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Studies on Transfer Ribonucleic Acids and Related Compounds. XLIV.[1] A Large-Scale Synthesis of the Anticodon Heptanucleotide of Formyl-methionine Transfer Ribonucleic Acid by Using 2′-*O*-Tetrahydrofuranylnucleosides

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2′-*O*-Tetrahydrofuranyl nucleosides were prepared by an improved procedure *via* 3′,5′-tetraisopropyldisiloxanylnucleosides. A large scale synthesis of the anticodon heptanucleotide C–U–C–A–U–A–A of *E.coli* formylmethionine tRNA was performed by the phosphotriester method involving condensation of oligonucleotides having 2′-*O*-tetrahydrofuranyl protecting groups.

Keywords——tRNA fragment; phosphotriester method; tetraisopropyldisiloxanylnucleosides; condensing reagent

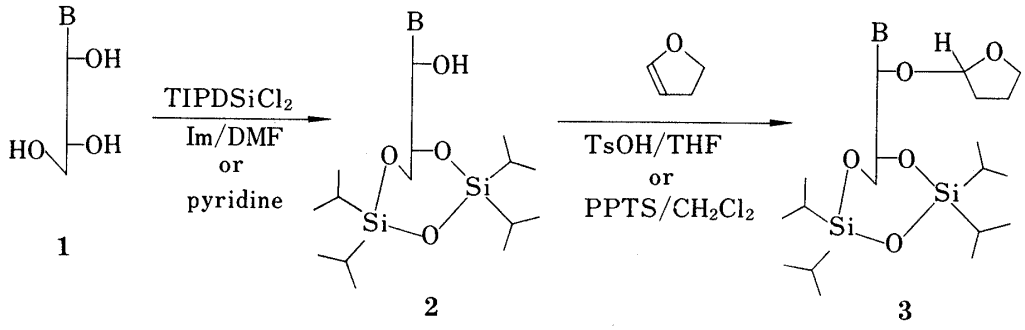
Ribooligonucleotides such as transfer ribonucleic acid (tRNA) fragments have been synthesized by various methods and can be used as substrates for RNA ligase to construct larger RNA molecules.[2] For studies on physicochemical properties of ribooligonucleotides, larger amounts of oligonucleotides have to be synthesized than for biochemical studies. In the preceding paper we reported the synthesis of ribooligomers by using the easily removable tetrahydrofuranyl group as a protecting group for the 2′-hydroxyl function.[1] In this paper we applied the method to a large scale synthesis of a heptanucleotide corresponding to the anticodon loop of *E.coli* $tRNA_f^{Met}$. An improved method for the preparation of the key intermediates, 2′-*O*-tetrahydrofuranyl-*N*-protected nucleosides has been employed in this synthesis. The condensations of 5′-phosphodiesters with the 3′-hydroxyl function of the protected four major nucleosides are also described and compared in this paper.

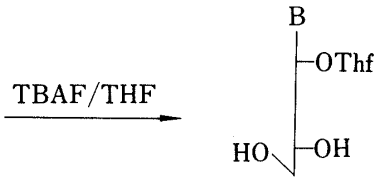
Preparation of 2′-*O*-Tetrahydrofuranylnucleosides

2′-*O*-Tetrahydrofuranylnucleosides have previously been prepared *via* 3′,5′-bis(*tert*-butyldimethylsilyl)nucleosides.[1] Since tetraisopropyldisiloxane dichloride ($TIPDSiCl_2$) was found to protect the 3′- and 5′-hydroxyl groups in high yield,[3] we prepared the 2′-*O*-tetrahydrofuranyl derivatives (**4**) by means of the procedure shown in Chart 1. Uridine (**1a**), *N*-benzoylcytidine (**1b**),[4] *N*-benzoyladenosine (**1c**)[5] and *N*-isobutyrylguanosine (**1d**)[6] were treated with $TIPDSiCl_2$ and the 3′, 5′ bis-substituted nucleosides (**2**) were reacted with 2,3-dihydrofuran in the presence of *p*-toluenesulfonic acid to yield **3**. The silyl groups of **3** were removed with tetra-*n*-butylammonium fluoride (TBAF). The overall yields of **4a**, **4b** and **4c** from **1** were 60, 45 and 49%, respectively. The 2′-*O*-tetrahydrofuranylation of **2d** did not proceed under the conditions described above. In the presence of pyridinium *p*-toluenesulfonate (PPTS)[7] in methylene chloride, **2d** reacted with 2,3-dihydrofuran nearly quantitatively and **3d** was converted to **4d** in an overall yield of 98%. Compounds **4a**, **4b** and **4c** were also obtained in yields of 90, 87 and 85%, respectively, from the silylnucleosides (**2a**, **b**, **c**).

Synthesis of a Heptaribonucleotide C-U-C-A-U-A-A (14)

The heptaribonucleotide (**14**) was synthesized by condensation of protected CUC (**7**, Chart 2) and pAUAA (**11**, Chart 3). The 5′-end of the trimer block (**7**) was protected with the monomethoxytrityl group and the chain was elongated in the 3′-direction by phosphorylation with *o*-chlorophenyl phosphoditriazolide[8] followed by condensation with the 3′,5′-

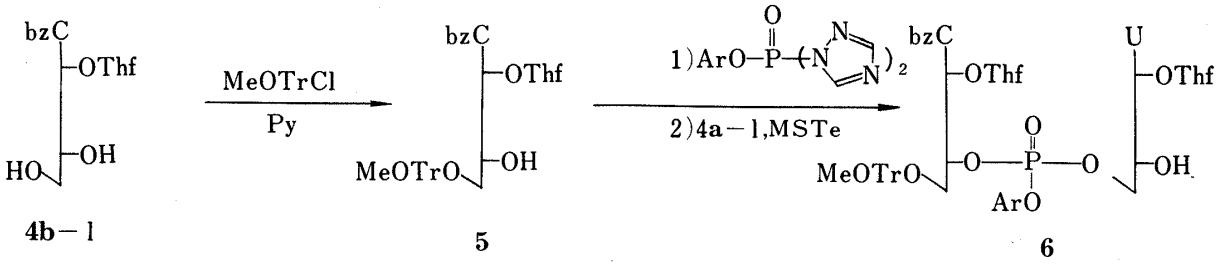


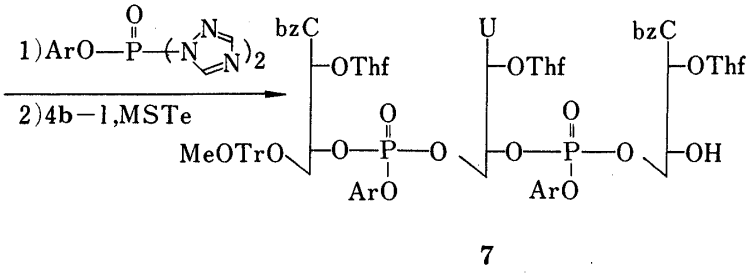


B : a = U
b = bzC
c = bzA
d = ibG
l : low isomer
h : high isomer

$TIPDSiCl_2$=1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane
Im=imidazole
PPTS=pyridinium *p*-toluenesulfonate
TBAF=tetra-*n*-butylammonium fluoride
Thf=tetrahydrofuranyl

Chart 1





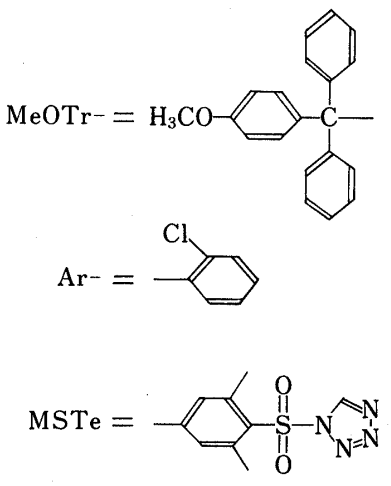


Chart 2

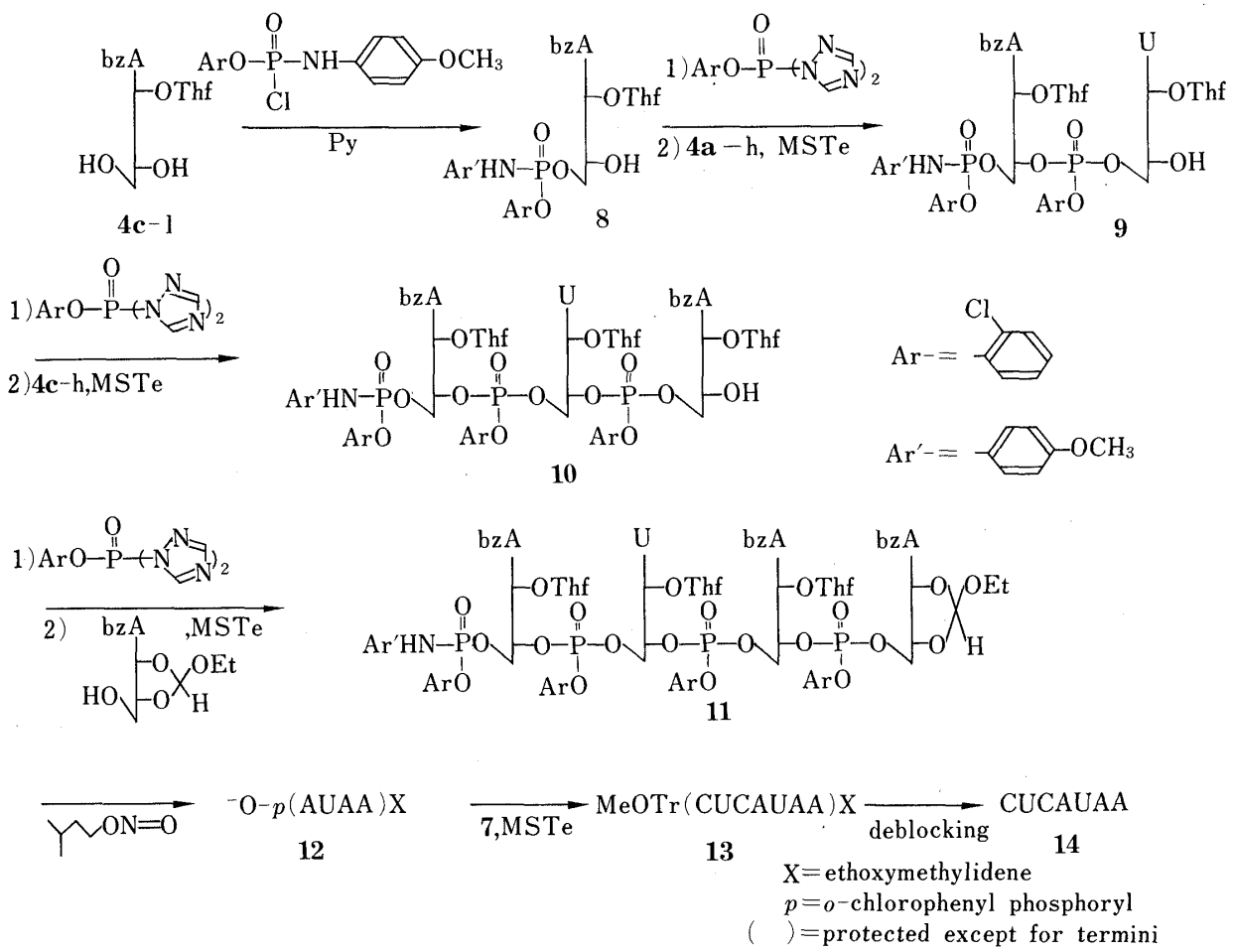


Chart 3

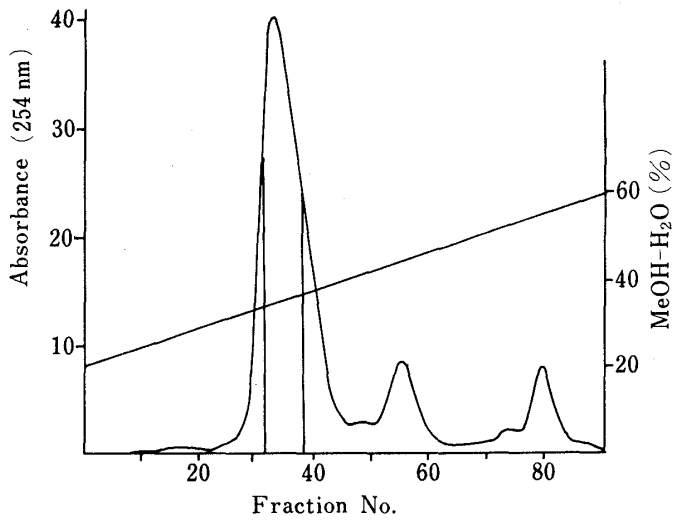


Fig. 1. Reversed Phase Chromatography of the Heptamer (**14**)

A column (1.0×20 cm) of C-18 silica gel (35—105 μ) was equilibrated with 1/15 M KH_2PO_4-Na_2HPO_4 (pH 7.5). Elution was performed with a linear gradient of methanol (10—30%, total volume 200 ml).

unprotected nucleosides **4a** and **4b**, successively. Mesitylenesulfonyl tetrazolide (MSTe)[9] was used as the condensing reagent. The overall yield of **7** from **5** was 40%. The tetramer block (**11**) was also synthesized by elongation in the 3′-direction. The 5′-end adenosine derivative (**4c**) was phosphorylated with *o*-chlorophenyl *p*-anisidophosphorochloridate and condensed with **4a**, **4c** and 2′,3′-ethoxymethylidene-*N*-benzoyladenosine similarly. The yields of these steps were 55, 71 and 67%, respectively. The 5′-phosphoro-*p*-anisidate of **11** was removed by treatment with isoamyl nitrite[10] and the tetranucleotide (**12**) was condensed with the trimer (**7**) to give the protected heptamer (**13**) in a yield of 76%. The protecting

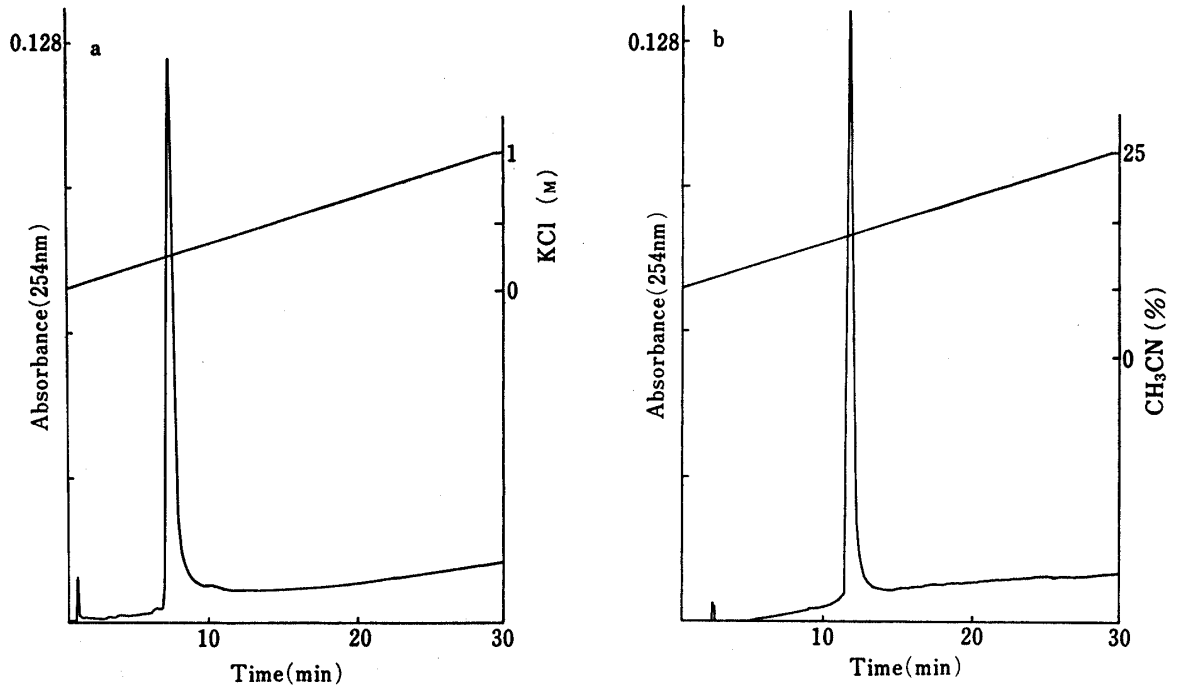


Fig. 2. HPLC of the Heptanucleotide (**14**)

a) Anion-exchange chromatography on Permaphase AAX (2.1×500 mm) was performed with a gradient formed from two solutions, 0.01 M KH_2PO_4 (pH 4.5) and 0.05 M KH_2PO_4 (pH 4.5) containing 1 M KCl during 30 min, b) reversed phase chromatography on μBondapack (C-18) (3.9×300 mm) was performed with a gradient of acetonitrile (5—25%, 30 min) in 0.1 M triethylammonium acetate.

groups were removed by treatment with alkali and acid as described previously.[1] The deblocked product (**14**) was purified by anion-exchange chromatography and reversed phase chromatography (Fig. 1). Characterization of C-U-C-A-U-A-A was performed by high pressure liquid chromatography (HPLC) (Fig. 2) and mobility shift analysis (Fig. 3). Physicochemical properties of this heptamer will be reported elsewhere.

Comparison of the Reactions of the 5′-Phosphodiester with the Four Major Nucleoside 3′-Hydroxyl Groups

The present condensation between **7** and **12** gave a satisfactory yield. The previous block condensation in the synthesis of oligonucleotides containing AUG, where condensation took place between the 5′-phosphodiester of an adenosine derivative and the 3′-hydroxy of protected guanosine did not proceed to completion.[1] To investigate the reaction of this type, reactions between

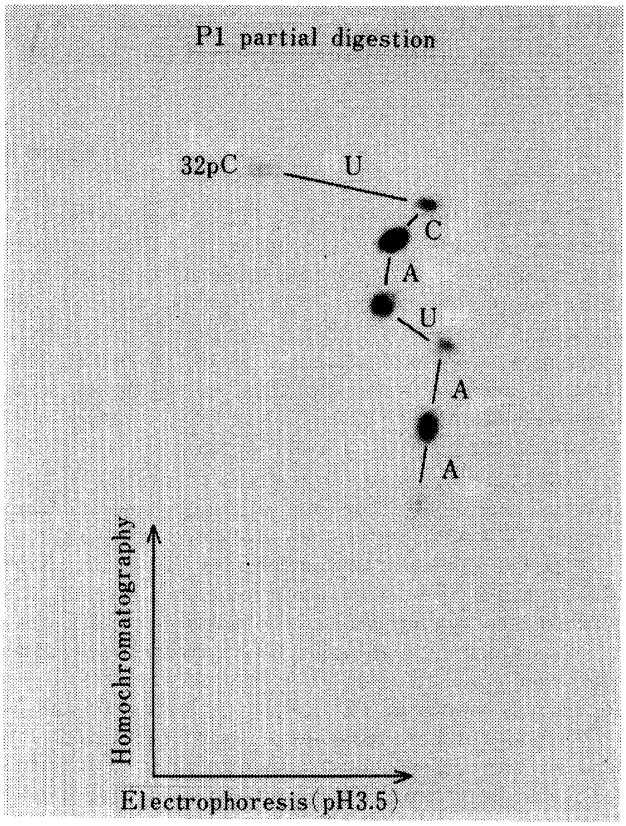


Fig. 3. Two-dimensional Homochromatography of the Products obtained by Partial Digestion of ^{32}pC–U–C–A–U–A–A with Nuclease P1

Homo-mix III[14] was used for the second run.

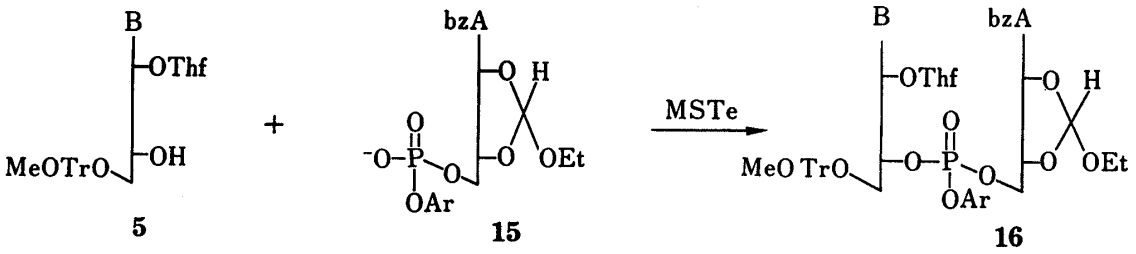


Chart 4

TABLE I. Condensation of Protected pA (**15**) with Different Nucleosides (**5**)

No.	**5** amnt (μmol)	**15** amnt (μmol)	MSTe amnt (μmol)	Yield %
1 B=U	150	151	453	63
2 B=bzC	149	150	465	68
3 B=bzA	150	150	450	87
4 B=ibG	127	127	381	58

the 5′-phosphodiester of 2′,3′-ethoxymethylidene-*N*-benzoyladenosine and the 3′-hydroxyl group of four major nucleoside were performed as shown in Chart 4. The results are summarized in Table I. The differences in yields of dimers with the different 3′-hydroxyl components were very small. The guanosine derivative gave the lowest yield. Since the condensation between a 3′-phosphodiester component and 3′,5′-unprotected guanosine occurred rather selectively, the 3′-hydroxyl group of protected guanosine might be hindered sterically. This effect may be enhanced when guanosine is involved in a large fragment. This may mean that block condensations can best be designed by avoiding oligonucleotides containing 3′-guanosine.

Experimental

Thin layer chromatography (TLC) was performed on plates of silica gel (Kieselgel HF_{254}, Merck) using a mixture of chloroform and methanol. For reversed phase thin layer chromatography (RTLC), silanized silica gel, high performance thin layer chromatography (HPTLC) RP-2 or RP-8 F_{254} (Merck) was used with a mixture of acetone–water. For columns, silica gel (60 or 60 H, Merck) was packed with chloroform and elution was carried out with a mixture of chloroform and methanol. For reversed phase column chromatography, alkylated silica gel (C-18, 35—105μ, Waters) was packed with 60—70% acetone and compounds in acetone were applied after addition of water until slight turbidity was apparent. Elution was performed with 60—80% acetone in 0.2% aqueous pyridine. High pressure liquid chromatography (HPLC) was carried out an Altex 332 MP apparatus.

Ethoxymethylidene derivatives of uridine[11] and *N*-benzoyladenosine[12] were prepared by using *p*-toluenesulfonic acid according to a slight modification of a published method.[13] Triethylammonium bicarbonate (TEAB) buffer (pH 7.5) used to wash organic layers containing protected nucleosides.

2′-*O*-Tetrahydrofuranylnucleosides (**4**)——a) *N*-Protected nucleoside (**1**) (10 mmol) was dissolved in DMF (15 ml) in the presence of imidazole (44 mmol) and the mixture was stirred with $TIPDSiCl_2$ (11 mmol) for 1 h to ensure completion of the reaction. DMF was removed by evaporation *in vacuo* and **2** was extracted with chloroform (200 mmol). The extract was washed twice with sat. sodium bicarbonate and once with water, then concentrated. The residue was dried by evaporation three times with pyridine then once with toluene. *Rf* values in TLC (9: 1) for **2a**, **2b**, **2c** and **2d** were 0.47, 0.66, 0.78 and 0.78, respectively.

Without further purification, **2a**, **b**, or **c** was dissolved in THF (50 ml) and 2,3-dihydrofuran (30 ml) was added in the presence of *p*-toluenesulfonic acid with cooling. The mixture was checked by TLC (9: 1) after it had been kept at room temperature for 2 h and neutralized with conc. ammonia (0.3—0.4 ml). The solution was concentrated and the residue was dissolved in chloroform (200 ml). The solution was filtered to remove insoluble salt, then washed twice with sat. sodium bicarbonate and twice with water. Chloroform was removed and the product (**3**) was dried by evaporation with pyridine and toluene.

3a, **b**, and **c** were each treated with 1 M TBAF in THF (30 ml) at room temperature for 10 min and the formation of **4a**, **b**, and **c** was checked by TLC (5: 1). The solution was concentrated and **4** was applied to a

column of silica gel (60 H, 60—70 g).

b) The Pyridinium *p*-Toluenesulfonate Method: **2b** (19 mmol), which had been isolated by silica gel chromatography, was dissolved in methylene chloride (130 ml) and the solution was stirred with 2,3-dihydrofuran in the presence of pyridinium *p*-toluenesulfonate (3.8 mmol) at room temperature for 14 h. Methylene chloride (100 ml) was added and the mixture was washed twice with sat. sodium bicarbonate and once with water. The product **3b** was dried by coevaporation with pyridine then with toluene, and treated with TBAF (1 M, 38 ml in THF) for 15 min. THF was removed and the residue was dissolved in methylene chloride–pyridine (3: 1, 200 ml). The mixture was washed twice with sat. sodium bicarbonate and the product was back extracted with methylene chloride–pyridine (3: 1, 50 ml). The combined organic solution was concentrated and applied to a column of silica gel (60H, 75 g) without being passed through a column of Dowex 50×2. Elution was performed with increasing concentrations of methanol (0—10%) in chloroform. Two diastereoisomers were separated. **4b**-h and **4b**-l were recrystallized from ethyl acetate and ethanol, respectively. The yields of **4b**-h and **4b**-l were 46% (3.61 g, 8.7 mmol) and 41% (3.25 g, 7.8 mmol).

2c, **2a** or **2d** (55.6 mmol) was reacted with 2,3-dihydrofuran (111 mmol) in methylene chloride (300 ml) in the presence of pyridinium *p*-toluenesulfonate (11.8 mmol) at 22°C for 12 h, and the mixture was worked up as above except that it was passed through a column of Dowex 50×2 (pyridinium) after treatment with TBAF (122 mmol). The column was washed with a mixture of pyridine: methanol: water (3: 1: 1, 500 ml) and the combined solution was concentrated. The product was extracted with chloroform. The extract was washed 3 times with sat. sodium bicarbonate, then with water (800 ml), and back extracted. The solution was concentrated and the residue was coevaporated with toluene, precipitated as a syrup with hexane from its solution in chloroform (100 ml), and applied to a column (10×7 cm) of silica gel (60 H, 200 g). Elution was performed with chloroform containing methanol (0—5%). The yields of **4c**-h and **4c**-l were 38% (9.34 g, 21.2 mmol) and 47% (11.64 g, 26.4 mmol). **4a**-h (47%), **4a**-l (43%), **4d**-h (49%) and **4d**-l (49%) were obtained by the same procedure.

Protected CUC (7)——**5c** (1.27 mmol) was phosphorylated with *o*-chlorophenyl phosphoroditriazolide, which was prepared from *o*-chlorophenyl phosphorodichloridate (2.5 mmol) as described previously.[1] The phosphodiester thus obtained was condensed with **4a**-l (1.52 mmol) using MSTe (2.54 mmol) at 30°C. After 30 min, aqueous pyridine (50%, 6 ml) was added with cooling, then 0.1 M TEAB (60 ml) was added. The product was extracted with chloroform (80 ml). The extract was washed twice with 0.1 M TEAB (60 ml) and applied to a column of C-18 silica gel. Elution was performed with acetone–0.2% aqueous pyridine (6: 4, 7: 3). Protected CU (**6**) was obtained in a yield of 60% (0.822 g, 0.75 mmol) as a powder. **6** (0.75 mmol) was phosphorylated as described above and condensed with **4b**-l (0.94 mmol) using MSTe (1.52 mmol) at 30°C. The trimer (**7**) was isolated by chromatography on C-18 silica gel (100 ml) in a yield of 67% (0.886 g, 0.50 mmol). Elution was performed with acetone–0.2% aqueous pyridine with increasing acetone content from 40% to 60%.

Protected pAUAA (11)——**4c**-l (2 mmol) was treated with *o*-chlorophenyl *p*-anisodophosphorochloridate (2.2 mmol) in pyridine (10 ml) at room temperature for 6 h. RTLC showed that no starting material remained. Water was added with cooling and the mixture was concentrated. The product (**8**) was isolated by chromatography on C-18 silica gel with 40 to 60% acetone in 0.2% aqueous pyridine in a yield of 50% (0.74 g, 1.0 mmol). **8** (1.1 mmol) was phosphorylated and condensed with **4a**-h (1.32 mmol) using MSTe (2.2 mmol) at 30°C to give protected AU **9** (734 mg, 0.6 mmol, 55%) after C-18 silica gel chromatography (40%—70% acetone). The trimer (**10**) was synthesized by the same procedure as described for the preparation of **9** using **9** (0.6 mmol) and **4c**-h (0.75 mmol). Isolation of **10** was performed by similar reversed phase chromatography with acetone–0.2% pyridine (acetone, 40—80%). The yield was 71% (782 mg, 0.42 mmol). **10** was then phosphorylated and condensed with 2′,3′-ethoxymethyliden-*N*-benzoyladenosine (0.51 mmol). **11** was isolated by reversed phase chromatography with 50—70% acetone. The yield was 67% (685 mg, 0.28 mmol).

C-U-C-A-U-A-A (14)——**11** (0.10 mmol) was treated with isoamyl nitrite (4.0 mmol) in pyridine–acetic acid (5: 4, 3 ml) at 30°C for 2 h and the product (**12**) was precipitated with pentane–ether (1: 1, 100 ml). The syrup thus obtained was dissolved in pyridine–chloroform (1: 2, 45 ml), and the solution was washed three times with 0.2 M TEAB (40 ml), then concentrated. The residue was dried by coevaporation with pyridine then with toluene. The product was precipitated with pentane–ether (3: 1, 80 ml) as a powder (234 mg, 0.1 mmol). 12 thus obtained was condensed with **7** (0.11 mmol) using MSTe (0.3 mmol) in pyridine at 30°C. After 1 h, aqueous pyridine (50%, 3 ml) was added with cooling. The product (**13**) was extracted with chloroform. The solution was washed with 0.1 M TEAB and applied to a column of C-18 silica gel (100 ml). Elution was performed with increasing concentrations of acetone (50—80%) in 0.2% aqueous pyridine. The yield of 13 was 313 mg, 76 μmol, 76%. An aliquot of **13** (123 mg, 30 μmol) was deblocked by treatment first with tetramethylguanidium pyridine-2-aldoximate (0.5 M, 14.4 ml) at room temperature for 3 d and the mixture was concentrated. The residue was dissolved in pyridine (3 ml) and treated with conc. ammonia (15 ml) at 50°C for 6 h. Ammonia was removed and the residue was passed through a column (1.8×6 cm) of Dowex 50×2 (pyridinium form) in 30% pyridine. The combined washings were concentrated and washed with ethyl acetate. The aqueous solution was concentrated, 0.01 N HCl (10 ml) was added to the residue, and the solution was adjusted to pH 2 by addition of 0.1 N HCl then kept at room temperature

for 5 h. Demonomethoxytritylation was confirmed by TLC and the solution was neutralized with 0.1 N ammonia. The solution was concentrated to *ca.* 100 ml, washed twice with ethyl acetate and applied to a column (2.8×7.5 cm) of DEAE-cellulose (bicarbonate). The column was washed with 0.05 M TEAB (250 ml) and the heptamer (1500 A_{260}) was eluted with 1 M TEAB. The product was purified by reverse-phase chromatography on a column (1.0×20 cm). of C-18 silica gel. Elution was performed with a gradient of methanol, (10—30%) in 1/15 M phosphate KH_2PO_4–Na_2HOP_4 (pH 7.5). The heptamer was desalted by absorption to a column (1.7×5 cm) of DEAE-cellulose. Salts were eluted with 0.05 M TEAB (300 ml) and the product was eluted with 1 M TEAB (130 ml). The product (520 A_{260}) was analyzed by HPLC using C-18 silica gel (μBondapak, 5 μ) and DEAE-silica gel (Pharmaphase AAX).

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