted three times with 0.1 M phosphate buffer pH 7.0 (2 mL). Some unreacted DMAD **3** could be isolated from the aqueous phase. After drying the organic phase over sodium sulfate, it was concentrated in

vacuum. The resulting residue was co-evaporated two times with toluene to give crude **4** as a white foam. Since the starting material was retained in the aqueous phase, isolated Dmt-DMAD **4** (140 mg) was virtually pure, and was used after characterization by mass spectrometry for succinylation without further purification. MS (FAB, NBA): 702.6 (M^+); M calculated: 701.99.

1-O-Dimethoxytrityl-2-[5-{3-[5-(2-dimethylaminopentyl) tetrahydrofuran-2-yl]-2-succinyloxy-1-methyl-butyl}tetrahydrofuran-2-yl]propanol (**5**). Dmt-DMAD **4** (ca. 135 mg, 0.2 mmol) was dried by co-evaporation with anhydrous pyridine and was dissolved in absolute pyridine (0.4 mL). To this solution *N,N*-dimethylaminopyridine (29 mg, 0.24 mmol) and succinic anhydride (24 mg, 0.24 mmol) were added, and the mixture was stirred at ambient temperature for a total of 48 hours. After 18, 27 and 42 hours, additional portions of succinic anhydride were added (30, 60 and 30 mg) to drive the reaction to completion. After concentration of the reaction mixture in vacuum, the resulting residue was dissolved in methylene chloride (4.5 mL). The organic phase was washed with 10% citric acid (2.5 mL) followed by water (three times 2.5 mL). The organic phase was dried with sodium sulfate, filtered and evaporated in vacuum. The crude Dmt-DMAD succinate **5** was purified by chromatography on a silica gel column (10 g, 35 - 70μm) using methylene chloride / methanol with 1% triethylamine (98/2 (25 mL); 96/4 (100 mL); 92/8 (100 mL); 90/10 (50 mL)). Homogeneous fractions containing **5** were combined, concentrated in vacuum and dried (106 mg). The Dmt-DMAD-succinate **5** was characterized by FAB mass spectrometry. MS (FAB, NBA, LiCl): 802.5 (M^+); M calculated: 802.06.

Preparation of CPG solid-support **6** *derivatized with Dmt-DMAD-succinate* **5**. The Dmt-DMAD-succinate **5** (12 mg, 0.015 mmol) was activated in DMF (1 mL) using TBTU (4.8 mg, 0.015 mmol) and *N*-ethylmorpholine (2.4 μL, 0.019 mmol). Aminopropyl-CPG (250 mg, 0.0125 mmol; 550 Å; 50 μmol/g amino functions; Fluka, batch # 302 934 990) was agitated with the preactivated succinate for 24 hours at ambient temperature. After removal of the excess succinate and washing of the CPG with methylene chloride, capping of unreacted amino functions was performed with acetic anhydride/*N*-methylimidazole/pyridine. The capping reagent was removed and the derivatized CPG support **6** washed successively with tetrahydrofuran, methylene chloride and diethyl ether, and finally dried in vacuum. Loading of the CPG support **6** with Dmt-DMAD-succinate was determined to be 26 μmol/g.

Solid-phase synthesis of DMAD-oligodeoxynucleotide conjugates **7 - 10.** Oligonucleotide conjugates were synthesized on a 1 μmol scale starting with Dmt-DMAD-succinate derivatized CPG support **6** on an Applied Biosystems 380B DNA synthesizer (Applied Biosystems, Inc., Foster City, USA) and applying standard phosphoramidite chemistry. However, in the first three coupling steps, the waiting time on incubation with phosphoramidite/tetrazole was increased from 180 (3 x 30 s plus once 90 s) to 240 seconds (3 x 40 s plus 1 x 120 s). After coupling, phosphorothioate linkages were introduced at the indicated positions by sulfurization with the Beaucage reagent [15] followed by capping with acetic anhydride and *N*-methylimidazole. Cleavage of DMAD-oligodeoxynucleotide conjugates from the solid support and final deprotection was achieved by treatment of the derivatized CPG with conc. ammonia for 18 hours at 50°C. After evaporation of the ammonia, solutions of the oligodeoxynucleotides were desalted and separated from solubilized CPG *via* PD10 Sephadex (Pharmacia) columns to give 95 to 120 OD_{260} of the desired DMAD-oligodeoxynucleotide conjugates **7 - 10**. All oligodeoxynucleotides were analyzed by HPLC on a Gen-Pak Fax column (Millipore-Waters) using a NaCl-gradient (buffer A: 10 mM NaH_2PO_4, 100 mM NaCl in acetonitrile/water = 1:4/v:v pH 6.8; buffer B: 10 mM NaH_2PO_4, 1.5 M NaCl in acetonitrile/water = 1:4/v:v; 5 to 40% B in 30 minutes). DMAD-oligodeoxynucleotide conjugates **7 - 10** were further analyzed by negative ion electrospray mass spectroscopy (Fisons Bio-Q) which in all cases confirmed the calculated mass (FIG. 2).

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