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Studies on Transfer Ribonucleic Acids and Related Compounds. XXXI.[1] Synthesis of the 3′,5′-Bisphosphorylated Hexanucleotide corresponding to Bases (72—77) of tRNA^{fMet} from *E. coli* and Its Base Transition Analog

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A hexanucleotide pC-A-A-C-C-Ap, which corresponds to the 3′-end of tRNA^{fMet}, was synthesized as a bisphosphorylated form, which should be a suitable donor substrate for joining with RNA ligase. 3′-Phosphorylated oligonucleotides were condensed by a triester method and the 5′-terminus of the protected hexanucleotide was phosphorylated with dianilidophosphorochloridate after demonomethoxytritylation. A site-directed base transition mutant sequence C-G-A-C-C-Ap was also synthesized by the triester method using suitably protected 3′-phosphorylated oligonucleotides.

Keywords——2′-O-(*o*-nitrobenzyl)nucleosides; 5′-phosphorylation; triester block condensation; *p*-chlorophenyl N-phenylchlorophosphoramidate; mesitylenesulfonyl triazolide; 2,4,6-triisopropylbenzenesulfonyl nitroimidazolide; ion-exchange chromatography; paper chromatography; paper electrophoresis

We have previously reported the synthesis of fragments of tRNA ^{fMet} from *E. coli*[3] using various approaches. A phosphodiester method was applied to bases 1—4,[4] 5—10[5] 41—46,[6] 47—54[6], 55—57[6] and a partial triester method was used for bases 58—60,[7] 61—65,[8] 66—71[8], 72—74[9] and 75—77.[9,10] Bases 11—20[11] and other 5′-fragments as well as their analogs have been synthesized by triester block condensation using 2′-O-(*o*-nitrobenzyl-nucleosides).[12] These oligonucleotides have been joined enzymically using bacteriophage T4 RNA ligase[13] to yield 3′-heptadeca[14] and 5′-eicosanucleotides.[1] In this paper we report a synthesis of the hexanucleotides C–A–A–C–C–Ap[15] and C–G–A–C–C–A, bases 72—77 and an analog. The 5′-phosphorylation of the hexanucleotide yielded the 3′,5′-bisphosphorylated hexanucleotide, p-C–A–A–C–C–Ap, which is a suitable donor substrate for the RNA ligase reaction. The 5′-hydroxyl group of oligonucleotides can be phosphorylated enzymically with polynucleotide kinase and ATP.[16] Chemical phosphorylation, however, would provide a

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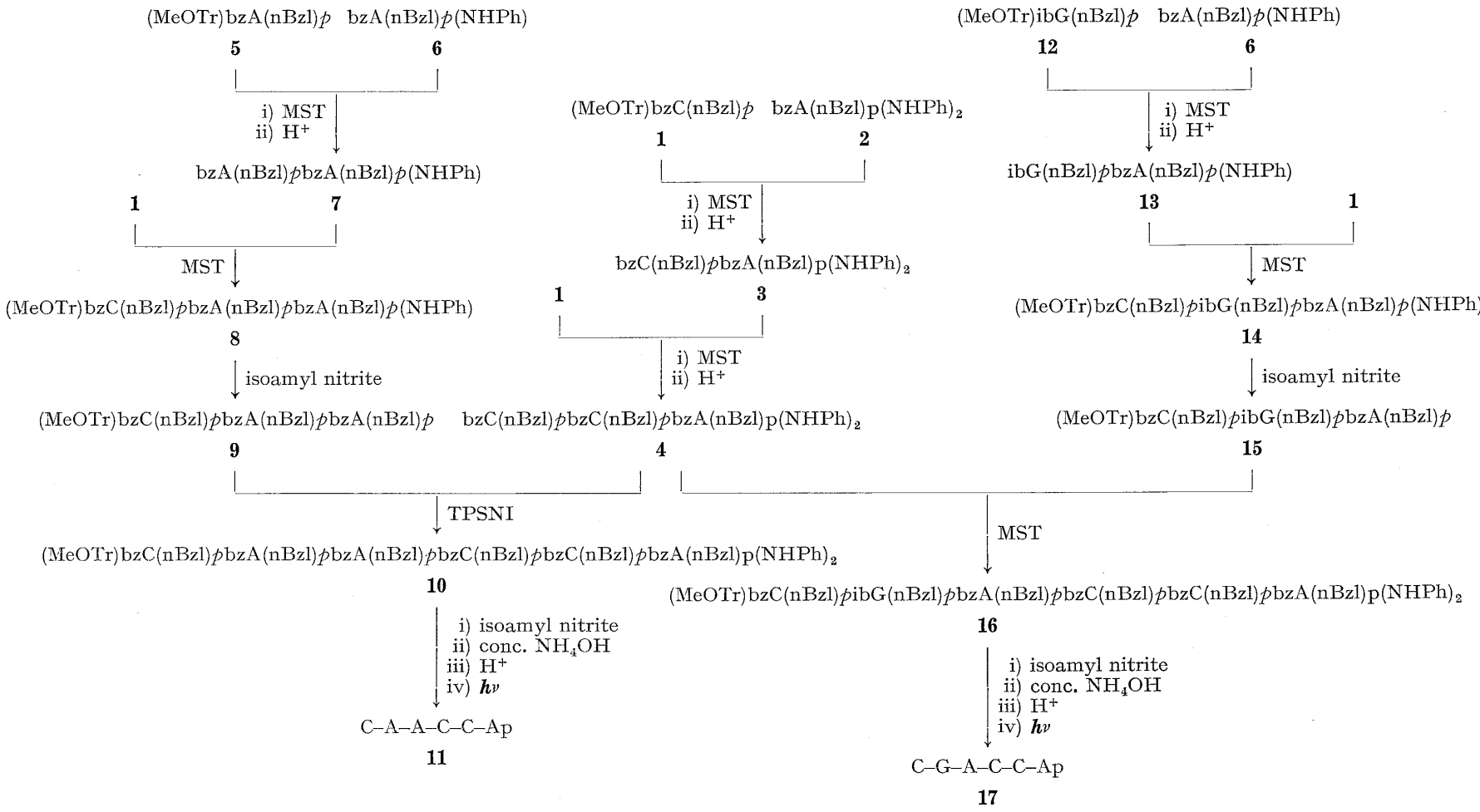

p=*p*-chlorophenyl phosphate MST=mesitylenesulfonyl triazolide
TPSNI=2,4,6-triisopropylbenzenesulfonyl 4-nitroimidazolide

Chart 1

larger quantity of phosphorylated oligonucleotides. Phosphorylation of triesterified di- and trinucleotides with 2,2,2-trichloroethyl phosphate and TPS has been reported as a post phosphorylation method.[17] Phosphomonoester insertion at the 5′-termini of oligodeoxynucleotides has also been used for the preparation of protected oligonucleotide blocks.[18]

Synthesis of C–A–A–C–C–Ap and C–G–A–C–C–Ap

The two hexanucleotides, C–A–A–C–C–Ap (**11**) and C–G–A–C–C–Ap (**17**), were synthesized by triester block condensation using 2′-O-(*o*-nitrobenzylnucleosides) (**12**) as shown in Chart 1. The 3′-terminal phosphates were protected as phosphorodianilidates[12,19] and mononucleotides for the synthesis of protected blocks were prepared by phosphorylation with

Table I. Reaction Conditions for Condensations

5′-Protected component	Amount (mmol)	3′-Protected component	Amount (mmol)	Reagent	Time (days)	Product	mg (mmol)	Yield (%)
5	0.95	6	0.7	MST 2.76	2	7	790.7 (0.54)	77
1	0.53	7	0.40	MST 1.57	1.5			
				MST 0.79	1	8	685 (0.29)	73
1	1.02	2	0.85	MST 3.06	2	3	928 (0.67)	79
1	0.50	3	0.33	MST 1.49	2	4	496 (0.24)	73
				MST 1.49	2			
12	0.93	6	0.73	MST 2.82	2	13	753 (0.53)	73
1	0.37	13	0.29	MST 1.13	2	14	479 (0.20)	69
				MST 1.13	2			
15	0.101	4	0.051	MST 0.30	2	16	182.5 (0.042)	82
				MST 0.15	2			
2	0.18	4	0.101	TPSNI 0.27	1.5	10	386 (0.089)	80

Table II. Elemental Analysis and UV Spectral Data for Di- and Trinucleotides

Compound	Elemental analysis	Calcd (Found) C	H	N	UV in 95% EtOH λmax (ε×10⁻⁴)	λmin (ε×10⁻⁴)
bzC(NB)*p*bzA(NB)p(NHPh)$_2$	$C_{65}H_{57}ClN_{12}O_{18}P_2$ H_2O (1409.78)	55.37 (55.54)	4.23 3.94	11.93 11.82)	263.5 (4.86)	241 (3.67)
ibG(NB)*p*bzA(NB)*p*(NHPh)	$C_{63}H_{57}Cl_2N_{13}O_{19}P_2$ $2H_2O$ (1469.25)	51.50 (51.55	4.19 3.89	12.40 12.18)	261.5 (3.82) 278 (3.77)	240.5 (2.77)
bzA(NB)*p*bzA(NB)*p*(NHPh)	$C_{66}H_{65}Cl_2N_{13}O_{18}P_2$ H_2O (1469.24)	53.95 (53.99	3.92 3.80	12.40 12.60)	279.5 (5.12)	247.5 (3.08)
bzC(NB)*p*bzC(NB)*p*bzA(NB)p-(NHPh)$_2$	$C_{94}H_{81}Cl_2N_{16}O_{28}P_3$ $2H_2O$ (2082.81)	54.20 (54.28	4.12 3.98	10.76 10.85)	263 (7.64)	245 (5.38)
(MTr)bzC(NB)*p*ibG(NB)*p*bzA-(NB)*p*(NHPh)	$C_{112}H_{97}Cl_3N_{17}O_{30}P_3$ $2H_2O$ (2396.62)	56.13 (56.20	4.26 4.02	9.94 9.83)	262.5 (6.95)	242.5 (5.52)
(MTr)bzC(NB)*p*bzA(NB)*p*bzA-(NB)*p*(NHPh)	$C_{115}H_{95}Cl_3N_{17}O_{29}P_3$ (2378.59)	58.07 (58.07	4.03 4.01	10.01 10.07)	274 (7.20)	246 (5.74)

p=*p*-chlorophenyl phosphate. NB=2′-O-*o*-nitrobenzyl. MTr=5′-O-monomethoxytrityl.

17) T. Neilson, K.V. Dengan, T.E. England, and E.S. Werstiuk, *Can. J. Chem.*, **53**, 1093 (1975).
18) H. Köster, H. Blöcker, R. Frank, S. Geussenhainer, W. Heidmann, W. Kaiser, and D. Skroch, *Ann.* **1978**, 854.
19) E. Ohtsuka, S. Tanaka, and M. Ikehara, *J. Am. Chem. Soc.*, **100**, 8210 (1978).

p-chlorophenyl N-phenylchlorophosphoramidate[20] or *p*-chlorophenyl phosphate plus dicyclohexylcarbodiimide (DCC).[19] The 3′-terminal trinucleotide **4** was synthesized by successive condensation and demonomethoxytritylation of the 3′-diesterified mononucleotide **1**. Mesitylenesulfonyl triazolide (MST)[21] was used as the activating reagent. The reaction conditions are summarized in Table I. Protected oligonucleotides were isolated by chromatography on silica gel. Elemental analysis and UV data are listed in Table II. **4** was used in both condensations, for the synthesis of **11** and **17**, as the common trinucleotide block. For the synthesis of the protected hexamer **10**, the protected C–A–Ap (**8**) was prepared by condensation of **5** with **6** and subsequent reaction with **1** as shown in Chart 1. Removal of the phosphoroanilidate of **8** was effected by treatment with isoamyl nitrite in pyridine–acetic acid[22] and the resulting phosphodiester (**9**) was activated with triisopropylbenzenesulfonyl 4-nitroimidazolide (TPSNI)[23] to yield **10**. A sample of **10** was deblocked by successive treatments with isoamyl nitrite; concentrated ammonia; 80% acetic acid; UV at a wavelength longer than 280 nm. The hexanucleotide **11** was characterized by RNase M[24] digestion to yield Cp and Ap in a 1:1 ratio and by mobility shift analysis[25] using two-dimensional homochromatography[26] after labeling at the 5′-hydroxyl group by phosphorylation with [γ-^{32}P] ATP and polynucleotide kinase. This hexanucleotide corresponds to the bases 72 to 77 of tRNA^{fMet} from *E. coli*. The other hexanucleotide C–G–A–C–C–Ap (**17**) was synthesized similarly and purified by ion-exchange chromatography on DEAE-cellulose in 7 M urea in a yield of 38%. **17** had a site-directed A to G transition mutation compared to **11** and should form a hydrogen bond with the 5′-terminal cytosine to construct a fully basepaired amino acid acceptor stem if used in place of **11**.

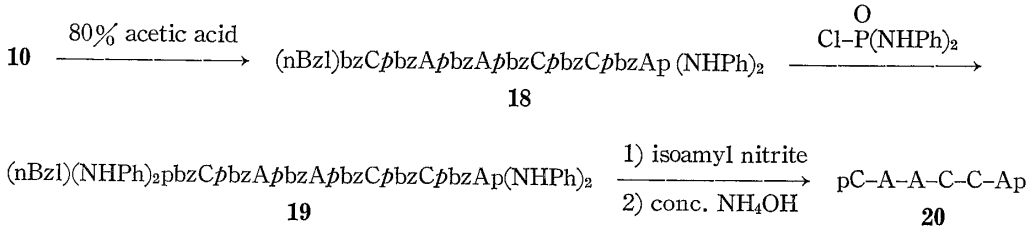

Chart 2

Insertion of Phosphomonoester at the 5′-End of a Hexanucleotide

The two hexanucleotides **11** and **17** were designed as donor substrates for RNA ligase, and the 3′termini were blocked with phosphomonoesters to prevent attack. Although the 5′-phosphate could be inserted by enzymatic phosphorylation, chemical synthesis of oligonucleotides with 3′—5′, bisphosphate would be desirable. With this aim, the protected hexanucleotide **10** was phosphorylated with dianilidophosphorochloridate after demonomethoxytritylation as shown in Chart 2. The product (**19**) was isolated by chromatography on silica gel in a yield of 36%. The deblocked hexanucleotide (**20**) was purified by ion-exchange chromatography on DEAE-cellulose in 7 M urea and identified by digestion with RNase M to yield pCp, Ap and Cp in the theoretical ratio. The yield of **20** from **19** was 56%. *Rf* values and relative mobilities in paper electrophoresis are collected in Table III.

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Table III. Paper Chromatography and Electrophoresis Data

Compound	Solvent A	Solvent B	Relative mobility pH 7.5
A	0.68	0.67	−0.27
G	0.40	0.51	0
C	0.57	0.58	−0.19
U	0.56	0.58	0.04
A>p			0.41
G>p			0.61
C>p	0.36		0.53
U>p	0.35		0.73
Ap	0.26	0.44	0.91
Gp	0.13	0.33	1.00
Cp	0.22	0.33	0.99
Up	0.22	0.43	1.07
C(nBzl)–A(nBzl)*p*	0.35	0.64	
C–Ap	0.12	0.35	0.93
G(nBzl)–A(nBzl)*p*	0.65		
G–A>p	0.26		0.72
G–Ap	0.07		0.92
A(nBzl)–A(nBzl)*p*	0.72		0.57
A–A>p	0.32	0.48	0.69
A–Ap	0.14	0.37	0.99
C(nBzl)–C(nBzl)–A(nBzl)*p*	0.30	0.73	0.81
C–C–Ap	0.06	0.26	0.97
C(nBzl)–G(nBzl)–A(nBzl)*p*	0.63		0.48
C–G–A>p	0.14		0.81
C–G–Ap	0.05	0.20	0.99
C(nBzl)–A(nBzl)–A(nBzl)*p*	0.65		0.53
C–A–A>p	0.15		0.71
C–A–Ap	0.08		0.95
C(nBzl)–G(nBzl)–A(nBzl)–C(nBzl)–C(nBzl)–A(nBzl)*p*		0.56	0.93
C–G–A–C–C–Ap		0.05	0.97
C(nBzl)–A(nBzl)–A(nBzl)–C(nBzl)–C(nBzl)–A(nBzl)*p*		0.66	0.86
C–A–A–C–C–Ap		0.09	0.97
pC(nBzl)–A(nBzl)–A(nBzl)–C(nBzl)–C(nBzl)–A(nBzl)*p*		0.42	
pC–A–A–C–C–Ap		0.03	

Experimental

General Methods——Paper chromatography was performed by a descending technique using solvents A, propan-2-ol–conc.ammonia–water (7:1:2, v/v), and B, propan-1-ol–conc.ammonia–water (55:10:35, v/v). Paper electrophoresis was performed using 0.05 M triethylammonium bicarbonate (pH 7.5) or 0.2 M morpholinium acetate (pH 3.5) at 900 V/40 cm. TLC was performed on plates of silica gel (Kieselgel HF_{254}, Merck) using a mixture of chloroform and methanol. For columns, silica gel G (Merck, type 60) was used. Procedures for removal of the 5′-O-monomethoxytrityl, 2′-O-(*o*-nitrobenzyl) and phosphorodianilidate moieties, and other general methods, were as described previously.[19] Enzymatic hydrolyses were described in ref. 27.

Condensation of Nucleotides——Reaction conditions for the condensation of protected nucleotides are summarized in Table I. 3′-Diesterified mononucleotides (*e.g.* **1**),[19] the 3′-dianilidate (**2**)[12] and 5′-deblocked mononucleotides (*e.g.* **6**)[11,20] were prepared by the cited methods.

C-G-A-C-C-Ap (17)——**15** (triethylammonium, 243 mg 7000 $A_{262.5}$, 0.101 mmol) and **4** (104 mg 0.051 mmol) were rendered anhydrous by addition and evaporation of pyridine. The mixture was treated with

27) E. Ohtsuka, M. Ubasawa, S. Morioka, and M. Ikehara, *J. Am. Chem. Soc.*, **95**, 4726 (1973).

MST (75.3 mg, 0.30 mmol) at 25—33° for s days. TLC showed a trityl-positive product **16** (*Rf* 0.34) overlapping the starting material **4** (*Rf* 0.40, 0.36, 0.34). The mixture was further treated with MST (37.7 mg) for 2 days then the reaction was stopped by addition of water (1 ml). Chloroform (20 ml) and 0.1 M triethylammonium bicarbonate (pH 7.5) (40 ml) were added to the mixture. The product was further extracted with chloroform (10 ml) twice. The organic layer was washed with water, then made anhydrous by addition and evaporation of pyridine then toluene, and the residue was applied to a column of silica gel (15 g). The product was eluted with 40: 1 chloroform–methanol and precipitated with *n*-pentane (50 ml) from its solution in chloroform (3 ml). The yield of **16** was 182.2 mg, 0.042 mmol (82%). An aliquot (43.1 mg) was treated with isoamyl nitrite (2.4 ml) in 1: 1 pyridine–acetic acid (10 ml) at room temperature for 24 hr. Water (10 ml) was added with cooling and the product was extracted with chloroform (15 ml) 3 times. The organic layer was washed with water concentrated, and the residue was dissolved in pyridine then treated with concentrated ammonia at 55° for 5 hr. Volatile materials were removed by evaporation and the residue was treated with 80% acetic acid (50 ml) at room temperature overnight. The extent of reaction was checked by TLC (10: 1) and acetic acid was removed by evaporation. The trityl alcohol was removed by extraction with ether and three-quarters of the aqueous solution was irradiated with UV light for 2 hr. The aqueous solution was concentrated to one-half of the original volume, washed with ether, concentrated, dissolved in 7 M urea (20 ml) and applied to a column (1.0×104 cm) of DEAE-cellulose (Cl^-) preequilibrated with 7 M urea–20 mM Tris–HCl (pH 8.0)–0.1 M NaCl. Elution was performed with a linear gradient of NaCl (0.15 M–0.35 M, total 1.21). Fractions of 3 ml were collected every 20 min. Fractions 125—138 were combined and desalted by absorption on DEAE-cellulose (bicarbonate). The yield was 162 A_{260} units (2.8 μmol calculated on the basis of 10% hypochromicity) (38%). An aliquot (3 A_{260}) was hydrolyzed with RNase M and subjected to paper electrophoresis (pH 3.5). Gp (0.21 A_{256}), Cp (0.820 A_{279}) and Ap (0.614 A_{257}) were obtained in a ratio of 1.00: 2.95: 1.90.

pC-A-A-C-C-Ap (20)——The protected hexanucleotide (**10**) was synthesized as above using the conditions shown in Table I. Demonomethoxytritylation was performed by treatment of **10** (217 mg, 0.05 mmol) with 80% acetic acid (30 ml) at 25—33° for 15 hr. TLC showed a new spot (*Rf* 0.29) and the disappearance of the trityl-positive spot (*Rf* 0.32). The product **18** (192 mg, 0.047 mmol) was precipitated with *n*-pentane (20 ml) from its solution in chloroform (2 ml) and treated with dianilidophosphorochloridate (25.1 mg, 0.094 mmol) in pyridine (1 ml) at 25—35° or 24 hr. Further reagent (25.1 mg) was then added and the extent of reaction was checked by deblocking except for the 2′-hydroxy group. The phosphorylated product (*Rf* 0.12) was contaminated with the starting material (*Rf* 0.07) and the mixture was treated with further reagent (25.1 mg) for 2 days. The reaction was stopped by adding water (1 ml) with cooling. After 1 hr the product was extracted with chloroform, washed with 5% potassium acetate (40 ml) then with water, and the organic phase was back-extracted. Chloroform was evaporated off and the residue was dried by addition and evaporation of pyridine, then toluene. The product (**19**) was applied to a column of silica gel (10 g), eluted with 30: 1 chloroform–methanol, and precipitated with *n*-pentane (20 ml) from its solution in chloroform (2 ml). The product (**19**) (123.3 mg) was contaminated with 41% of **18**. Compound **19** was deblocked as described above except for the ommission of acid treatment and purified by ion-exchange chromatography on DEAE-cellulose with the same column used for **17**. Elution was performed with a linear gradient of sodium chloride (0.15—0.4 M, total 1.2 l). Fractions of 3 ml were collected every 13 min. Fractions 126—139 were combined and desalted. The yield was 202 A_{260} units (3.3 μmol, calculated on the basis of 10% hypochromicity) (56%). A sample (3 A_{260}) was hydrolyzed with RNase M and analyzed by paper electrophoresis (pH 3.5). pCp (0.239 A_{279}) Cp (0.443 A_{279}) and Ap (0.775 A_{257}) were obtained in a ratio of 1.00: 1.92: 2.88.