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SOLID-PHASE SYNTHESIS OF CARBOHYDRATE AND PHOSPHODIESTER MODIFIED 2'-5' OLIGOADENYLATE ANALOGS

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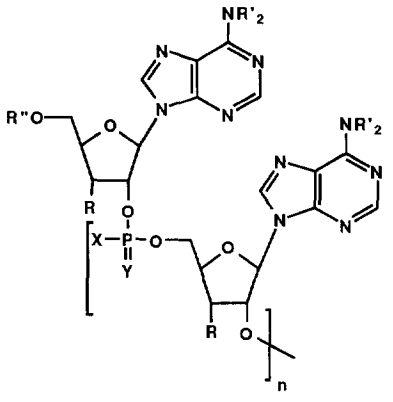
Abstract: A series of 2'-5' oligoadenylate analogs containing internucleotide and ribose modifications were prepared by solid-phase methods as potential interferon mimetics. All syntheses were carried out using automated methodologies with precursors that allow for the generation of multiple combinations of modification.

Interferons mediate diverse and important cellular responses such as the induction of an antiviral state, motility, proliferation, and various immunological processes.[1] One extensively characterized mechanism of interferon action is the (2'-5')$(A)_n$ Synthetase-RNase L pathway.[1,2] Oligoadenylates that are linked 2' to 5' [Figure 1, **1**, (2'-5')$(A)_n$] are produced by (2'-5')$(A)_n$ Synthetase in response to interferon and double-stranded RNA. These molecules activate a latent endoribonuclease (RNase L) that catalyses the degradation of all cellular and viral RNA. The third member in the pathway is the 2'-5' phosphodiesterase that rapidly degrades (2'-5')$(A)_n$. Oligoadenylate analogs with nuclease stabilizing modifications that retain their ability to act as effectors in this pathway may prove to be potent affectors of numerous biological phenomena. Molecules bearing desirable properties may find therapeutic use for their antiviral, antineoplastic or antimitogenic effects. By either activating or inhibiting the various enzymes involved in this pathway one can imagine indications for (2'-5')$(A)_n$ analogs as small molecule substitutes for interferon or adjuvants in interferon therapy. The potential utility of this class of compounds has prompted the synthesis of several modified 2'-5' oligoadenylates with agonist, antagonist or antiviral activities.[2-6] Here we report the solid-phase synthesis of several analogs of (2'-5')$(A)_n$ in which the ribose and internucleotide linkages have been modified. Solid-phase methodologies facilitate the rapid preparation of known and novel analogs allowing for their assessment as effectors of the interferon pathway.



	R	R'	R"	X	Y	n
1	OH	H	$(PO_3)_3$	O	O	2-3
2	OH	H	H	S	O	3
3	OH	H	H	S	S	3
4	H	H	H	O	O	3
5	H	H	H	S	O	3
6	H	H	PO_3	S	O	3
7	H	H	H	S	S	3
8	H	H	PO_3	S	S	3
9	H	H	H	S	S	2
10	OCH_3	H	H	O	O	3
11	OCH_3	H	PO_3	O	O	3
12	OCH_3	H	H	S	O	3
13	OCH_3	H	PO_3	S	O	3
14	NH_2	H	H	O	O	3
15	NH_2	H	H	O	O	2
16	NH_2	H	PO_3	O	O	3
17	NH_2	CH_3	H	O	O	3

Figure 1. Structure of 2'-5' oligoadenylate and analogs.

In the design of the analogs, we sought to incorporate several modifications that have been shown to stabilize nucleic acids to degradation by nucleases[7] while maintaining a structural similarity to the natural product. Therefore, in addition to the 3'-OH we targeted the 3'- deoxy, O-methyl, and amino ribose modifications and the phosphorothioate and phosphorodithioate internucleotide phosphodiester analogs. All of the targeted oligomers were synthesized using phosphoramidite chemistry on solid support.[8] Using this approach, a common nucleoside precursor could be derivatized to the phosphoramidite or phosphorothioamidite and used in automated solid-phase synthesis with standard oxidation or sulfurization techniques to lead to the phosphodiester, phosphorothioate, or phosphorodithioate derivatives (Figure 2). This convergent approach allowed for the generation of several novel analogs with a combination of modifications.

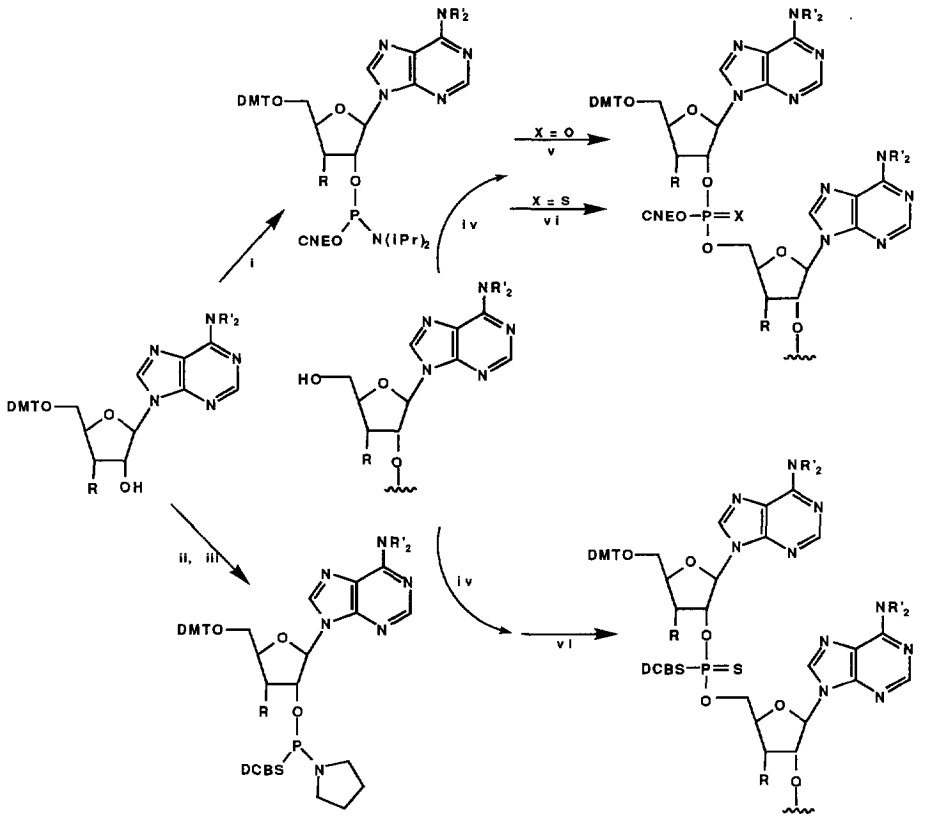


Figure 2. Synthesis of multiple 2'-5' oligoadenylate analogs from a common precursor: (i) 2-cyanoethyl-N,N-diisopropyl-chlorophosphoramidite/2,4,6-collidine; (ii) tris(pyrrolidino)phosphine/tetrazole; (iii) 2,4-dichlorobenzylmercaptan/tetrazole; (iv) tetrazole; (v) iodine/water/2,6-lutidine; (vi) phenylacetyl disulfide/triethylamine; R, R' - as described in synthesis of nucleoside precursors, CNE - 2-cyanoethyl, iPr - isopropyl, DCB - 2,4-dichlorobenzyl

Synthesis of nucleoside precursors

3'-Deoxyadenosine (cordycepin) was synthesized according to literature procedures.[9] The resultant nucleoside was protected with dimethoxytrityl at the 5'-OH position and benzoyl at the N^6 position of the base.[10]

3'-O-methyl-3'-deoxyadenosine was synthesized using a variation of a published procedure by Robins et al.[11] The nucleoside was protected with dimethoxytrityl at the 5'-OH and benzoylated at N^6. 3'-Azido adenosine was prepared by glycosidation[12] of N^6-benzoyl adenine with 5'-O-methoxycarbonyl-3'-azido-1',2'-di-O-acetylribofuranose.[13] The nucleoside thus generated was deprotected using 8 M ammonia in methanol and HPLC purified as described.[11b] The azido group was reduced using H_2/10% palladium on carbon. The resultant amino group was Fmoc protected with fluorenyl (methoxycarbonyl)-succinimide.[14] The nucleoside was protected at the 5' position with dimethoxytrityl and benzoylated at N^6. Puromycin nucleoside (3'-amino-3'-deoxy-(N^6-dimethyl)adenosine) was Fmoc protected at the 3'-NH_2 position using fluorenyl(methoxycarbonyl)-succinimide.

Synthesis of phosphoramidites and phosphorothioamidites

Phosphoramidites of the nucleoside derivatives were achieved using standard procedures.[8] Appropriately protected nucleosides were phosphitylated using 2-cyanoethyl-N,N-diisopropyl chlorophosphoramidite and purified by flash chromatography (silica gel washed with 1% TEA; ethyl acetate/hexane). The phosphorothioamidite synthons were prepared according to Beaton et al. with slight modifications.[15] Tris(pyrrolidino)phosphine was treated with the appropriately protected nucleoside in the presence of tetrazole to yield the bis(pyrrolidino)phosphoramidite. 2,4-Dichlorobenzylmercaptan and tetrazole were added, and the synthesis was monitored by ^{31}P NMR. The kinetics of this reaction need to be carefully monitored to avoid disubstituted mercaptophosphine nucleoside or residual bis(pyrrolidino)amidite nucleoside in the final product. The reaction time required for optimal production of the 3'-O-pyrrolidino-S-(2,4-dichlorobenzyl)-phosphorothioamidite was as follows: N^6-benzoyl-5'-O-dimethoxytrityl-3'-deoxyadenosine, 110 s; N^6-benzoyl-5'-O-dimethoxytrityl-3'-O-TBDMS-adenosine, 120 s; and N^6-benzoyl-5'-O-dimethoxytrityl-3'-O-methyl-3'-deoxyadenosine, 180 s. The reactions were quenched by addition of a large excess of 1% triethylamine in CH_2Cl_2 and worked up as previously described.[15]

Solid-phase synthesis of oligomers

All of the oligonucleotides were prepared by automated chemical synthesis on an Applied Biosystems 394 DNA/RNA synthesizer at 1.0 μmol scale using standard nucleic acid synthesis cycles with minor modifications. For the 3'-deoxy, O-methyl, and amino analogs, the 2-cyanoethyl-N,N-diisopropyl phosphoramidite of the desired nucleoside was coupled to a universal support,[16] then capped and oxidized under standard conditions. The universal support allows for the assembly of uniformly modified oligomers without the need to individually load resins. In the case of species containing a 3'-OH, commercially available 2'-O-TBDMS-N^6-benzoyl adenosine-3'-O-CPG was used. In the coupling reactions, a 13-fold excess of phosphoramidite or phosphorothioamidite compared to resin bound hydroxyl was used with extended coupling times. Double coupling was utilized with phosphorothioamidites. For oligomers containing unmodified phosphodiesters; capping, oxidation, and detritylation followed under standard conditions. In the case of phosphorothioates and phosphorodithioates, sulfurization immediately followed coupling and was achieved with 0.2 M phenylacetyl disulfide/5% triethylamine in CH_2Cl_2.[17] Capping and detritylation followed using standard procedures. Chemical

phosphorylation of the 5' terminus could be achieved using 2-[2-(4,4'-dimethoxytrityloxy)ethylsulfonyl]ethyl-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite.[18]

Deprotection, analysis, and purification

Removal of the 2,4-dichlorobenzyl protecting group from the phosphorodithioate thiol was achieved by treating resin bound oligomer with 1 M dithiolate[19] in DMF at room temperature for at least 4 h. For oligomers synthesized on universal support, the resin was treated with 30% aqueous NH_4OH at 75 °C for at least 12 h. Oligomers synthesized on standard CPG were treated with 30% aqueous NH_4OH at 65 °C for at least 12 h. The resultant solution was evaporated to dryness. The 3'-O-TBDMS protected analogs were taken up in tetrabutylammonium fluoride (1 M in THF) and treated overnight at room temperature. Oligomers were quantitated by UV absorbance and characterized by ^{31}P NMR, HPLC and matrix assisted laser desorption- time of flight (MALDI-TOF) MS. Reverse phase HPLC was used to purify the products to homogeneity.[20] NMR and HPLC analysis of a 3'-deoxy-(2'-5' dithiophosphate)-tetraadenosine (**7**) is shown in Figure 3. The MALDI-TOF MS obtained for the 5'-phosphoryl-3'-amino-3'-deoxy-(2'-5' phosphodiester)-tetraadenosine (**16**) is shown in Figure 4.

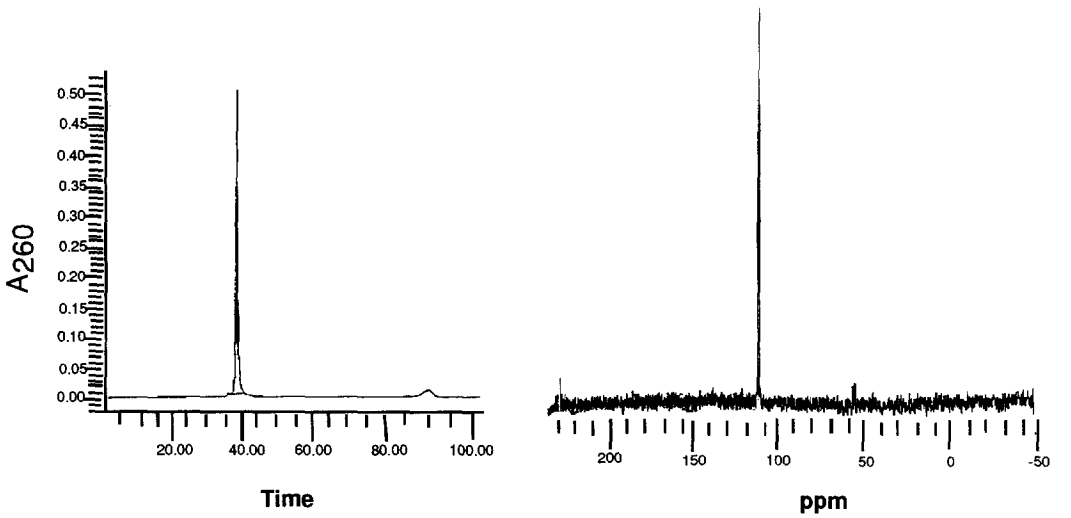


Figure 3. Reverse-phase HPLC and ^{31}P NMR analysis of **7**.[20]

Results and Discussion

Solid-phase synthesis allows for the efficient assembly of a variety of $(2'\text{-}5')(A)_n$ analogs that may have therapeutic utility. Using the methodology we were able to synthesize certain previously reported analogs[5,6] as well as several novel species. Stepwise coupling yields obtained for the phosphodiester and phosphorothioate species were in excess of 98%. Phosphorodithioate linked oligomers were produced at high efficiency for the cordycepin analog (96%); however, coupling times up to 80 min (2 x 40 min) were necessary to elaborate the dithiophosphate linked 3'-OH derivatives. Even under these conditions, the average stepwise coupling yields were approximately 85%. This is in contrast to previous literature reports on the synthesis of these derivatives

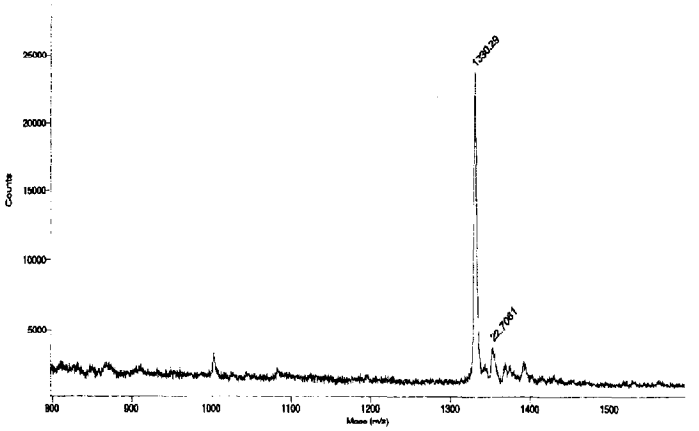


Figure 4. MALDI-TOF MS spectrum obtained for **16**.[21]

that indicated nearly quantitative coupling yields using a closely related but different system (2'-S-(ß-thiobenzoylethyl)pyrrolidinophosphorothioamidite).[6] However, in multiple attempts to make these derivatives and in our attempts to synthesize phosphorodithioate linked RNA, we found that the phosphorothioamidite approach was not effective. We suspect that a better approach to 2',5'-phosphorodithioate linked 3'-OH derivatives is the H-phosphonothioate method that was recently reported for the construction phosphorodithioate linked RNA.[22] Phosphorodithioate linked products contained approximately 5% phosphorothioate (Figure 3), consistent with previous reports.[15,22] The masses obtained by MALDI-TOF MS for all of the oligomers after purification were within 0.1% of the calculated value. While the synthesis of uniformly modified oligomers are reported here, the methods allow for the generation of large numbers of chimeric analogs containing any mixture of modification. A library of this type can be useful in rapidly defining important structure activity relationships. An examination of the potential utility of several previously reported and novel compounds as interferon mimetics has been initiated. Detailed enzymological investigations and experiments to assess and/or facilitate the cellular uptake of these molecules will be necessary to fully elucidate their utility in effecting this important biochemical pathway.

Acknowledgements

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20. Reverse-phase HPLC conditions: solid-phase, Hamilton PRP-1; mobile-phase; A, 100 mM triethylammonium acetate pH 7.5/B, acetonitrile. Chromatogram shown in Figure 3 is of purified product, time in min, the gradient was 2-50% B over 72.5 min. NMR conditions: accumulated in D_2O with phosphoric acid as external standard; phosphorodithioate, 113 ppm; phosphorothioate, 56 ppm.
21. Matrix consisted of 3-hydroxypicolinic acid:picolinic acid:ammonium citrate (10:1:1). Approximately 150 pmol of oligomer was used. Calculated mass: 1330.92 ($C_{40}H_{54}N_{24}O_{21}P_4$), actual mass 1330.29.
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