AN APPROACH TO THE SYNTHESIS OF INTERMEDIATE SIZED OLIGORIBONUCLEOTIDES

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A mixed phosphodiester-phosphotriester stepwise addition method for the synthesis of oligoribonucleotides is described. Using this method CpCpCpCpCpG and UpCpCpGpG which correspond to bases 66 to 71 and 61 to 65 of E. coli $tRNA_f^{Met}$ have been synthesized.

Although phosphodiester or phosphotriester methods for the chemical synthesis of oligodeoxyribonucleotides have been applied with considerable success to obtain fairly large oligomers,[1] chemical synthesis of oligoribonucleotides with naturally occurring sequences to be a more difficult proposition and the largest blocks reported in the literature remain nonanucleotides.[2]

* This paper is dedicated to Professor Shigehiko Sugasawa on the occasion of his 80th birthday.

The recent isolation of RNA ligase from T4 infected E. coli and its use in the joining of preformed ribo-oligomers[3] suggest that medium sized blocks (larger than perhaps the pentamer) will prove sufficiently large to allow efficient chemico-enzymatic syntheses of bigger RNA tract. Although the enzyme will catalyze the joining of shorter oligomers, such as two dimers, in high yields,[4] the number of kination/ligation/purification steps becomes prohibitively high in the synthesis of large molecules. As well as suitability as RNA ligase substrates, many intermediate-sized oligoribonucleotides are of particular biochemical interest in their own right.[5,6] In this communication we describe the use of a mixed phosphodiester-phosphotriester stepwise addition method for the synthesis of ribo blocks with separation of oligomers longer than trimers from incoming nucleotide and its pyrophosphate by gel filtration. This approach combines the most attractive feature of the "solid support" method - ease of separation of monomer component from growing chains - with the high yields possible in condensation in homogeneous solution.

Fig. 1 illustrates the application of this approach to the synthesis of the hexamer CpCpCpCpCpG* (III) which corresponds to bases 66 to 71 of E. coli $tRNA_f^{Met}$ whose total synthesis we are currently attempting. We will comment in detail elsewhere on the synthesis of the intermediate[7] (II) but in brief it was prepared

* Abbreviations are principally as suggested by the IUPAC-IUB commission. For nomenclature see Bichim. Biochys. Acta, 247, 1 (1971).

Fig.1

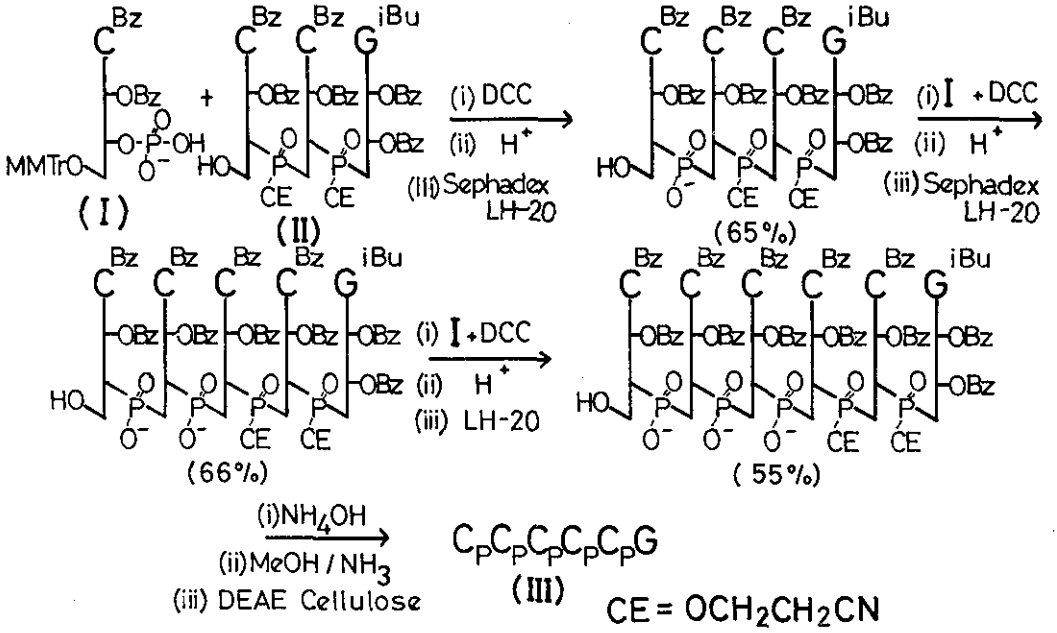
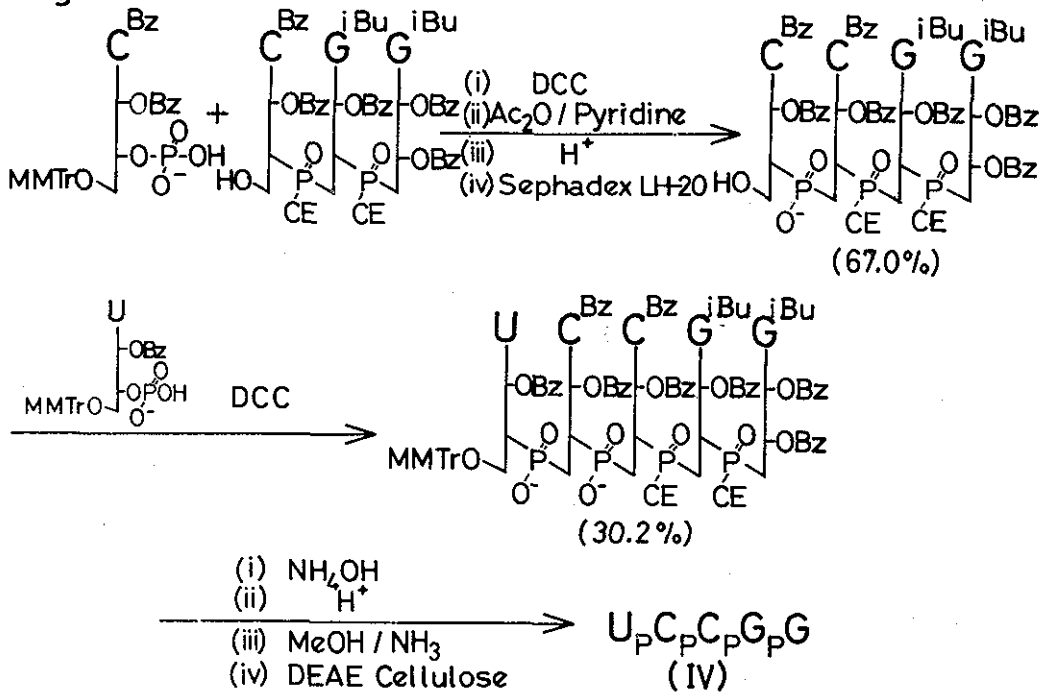

(I)
(II)
(i) DCC
(ii) H+
(iii) Sephadex LH-20
(65%)
(i) I +DCC
(ii) H+
(iii) Sephadex LH-20
(66%)
(i) I +DCC
(ii) H+
(iii) LH-20
(55%)
(i) NH4OH
(ii) MeOH / NH3
(iii) DEAE Cellulose
CpCpCpCpCpG
(III)
CE = OCH2CH2CN

Fig. 2


(i) DCC
(ii) Ac2O / Pyridine
(iii) H+
(iv) Sephadex LH-20
(67.0%)
DCC
(30.2%)
(i) NH4OH
(ii) H+
(iii) MeOH / NH3
(iv) DEAE Cellulose
UpCpCpGpG
(IV)

by 2,4,6-triisopropylbenzenesulfonyl chloride (TPS) catalyzed β-cyanoethylation of the 5'-monomethoxytritylated trinucleoside diphosphate followed by de-monomethoxytritylation.

Conditions for the stepwise additions of mononucleotide to the growing chain were as follows. A twofold excess of 5'-monomethoxytrityl-N,2'-O-dibenzoylcytidine 3'-phosphate (I) (threefold in the final step) plus the growing chain as their pyridinium salts were precipitated from their solution in dry pyridine and washed three times with ether/n-hexane (3 : 2). After drying in vacuo over P_2O_5 the precipitate was dried further by evaporation of added pyridine (5 times) then treated in pyridine solution with 10 equivalents of dicyclohexylcarbodiimide (DCC) in the presence of Dowex 50X2 (pyridinium form) as an amine scavenger. After ca. 30 min, the solution was evaporated to a viscous gum with exclusion of moisture at 27° for 4 days. Cold (0°C) 50% aqueous pyridine was added and the resulting solution extracted with n-hexane to remove DCC. After 12 hr at room temperature Dowex resin and dicyclohexylurea was filtered off and the filtrate and washings were evaporated to dryness. The residue was treated with 80% aqueous acetic acid for 80 min at room temperature and acetic acid was removed by coevaporation with aqueous n-butanol. The residue was dissolved in the minimum volume of pyridine and applied to a column of the alkylated Sephadex LH-20 packed in 95% ethanol. Elution was performed with 95% ethanol. Satisfactory resolutions of the longer oligomers with 5'-hydroxyl groups and the mononucleotide were achieved and these oligomers could be used for further condensation.

Some advantages of this approach are worthy of mention. The recovery of material after gel filtration is essentially quantitative so that substantial amounts of material are not lost in each step. This is not the case where ion exchange chromatography is used and considerable irreversible adsorption occurs. Also there is no problem of removing the less volatile eluting buffers necessary for the alkaline labile protected oligoribonucleotides on ion exchangers. This means in practice that either larger amounts of oligomers can be readily obtained or that one can work with quite small amounts of the relatively inaccessible starting trimer without encounting handling problems at later stages. Although yields are the order of 60%, the product always contains an extra nucleotide residue so that the actual quantities involved remain almost constant.

The advantage of basing the synthetic approach on the triesterified trimer can be appreciated by comparing the yields in condensation steps to the tetramer, pentamer and hexamer with those previously achieved in a similar series of stepwise phosphodiester addition leading to a pentamer sequence UpApm^{2}GpCpC from yeast tRNA^{Tyr}.[8] Successive yields there were 75, 38, 31 and 21% respectively. It is difficult to decide wether further triesterification would be justified in these types of synthesis. Although the yields in subsequent condensations might be higher, and although almost quantitative triesterification on single phosphodiesters can be achieved, the inevitable losses of material during purifications on silica gel mean that overall yields would probably be similar. Further triesterifications

would probably be worthwhile in syntheses of longer blocks and we are investigationg such approaches.

In fact it is not absolutely essential to separate mixtures of oligomers after condensations as the desired compound will always be the longest one at the final stage and is easily separated by DEAE-cellulose chromatography in the presence of 7 M urea.[9] However, there is a danger that internal pyrophophphates such as protected $Cp_{\underset{Cp}{O}}CpCpG$ could survive aqueous pyridine treatment, leading to multiple addition in subsequent condensation steps. In the case in Fig. 1, this phenomenon is not a problem as the same nucleotide was to be added throughout. However, as shown in Fig. 2 we overcome this problem in the mixed sequence by cleavage of pyrophosphate at each stage with acetic anhydride-pyridine treatment followed by aqueous pyridine, before de-monomethoxytritylation. This has further advantage that unreacted oligomer 5'-hydroxyl groups are acetylated and if not fully resolved on LH-20 take no further part in subsequent condensation.[10] Thus sequence UpCpCpGpG (IV) (*E. coli* $tRNA_f^{Met}$ bases 61-65) could be isolated by DEAE-cellulose chromatography after deprotection by sequential treatments with dilute aqueous ammonia for 5 min at 0°, 80% acetic acid for 4hr at room temperature and 15 N methanolic ammonia for 20 hr. The overall yields of the hexamer (III) and pentamer (IV) in deblocking, chromatography and desalting were ca. 40%. All compounds described were fully characterized by enzymatic digestions (RNase A, base non-specific RNase M and venom phosphodiesterase). Satisfactory ratio of nucleotidic components were obtained and no

nuclease-resistant 2'-5' linked compounds could be detected. The joining of these oligonucleotides to the previously synthesized oligonucleotides[11] with RNA ligase are under investigation.

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