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SIMPLE SYNTHESIS AND SEPARATION OF THE DIASTEREOMERS OF α-THIO ANALOGS OF RIBO- AND DEOXYRIBO- DI- AND TRIPHOSPHATES

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ABSTRACT

Nucleoside 5'-O-(1-thiodi-) and triphosphates can be obtained in yields of up to 45% directly from the nucleosides. Their diastereomers can be separated by preparative reversed phase chromatography.

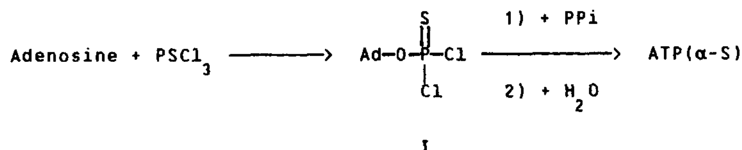
In the period since their introduction, thiophosphate analogs of nucleotides have been extremely useful in a wide variety of biochemical studies (for a recent review see ref. 1). While their most important applications initially were in the field of enzyme mechanisms, there has been increasing use of them in the past few years as tools in gene technological methods (e.g. 2,3). In a further contribution in this area, we have recently introduced a new method for sequencing DNA using the α-thiophosphates of deoxynucleotide triphosphates to introduce exonuclease III resistant residues into DNA (4).

In order to facilitate applications such as those described in refs. 2-4, which require the α-thiotriphosphates of deoxynucleosides, we have developed a new and general method for the rapid synthesis of these substances and of their ribo counterparts and of the corresponding diphosphates. In addition to this, we describe a general method for the chromatographic separation of the diastereomers of the α-thiotriphosphates.

The α-thiophosphate analogs of nucleoside di- and triphosphates were first prepared in 1969 (5), but the method described (reaction of a nucleoside monophosphorothioate imidazolide with pyrophosphate) has not proved to be convenient for routine syntheses. The currently used method (6) involves thiophosphorylation of the appropriate nucleoside using thiophosphoryl chloride followed by hydrolysis and isolation of the nucleoside monophosphorothioate (an alternative procedure for this step has also been described; ref. 7). This is then activated by reaction with diphenyl phosphorochloridate to form the mixed anhydride between the nucleotide analog and diphenyl phosphate, and the latter residue is then displaced by phosphate or pyrophosphate to give the corresponding nucleoside α-thiodi- or triphosphate. Although this method has been used to prepare the thiophosphate analogs of

all the naturally occurring nucleoside and deoxynucleoside di- and triphosphates, yields are not always satisfactory and the procedures are relatively difficult and time consuming.

In considering a possible new method for the syntheses of the α-thiopolyphosphates, it seemed reasonable to attempt to begin as in the established method but to avoid the step in which the intermediate thiophosphorodichloridate (I) of the nucleoside is hydrolysed to the corresponding monophosphorothioate.



In the new procedure, I is not hydrolysed but is allowed to react directly with pyrophosphate or phosphate in the same reaction vessel to produce the corresponding nucleoside 5'-tri- or diphosphate. The advantage of the new method is that all chemical manipulations can be completed in ca. 2 h, and that only one ion-exchange column is needed to obtain the mixed diastereomers uncontaminated with other products. The procedure is essentially the same for all the nucleosides and deoxynucleosides, except for minor differences in the preparation for the initial thiophosphorylation step (see Table 1). Yields can be as high as 45% (overall starting from the nucleoside) (i.e. much better than the yield based on the nucleoside monophophorothioate using the older procedure), and if done on a scale of ca. 1-5 mmoles, can be completed (including the column separation) in 24 h. A yield of 25% starting with 1 mmole of a deoxynucleoside produces enough product for ca. 10^8 DNA sequencing reactions according to the method described in (4), and would also be enough for a very large number of investigations of other types cited above.

A typical synthesis is described here. The first step is similar to that described by Moran and Whitesides (8) for the preparation of adenosine 5'-monophosporothioate. Dry deoxyadenosine (1 mmole) was dissolved in dry triethyl phosphate (2.4 ml) in a 25 ml double necked pointed flask equipped with a reflux condensor and argon gas inlet by heating at 180°C for 2 min with stirring under argon. The flask was quickly cooled in ice, 2,6 lutidine (0.408 ml) and $PSCl_3$ (0.187 ml) were added and the mixture was left to stand at 4°C. In the meantime, pyrophosphoric acid (5 mmole of the free acid) was treated with tri-n-octylamine (4.5 ml) and dried by evaporating three times from dry pyridine (after the second evaporation, 1 ml of tri-n-butylamine was added) using a rotary evaporator equipped with an oil pump. After 45 min at 4°C, the thiophosphorylation mixture was evaporated for ca. 4 min (oil pump) to remove excess $PSCl_3$, the dried tri-n-octylammonium pyrophosphate was added to this flask after dissolving in ca. 1.5 ml dry DMF (the flask was washed with a further 2 ml of DMF), and the combined reaction mixture was allowed to evaporate on the rotary evaporator (oil pump) for 2-3 min before stopping the reaction by addition of 4 ml of 2 M pH 7.6 triethylammonium bicarbonate (longer reaction times lead to lower yields due

to formation of higher polyphosphates). The mixture was evaporated on a rotary evaporator, the pH adjusted to 7.5 and extracted with diethyl ether (2 x 50 ml) to remove tri-n-octylamine. After brief evaporation to remove residual ether, the product mixture was separated on a QAE-Sephadex A-25 column (2 x 20 cm) using a linear gradient of triethylammonium bicarbonate (0.35-0.5 M over 3 l). The major peak (fourth large peak) was dATP(α-S) (0.35 mmole), which was identified by comparison with authentic dATP(α-S) in a variety of thin layer and HPLC chromatographic systems. The diastereomers could be separated easily for analytical purposes on reversed phase HPLC (see e.g. 9,10).

Table 1

Conditions for dissolving nucleosides in triethylphosphate. Longer heating times lead to decomposition, especially of the deoxynucleosides. The reaction time with $PSCl_3$ was 45 min when complete solution had been obtained, but 90 min if it was only partial.

Nucleoside	Temperature (^oC)	Heating time	Solution
A	216	2 min	complete
G	180	2 min	partial
dA	180	2 min	complete
dG	185	2 min	partial
T	180	ca. 10 sec	complete
dC	180	2 min	partial

For many purposes, the mixture of diastereomers obtained after purification of the products on QAE-Sephadex can be used directly, i.e. without separation of the isomers. Enzymatic methods have been developed for the separation of diastereomers where this is required (see 1 for references), but since not all the methods can be applied to all nucleoside analogs (because of base specificity of some of the enzymes), a general method has not yet been described. We report here that this aim can be achieved on a preparative scale using medium high pressure chromatography (MPLC) on reversed phase (C_{18}) materials. The apparatus for these separations can be set up at reasonable, partially making use of components available in many biochemical laboratories. HPLC quality pumps, detectors and injection systems need not be used, and the 45-60 u C_{18} material (Organogen, Heidelberg) used for the glass columns (Labomatic, Heidelberg) is much less expensive than that used for preparative HPLC. Separation of the diastereomers is achieved by isocratic elution using low concentrations of acetonitrile (2-6%, depending on the nucleoside; established by trial separations on analytical HPLC columns using the same system) in 50 mM tri-n-ethylammonium acetate (pH 7.5). For separation of 0.5-1 mmole of diastereomers, a column of dimensions 40x3 cm was used at flow rates of 25-40 ml/min. Due to ion pair effects in the buffer used, triphosphates are eluted later, or at higher acetonitrile concentration, than

diphosphates. The two fractions (the Sp diastereomer is always eluted before the Rp isomer) can usually be obtained pure in one step. Columns showing significant peak tailing usually allow the Sp isomer to be obtained in pure form, but thé Rp isomer requires a further column purification under the same conditions. Solvent and buffer are removed by freeze-drying.

The method described above for the preparation of the α-thiotriphosphates is highly suitable for the synthesis of radioactively labelled analogs, since the chemical manipulations involved are kept to a minimum and only one column is required to obtain the mixed isomers. The method can also be modified to allow the introduction of oxygen isotopes at the α-phosphate group. This is most easily accomplished by stopping the reaction (after mixing with pyrophosphate or phosphate) by addition of oxygen isotope labelled water.

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