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SOLID PHASE SYNTHESIS OF OLIGONUCLEOTIDES CARRYING PUROMYCIN AT 3'-TERMINAL

Agnes Nyilas, Sudhir Agrawal* and Paul Zamecnik

Worcester Foundation for Experimental Biology
222 Maple Avenue, Shrewsbury, MA 01545

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Abstract: For incorporation of puromycin at 3'-end of oligonucleotides or their analogs, suitably protected puromycin has been attached to controlled pore glass (CPG).

The use of antisense oligonucletides and their analogues to modulate gene expression is becoming an important tool in basic as well as applied science. The antisense oligonucleotides have been found to regulate cellular as well as viral gene expression (1-3).

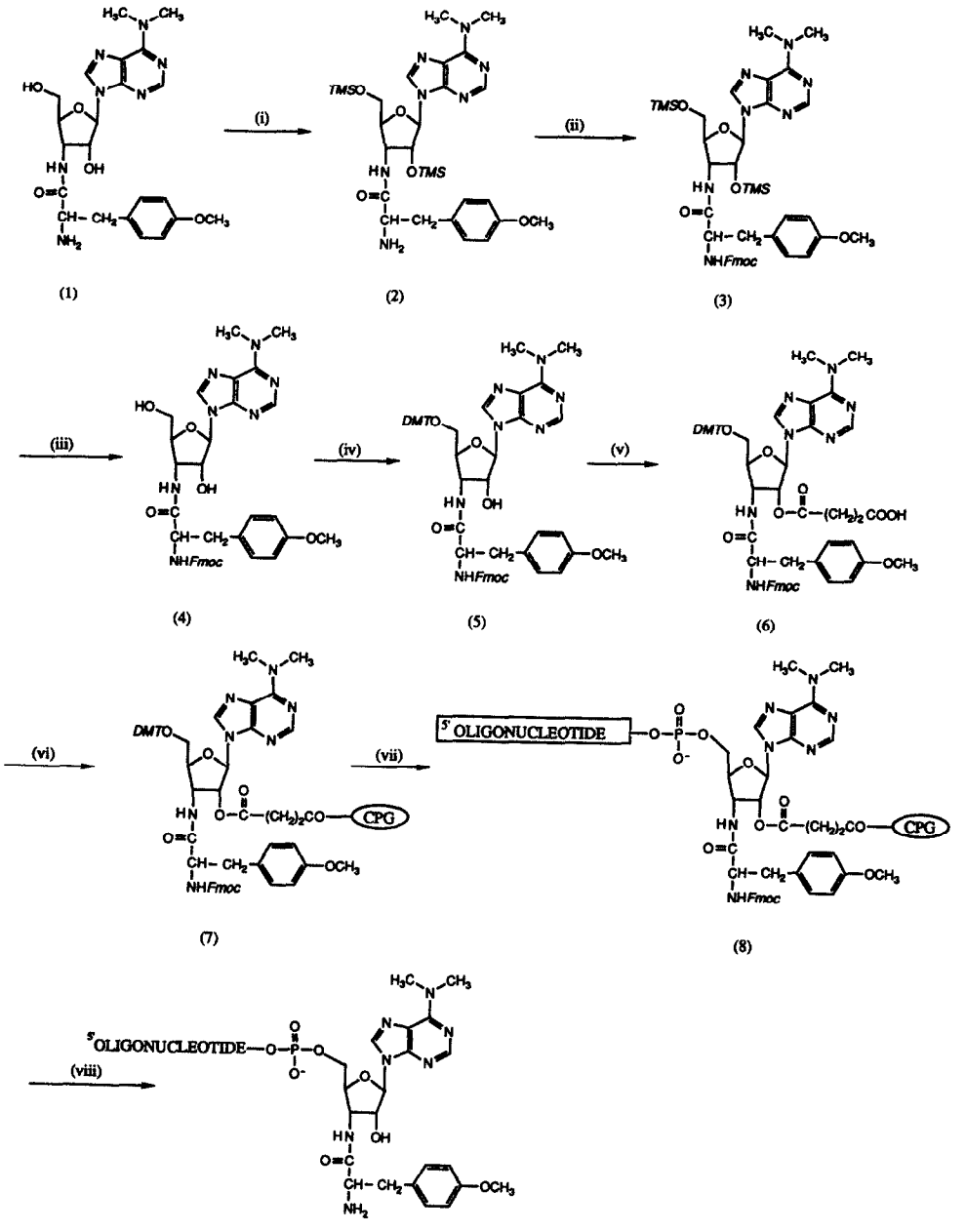
Several oligonucleotide conjugates have been studied to improve the gene regulation activity of oligonucleotides. Oligonucleotide conjugates which have been studied include cholesterol (4), lipids (5,6), alkyl chains (7) and others (8). Here we have studied the oligonucleotides carrying puromycin as terminal unit at the 3'-end. Puromycin is known to inhibit protein synthesis by binding to the aminoacyl (A) site of ribosomes and inhibiting the entry of aminoacyl-tRNA at this site (9). In a previous study, puromycin has been attached to the 3'-hydroxyl moiety of thymidine residue in solution phase (10).

For incorporation of puromycin at the 3'-end of an oligonucleotide, puromycin was first suitably protected and then attached to CPG. The synthetic steps are shown to *scheme I.*

Puromycin dihydrochloride hydrate (0.1 g; 0.18 mmole) was co-evaporated with toluene and dissolved in dry dichloromethane (1.8 ml). To the solution, trimethylsilylchloride (6 equiv.; 0.15 ml) and triethylamine (6 equiv.; 0.16 ml) were added and the reaction mixture was stirred for 90 minutes under nitrogen. The reaction mixture was then poured into saturated sodium bicarbonate solution (20 ml) and extracted with CH_2Cl_2 (20 ml). The organic layer was dried over $MgSO_4$, filtered, evaporated and co-evaporated with toluene, and product **2** was taken up in dry pyridine (1 ml). To the solution was added fluorenylmethyl chloroformate (1.2 equiv.; 0.06 g) dissolved in dry CH_2Cl_2 (0.8 ml). The reaction mixture was stirred for 30 minutes and then mixed with CH_3OH (0.5 ml). The reaction mixture was co-evaporated with toluene and applied to silica gel for chromatography. The column was eluted with 10% CH_3OH - CH_2Cl_2 to obtain product **3**. Trimethylsilyl groups were hydrolyzed with 80% aq. CH_3COOH to obtain product **4**. A dimethoxytrityl group was introduced to product **4** to obtain product **5** by a known procedure (11). The overall yield of the above four steps of the reactions was 78%.

Product **5** (0.17 g; 0.17 mmole) was dried, dissolved in dry pyridine (2 ml), mixed with succinic anhydride (0.02 g; 0.17 mmole) and dimethylaminopyridine (0.01 g; 0.09 mmole). The reaction mixture was stirred overnight and progress of the reaction was followed by thin layer chromatography (5% CH_3OH-CH_2Cl_2).

Scheme 1



Steps for derivatizing CPG with puromycin. (I) Trimethylsilyl chloride (TMSCl) in triethylamine; (II) fluorenylmethyl chloroformate (Fmoc Cl) in CH_2Cl_2/pyridine; (III) Aqueous CH_3COOH; (IV) Dimethoxytritylchloride (DMTCl) in pyridine; (V) Succinic anhydride, dimethylaminopyridine in pyridine; (VI) Dicyclohexylcarbodiimide in dimethylformamide and pyridine (9:1); (VII) Oligonucleotide synthesis using H-phosphonate approach and; (VIII) Deprotection in conc. ammonia.

The reaction mixture was poured into ice cold citric acid solution (20 ml) and extracted with CH_2Cl_2 (20 ml). The organic phase was evaporated and product **6** was purified using silica gel chromatography (2% CH_3OH - CH_2Cl_2). Yield - 0.15 g., 78%.

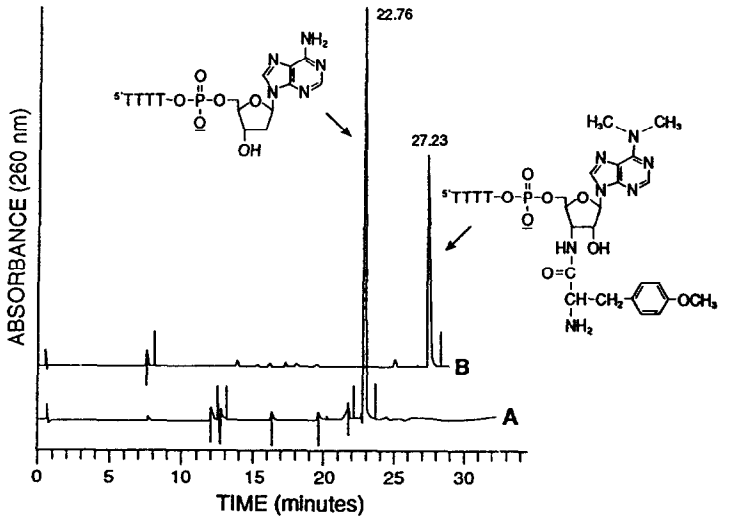


Figure 1.

Reversed phase HPLC traces of oligonucleotides A and B (for HPLC condition - see reference 12).

Product **6** (0.04 g; 0.04 mmole) and long chain alkyl amine CPG (1 g) were suspended in dimethylformamide/pyridine (2.5 ml; 9:1), and mixed with DCC (0.1 g; 0.4 mmole). The reaction mixture was stirred for 48 hours at room temperature. Puromycin derivatized CPG **7** was washed and dried. An aliquot of the CPG was checked for loading and found to be 20.9 μmole/g.

The following oligonucleotides were synthesized.

A. ^{5}TTTTA

B. ^{5}TTTTP

The oligonucleotides were synthesized on a 1 μM scale using H-phosphonate chemistry. Support bound oligonucleoside H-phosphonate intermediate was then oxidized with 2% iodine in pyridine/H_2O (98:2 v/v) to generate the phosphodiester backbone. Deprotection was carried out with concentrated ammonia at 55° for 6 hours.

Oligonucleotide **A**, T T T T A, has been synthesized as a control in which the 3'-terminal nucleoside is adenosine. Oligonucleotide **B**, T T T T P, was synthesized in which the 3'-terminal nucleoside is puromycin. Reversed phase HPLC analysis of oligonucleotide **A** and **B** showed that oligonucleotide **B** was retained more on the column compared to oligonucleotide **A**, possibly because of increased hydrophobicity due to puromycin (figure 1). However, ion exchange HPLC analysis showed that both oligonucleotides **A** and **B** had almost the same retention time. Base composition analysis of oligonucleotide **A** and **B**, after digestion with spleen phosphodiesterase was in agreement. Oligonucleotide **B** yielded puromycin as the digestion product, the identity of which was confirmed by reversed phase HPLC using an authentic sample.

Puromycin is an antibiotic whose resemblance to a foreshortened transfer RNA (11) results in covalent binding of a growing peptide chain to the p-methoxytyrosyl residue attached to a 3'-amino residue of the ribosyl moiety of puromycin. The peptidyl puromycin then separates from the A site on the ribosome, since there is no segment of this molecule capable of hybridizing with the 50S ribosome. No further peptide synthesis occurs, but instead a premature termination of the chain. Toxicity of puromycin has been a barrier in its clinical use. However, it is possible that a puromycin residue may be targeted to the initiation area of a viral or other pathogenic protein by an appropriate antisense oligonucleotide sequence. In this way, its abortive effect on peptide chain growth may be localized, and its general toxicity to protein sythesis minimized. With this thought in mind, several oligodeoxynucleotide heterosequences (phosphodiester as well as phosphorothioate) containing 3'-terminal puromycin have been synthesized and are being studied for their gene regulatory activity in cell-free as well as in tissue culture systems.

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* Present address: Hybridon, Inc., One Innovation Drive, Worcester, MA 01605

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12. HPLC was carried out using Waters 600E Gradient System, Waters 684 UV detector, 7125 Rheodyne injector and 746 data module. Buffers used were (A) 100 mM NH_4OAc and (B) 20% buffer A containing 80% CH_3CN. Flow 1.5 ml min^{-1}. Gradient 0% B for 2'. 0-20% B in 40 minutes. Detector 260 nm.