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Synthesis and Properties of 2′,5′-Adenylate Trimers Bearing 2′-Terminal 8-Bromo- or 8-Hydroxyadenosine

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Trimers of 2′,5′-oligoadenylic acids bearing 2′-terminal 8-bromo- or 8-hydroxyadenosine have been synthesized by the phosphotriester method. Some properties of these compounds were studied by circular dichroism (CD) spectroscopy. The 2′,5′-adenylate trimer, A2′p5′A2′p5′HOA (**10a**) was found to have a less stacked structure than the 2′,5′-adenylate trimers A2′p5′A2′p5′BrA (**11**) and A2′p5′A2′p5′A (**10b**).

Keywords——modified 2′,5′-adenylate trimer; N^6,N^6,3′-*O*-tribenzoyladenosine; 8-bromoadenosine; coupling agent; CD spectrum; HPLC; reversed-phase liquid chromatography

A mixture of oligomers (2-5 oligo A) with the general formula ppp5′A2′p5′(A2′p)$_n$5′pA, is synthesized in extracts of interferon-treated cells in the presence of double-stranded ribonucleic acid (dsRNA).[1] This mixture may take part in a mechanism of interferon antiviral action by acting as an activator of endonuclease L, which degrades messenger and ribosomal RNAs, and in this way inhibits translation.[2-4] The possibility of using 2-5 oligo A as a drug is limited because of its highly ionic character, which does not permit it to enter intact cells, and its fast hydrolysis by a 2′,5′ bond-specific phosphodiesterase.[5] From this point of view, less polar and more lipophilic analogs with increased stability may be important.[6] Recently, Lesiak *et al.* have reported[7] that 8-bromo-substituted 2-5 oligo A trimer is active as a protein synthesis inhibitor in mouse L-cells and is more resistant to degradation in the presence of cytoplasmic enzymes. It would be of interest to determine whether all 8-bromoadenosine residues are equally important for the activation of the endoribonuclease L. The use of analogs with different numbers or positions of modified groups is of growing importance in this connection. In this paper, we wish to report a simple synthetic method and some properties of 2′,5′-adenylate trimers bearing 2′-terminal 8-bromo- or 8-hydroxyadenosine.

Synthesis of 2′,5′-Adenylate Trimer Analogs

Synthesis of the 2′-terminal fragment 2′,3′-*O*-N^6,N^6-tetrabenzoyl-8-bromoadenosine (**1a**) was achieved according to the method of Khorana[8] in 79% yield. The second component **5** was prepared simply as follows. 3′-*O*-Benzoyladenosine (**2**)[9] was treated first with trimethylsilyl chloride in dry pyridine at room temperature for 1 h, then with benzoyl chloride for 6 h, and finally with a 5% $NaHCO_3$ solution at 0 °C for 1 h. N^6,N^6,3′-*O*-Tribenzoyladenosine (**4**) could be isolated in 60% yield by chromatography on silica gel. Tritylation of **4** with dimethoxytrityl chloride in dry pyridine gave the tritylated product **5** in 91% yield.

The phosphorylation of **5** was carried out by using 5-chloro-8-quinolyl phosphate (pQCl)[10] in the presence of 8-quinolinesulfonyl chloride (QsCl)[11] to give the corresponding phosphodiester derivative **6** in 98% yield after chromatography on a short column of silica gel. The condensation of **6** (1.5 molar equiv.) and **1a** (1.0 molar equiv.) was performed by

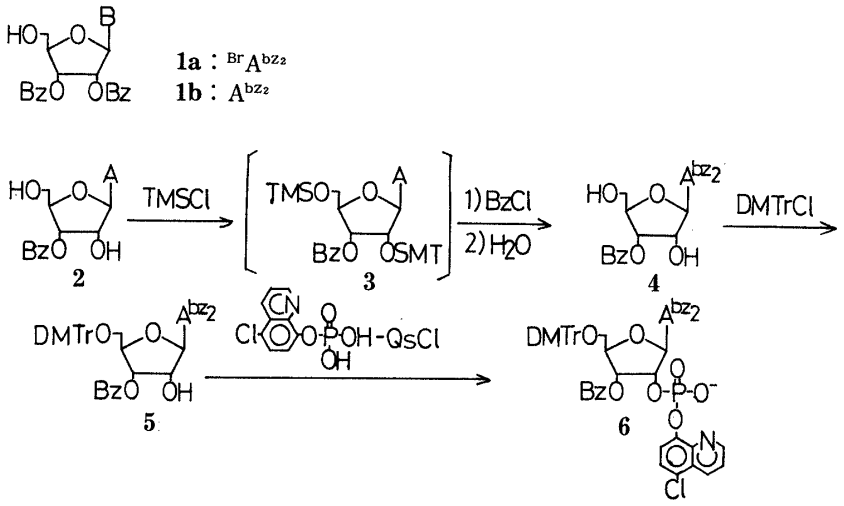


Chart 1

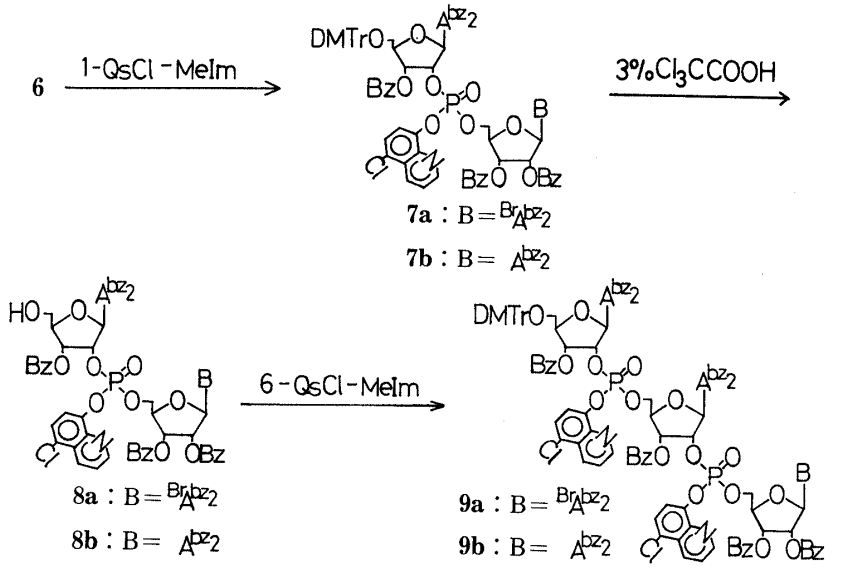


Chart 2

using QsCl (4.5 molar equiv.) and *N*-methylimidazole (MeIm)[12] (9.0 molar equiv.) at room temperature. The reaction was complete within 1 h and the usual work-up gave the fully protected dinucleoside monophosphate (**7a**) in 90% yield. Removal of the dimethoxytrityl group from the dimer **7a** thus obtained was performed by treatment with 3% Cl_3CCOOH in CH_3NO_2–MeOH (95:5 (v/v)) at room temperature for 5 min.[13] The usual work-up and purification of the reaction mixture gave the detritylated product **8a** in 98% yield. The 5′-hydroxyl component **8a** (1.0 molar equiv.) thus obtained was treated with **6** (1.5 molar equiv.) in the presence of QsCl (3.0 molar equiv.) and MeIm (6.0 molar equiv.) in dry pyridine for 1 h to give the fully protected trinucleoside diphosphate (**9a**) in 92% yield. In a similar manner, the 2′,5′-adenylate trimer (**9b**) was obtained in 87% yield.

Deprotection of All the Protecting Groups from 9

We first examined complete deblocking for the fully protected trimer **9a**. The trimer **9a** was treated with a large amount of zinc acetate in aqueous pyridine at room temperature to cleave the 5-chloro-8-quinolyl groups. After 2 d, thin-layer chromatography (TLC) analysis (solvent A) showed complete conversion of **9a** into base line material.[14] The mixture was treated with Dowex 50W-X2 (pyridinium form) and the resin was removed by filtration. The

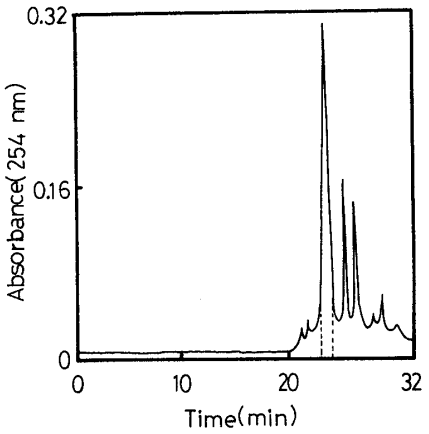


Fig. 1. Reversed-Phase Chromatography of **10a** on a Column of Finepak (SIL C-18)

Elution was performed with a gradient of MeOH–H_2O (1 : 1 (v/v), 0—70%) in 0.05 M ammonium phosphate (pH 7.0). The flow rate was 2 ml/min.

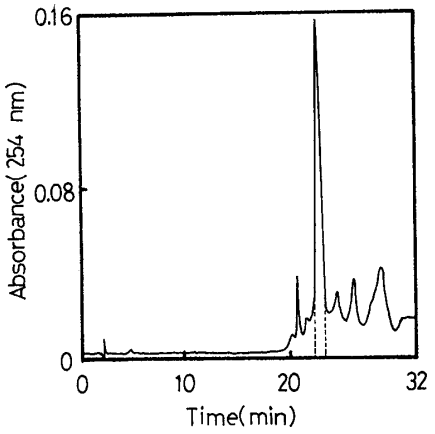


Fig. 2. Reversed-Phase Chromatography of **11** on a Column of Finepak (SIL C-18)

Elution was performed with a gradient of MeOH–H_2O (1 : 1 (v/v), 0—70%) in 0.05 M ammonium phosphate (pH 7.0). The flow rate was 2 ml/min.

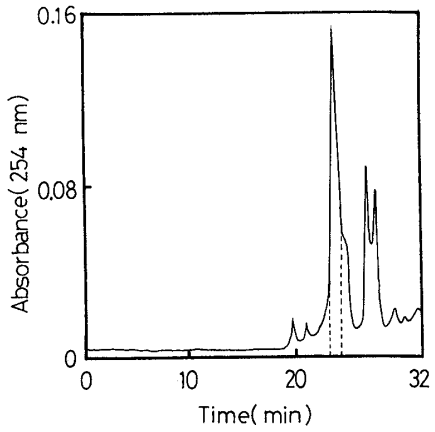


Fig. 3. Reversed-Phase Chromatography of **10b** on a Column of Finepak (SIL C-18)

Elution was performed with a gradient of MeOH–H_2O (1 : 1 (v/v), 0—70%) in 0.05 M ammonium phosphate (pH 7.0). The flow rate was 2 ml/min.

filtrate was concentrated and the residue was dissolved in conc. ammonia–pyridine (9 : 1 (v/v)). The mixture was kept at room temperature for 2 d to remove the benzoyl groups. The solution was concentrated and 80% AcOH was added to split off the dimethoxytrityl group. After 15 min, the solution was evaporated *in vacuo*, and the residue was dissolved in water and washed with ether. The 2′,5′-adenylate trimer analog was then purified from the mixture by high-pressure liquid chromatography (HPLC) on reversed-phase column (Finepak SIL C-18) with a gradient of MeOH–H_2O (1 : 1, 0—70%) in 0.05 M ammonium phosphate (pH 7.0) (Fig. 1). The 2′,5′-adenylate trimer analog thus obtained was hydrolyzed with snake venom phosphodiesterase, which yields component nucleoside and nucleoside 5′-phosphates. Paper chromatography of the incubation mixture showed three spots corresponding to adenosine, adenosine 5′-phosphate, and 8-hydroxyadenosine 5′-phosphate (pHOA) in a ratio of 1.00 : 1.03 : 1.03. Thus, the 2′,5′-adenylate trimer analog was confirmed to be adenylyl-(2′-5′)-adenylyl-(2′-5′)-8-hydroxyadenosine (A2′p5′A2′p5′HOA) (**10a**).

As we failed to obtain adenylyl-(2′-5′)-adenylyl-(2′-5′)-8-bromoadenosine (A2′p5′A2′p5′BrA) (**11**) under the above conditions, we tried modified deblocking procedures and found that when methanolic ammonia was used in place of conc. ammonia–pyridine (9 : 1, (v/v)), the desired 2′,5′-adenylate trimer analog **11** was isolated in 39% yield after separation on an HPLC reversed-phase column (Fig. 2). The 2′,5′-adenylate trimer analog **11** was hydrolyzed by snake venom phosphodiesterase to give adenosine, adenosine 5′-

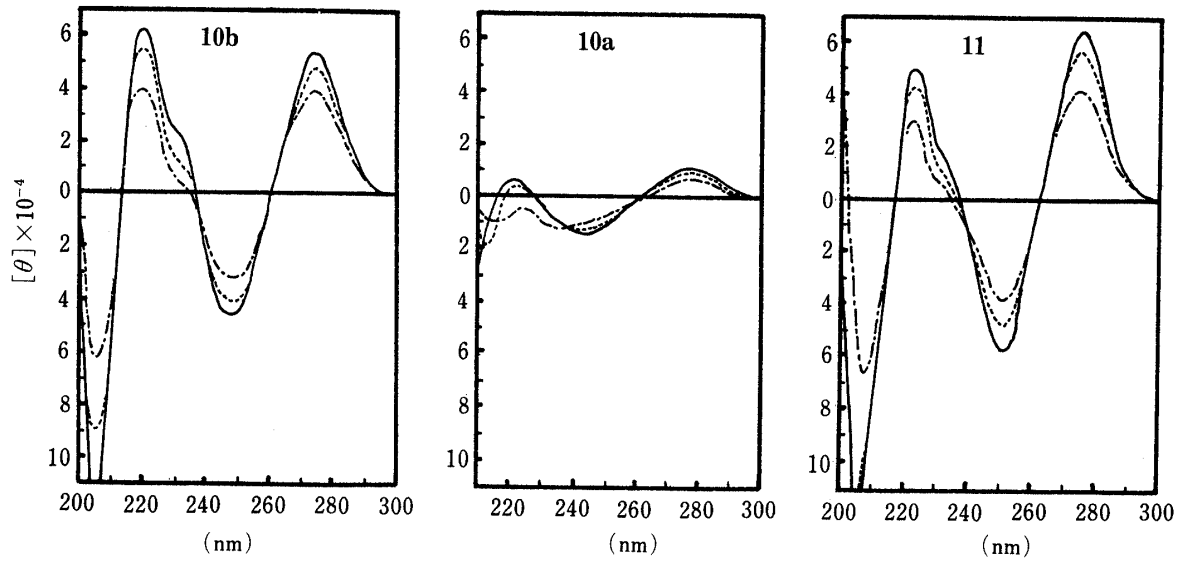
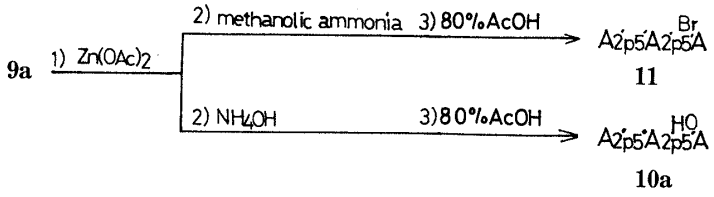


Fig. 4. CD Spectra of **10a**, **10b**, and **11** in 0.1 M Phosphate Buffer (pH 7.0) at Various Temperatures

——, +4 °C; – – – –, +21 °C; – – – – –, +62 °C.



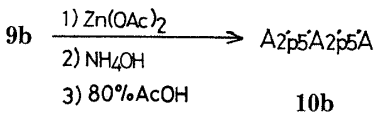


Chart 3

phosphate, and 8-bromoadenosine 5′-phosphate (pBrA) in a ratio of 1.00 : 0.97 : 1.01.

Treatment of **9b** by the same procedure as described in the case of **10a** gave the 2′,5′-adenylate trimer (A2′p5′A2′p5′A) (**10b**) in 38% yield. Compound **10a** was hydrolyzed by snake venom phosphodiesterase to give adenosine and adenosine 5′-phosphate in a ratio of 1.00 : 1.90. However, **10a**, **b** and **11** were resistant to the actions of nuclease P1 and ribonuclease (RNase) T_2, which are specific for 3′-5′ phosphodiester bonds.

Circular Dichroism (CD) Spectra of 2-5 Oligo A (10a, b and 11)

The CD spectra of the 2-5 oligo A (**10a**, **b** and **11**) at several temperatures are presented in Fig. 4. There are some interesting features. The Cotton effect amplitude of **10a** is smaller than those of **10b** and **11**, which suggests a decreased stacking. On the other hand, no significant difference was observed between the CD spectra of **10b** and **11**. Also, 2-5 oligo A, **10a**, **b** and **11** exhibit decreased Cotton effect amplitudes at elevated temperature. These results suggest that the 2-5 oligo A analog **10a** has a different conformation from the 2-5 oligomers **10b** and **11**.

Experimental

Ultraviolet (UV) spectra were recorded on a Shimadzu UV-200 spectrometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a JEOL JNMPS 100 spectrometer with tetramethylsilane (TMS) as an internal

standard. TLC was performed on precoated TLC plates of Silica gel 60 F_{254} (Merck, Art. No. 5715) and the *Rf* values of the nucleoside and nucleotide derivatives were measured after development with solvent A (CH_2Cl_2–MeOH, 9 : 1 (v/v)) or solvent B (CH_2Cl_2–MeOH, 95 : 5 (v/v)). Column chromatography was performed with Silica gel (300 mesh) from Kanto Chem. Co., Ltd. Paper chromatography was performed by the descending technique with Whatmann 3 MM paper using solvent C (1-PrOH–conc. NH_4OH–H_2O, 55 : 35 : 10 (v/v)). HPLC was performed on a Finepak SIL C-18 reversed-phase column, and elution was done with a gradient of MeOH–H_2O (1 : 1 (v/v) 0—70%) in 0.05 M ammonium phosphate (pH 7.0) over 32 min at the flow rate of 2 ml/min. Paper electrophoresis was performed with 0.05 M triethylammonium bicarbonate (TEAB) (pH 7.5) at 1100/40 cm. 8-Bromoadenosine was prepared by the published procedure.[15] 8-Quinolinesulfonyl chloride, dimethoxytrityl chroride (DMTrCl), and *N*-methylimidazole were obtained commercially. Snake venom phosphodiesterase was from Boehringer Mannheim GmbH and nuclease P1 was from Yamasa Co.

Enzyme digestion was carried out as described previously.[16,17] CD spectra were measured with a Jasco J-500A spectrometer using a 2-mm cell in 0.1 M phosphate buffer (pH 7.0).

N^6,N^6,2′,3′-*O*-Tetrabenzoyl-8-bromoadenosine (1a)——8-Bromoadenosine (1.73 g, 5.0 mmol) was treated with DMTrCl (2.20 g, 6.5 mmol) in dry pyridine (25 ml) at room temperature for 2 h. The mixture was quenched with ice-water (50 ml) and extracted with CH_2Cl_2 (150 ml). The CH_2Cl_2 extract was washed with water (2 × 100 ml), dried over Na_2SO_4, and evaporated *in vacuo*. The residue was dissolved in dry pyridine (25 ml) and benzoyl chloride (5.8 ml, 50 mmol) was added. The mixture was stirred for 3 h, quenched with ice-water (50 ml), then extracted with CH_2Cl_2 (2 × 70 ml). The organic extracts were combined, washed with 5% $NaHCO_3$ (3 × 100 ml), dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was treated with 80% AcOH at room temperature for 2 h. The solution was concentrated and coevaporated several times with water to remove the last traces of AcOH and then chromatographed on a silica gel column using CH_2Cl_2–MeOH (97 : 3 (v/v)) to give **1a** (30.5 g, 79%) as a white powder, which was precipitated from its CH_2Cl_2 solution into hexane to give an analytically pure sample. mp 121—123 °C. UV $\lambda_{max}^{methanol}$ nm: 278. NMR (DMSO-d_6) δ: 3.85 (m, 2H, C-5′), 4.55 (m, 1H, C-4′), 5.00—5.30 (m, 1H, 5′-OH), 6.17 (t, 2H, C-2′, C-3′), 6.57 (d, 1H, $J_{1',2'}$ = 3 Hz, C′-1), 7.40—8.10 (m, 20H, Ar), 8.82 (s, 1H, C-2). *Anal.* Calcd for $C_{38}H_{28}BrN_5O_8 \cdot 1/2\ C_6H_{14}$: C, 60.76; H, 4.08; N, 8.86. Found: C, 60.98; H, 3.83; N, 9.11.

N^6,N^6, 3′-*O*-Tribenzoyladenosine (4)——3′-*O*-Benzoyladenosine (**2**) (3.65 g, 9.8 mmol) was treated with trimethylsilyl chloride (TMSCl) (12.5 ml, 98 mmol) in dry pyridine (50 ml) at room temperature. After 1 h, benzoyl chloride (2.7 ml, 24 mmol) was added. The reaction mixture was stirred for 4 h, quenched with ice-water (10 ml) and extracted with CH_2Cl_2 (2 × 150 ml). The organic extracts were combined, washed with 5% $NaHCO_3$ (3 × 100 ml), dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was recrystallized from ethanol to give pure **4** (3.41 g, 60%), mp 175—177 °C. *Rf* 0.67 (solvent A). UV $\lambda_{max}^{methanol}$ nm: 273, 230. NMR ($CDCl_3$) δ: 4.08 (br s, 2H, C-5′), 4.67 (br s, 1H, C-4′), 5.23, 5.50 (m, 2H, 5′-OH, C-2′), 5.65 (d, 1H, 2′-OH), 5.90 (d, 1H, C-3′), 6.05 (d, 1H, $J_{1',2'}$ = 5 Hz, C-1′), 7.30—8.15 (m, 15H, Ar), 8.20 (s, 1H, C-2), 8.68 (s, 1H, C-8). *Anal.* Calcd for $C_{31}H_{25}N_5O_7 \cdot 5/3\ C_2H_5OH$: C, 62.83; H, 5.37; N, 10.67. Found: C, 62.57; H, 5.17; N, 10.51.

N^6,N^6,3′-*O*-Tribenzoyl-5′-*O*-dimethoxytrityladenosine (5)——Compound **4** (337 mg, 0.56 mmol) was rendered anhydrous by repeated coevaporations with dry pyridine and then dissolved in dry pyridine (28 ml). DMTrCl (280 mg, 0.64 mmol) was added and the mixture was kept for 1 h. The usual work-up followed by chromatography gave pure **5** (450 mg, 91%). *Rf* 0.75 (solvent A). UV $\lambda_{max}^{methanol}$ nm: 273, 234. NMR ($CDCl_3$) δ: 3.45 (br s, 2H, C-5′), 3.70 (s, 6H, OCH_3), 4.50 (br s, 1H, C-4′), 5.21 (t, 1H, C-2′), 5.63 (m, 2H, 2′-OH, C-3′), 6.10 (d, 1H, $J_{1',2'}$ = 6 Hz, C-1′), 6.58—8.10 (m, 28H, Ar), 8.27 (s, 1H, C-2), 8.53 (s, 1H, C-8).

2′-(5′-*O*-Dimethoxytrityl-N^6,N^6,3′-*O*-tribenzoyladenosyl)-8-(5-chloro-8-quinolyl) Phosphate (6)——Nucleoside **5** (2.37 g, 2.7 mmol) and pQCl (1.06 g, 4.1 mmol) were dried in the same manner as described above and then treated with QsCl (1.06 g, 1.04 mmol) in dry pyridine (27 ml) for 1 h. Extractive work-up followed by chromatography using CH_2Cl_2–MeOH–Et_3N (95 : 5 : 5 (v/v)) as the solvent for elution gave the triethylammonium salt of **6** (3.22 g, 97%). *Rf* 0.15 (solvent A). UV $\lambda_{max}^{methanol}$ nm: 280, 237. ^{31}P-NMR ($CDCl_3$, 85% H_3PO_4) δ: −4.68.

Synthesis of 2′,5′-Adenylate Trimer Analog (9)——The phosphodiester **6** (1.22 g, 1.0 mmol) and **1a** (0.46 g, 0.67 mmol) were dried in the same manner as previously described and then treated with QsCl (0.68 g, 3.0 mmol) and MeIm (0.48 ml, 6.0 mmol) in dry pyridine (3.5 ml) for 1 h. The reaction mixture was quenched with aqueous pyridine (1 ml) and extracted with CH_2Cl_2 (2 × 25 ml). The organic extracts were combined, washed with 0.1 M TEAB solution (3 × 15 ml), dried over Na_2SO_4, filtered, and evaporated *in vacuo*. The residue was redissolved in CH_2Cl_2 and chromatographed on a silica gel column. The appropriate fractions [eluted with CH_2Cl_2–MeOH (97 : 3 (v/v))] were evaporated to give the fully protected dimer **7a**, which was isolated as a solid (1.18 g, 90%) by precipitation from hexane. *Rf* 0.77 (solvent A). UV $\lambda_{max}^{methanol}$ nm: 278, 232.

The dimer **7a** (1.18 g, 0.63 mmol) was treated with 3% Cl_3CCOOH in CH_3NQ_2–MeOH (95 : 5 (v/v) 15 ml) at room temperature for 15 min. The mixture was quenched with aqueous pyridine (5 ml) and extracted with CH_2Cl_2 (25 ml). The CH_2Cl_2 extract was washed with water (2 × 10 ml), dried over Na_2SO_4, filtered, and evaporated *in vacuo*. The residue was dissolved in CH_2Cl_2 and added dropwise with stirring to hexane–ether (9 : 1 (v/v)). The precipitate was collected by filtration and dried over P_4O_{10} *in vacuo* to give **8a** (0.91 g, 92%). *Rf* 0.70 (solvent A). UV $\lambda_{max}^{methanol}$ nm: 280, 232.

A mixture of **6** (1.07 g, 0.87 mmol) and **8a** (0.91 g, 0.58 mmol) in dry pyridine (3 ml) was then condensed in the presence of QsCl (0.59 g, 2.61 mmol) and MeIm (0.42 ml, 5.22 mmol) for 2 h. The same work-up as described in the case of **7a** gave **9a** (1.43 g, 92%). *Rf* 0.75 (solvent A). UV $\lambda_{max}^{methanol}$ nm: 278, 232.

In the same way, trimer **9b** was isolated as a solid (1.26 g, 87%). *Rf* 0.83 (solvent A). UV $\lambda_{max}^{methanol}$ nm: 274, 232.

Deprotection of All the Protecting Groups from 9——a) **9a** (83 mg, 0.03 mmol) was treated with zinc acetate (690 mg, 3.1 mmol) in pyridine–H_2O (9 : 1 (v/v) 1.5 ml) at room temperature for 2 d. The solution was passed through a column (2 × 15 cm) of Dowex 50W-X2 (pyridinium form) in 30% pyridine. The combined washings were concentrated. The residue was dissolved in conc. NH_4OH (10 ml) and the mixture was kept for 2 d. Ammonia was removed and the residue was treated with 80% AcOH (5 ml) for 30 min. The solution was concentrated and the residue was redissolved in 0.01 M TEAB (20 ml). This solution was washed with ether (2 × 10 ml). The deblocked product (**10a**) was isolated by HPLC on a reversed-phase column (Finepak SIL C-18). Elution was performed with a gradient of MeOH–H_2O (1 : 1 (v/v) 0—70%) in 0.05 M ammonium phosphate (pH 7.0) over 32 min (Fig. 1). The trimer was desalted by absorption on a column (2 × 20 cm) of Sephadex G-10. The product was eluted with 0.01 M ammonium carbonate. The product **10a** (38%, 398 A_{254}) was characterized by base composition analysis by HPLC after complete digestion with snake venom phosphodiesterase. The ratio of A : pA : pHOA was 1.00 : 1.03 : 1.03. *Rf* 0.92 (to pA, solvent C), *Rm* 0.58 (to pA). UV $\lambda_{max}^{H_2O}$ (pH 7.0) nm: 257.

In the same way, **10b** was isolated in 26% (264 A_{254}) after separation by HPLC on a reversed-phase column (Fig. 3). The product **10b** was hydrolyzed by using snake venom phosphodiesterase to give A and pA in a ratio of 1.00 : 1.98. *Rf* 0.95 (to pA, solvent C), *Rm* 0.56 (to pA). UV $\lambda_{max}^{H_2O}$ (pH 7.0) nm: 258.

Compound **9a** (267 mg, 0.1 mmol) was dissolved in pyridine–H_2O (9 : 1 (v/v) 4 ml) and zinc acetate (1.40 g, 6.4 mmol) was added. The reaction mixture was stirred at room temperature for 3 d, then treated with Dowex 50W-X2 (pyridinium form) as above. The phosphodiester product was dissolved in methanolic ammonia (30 ml) and the mixture was kept for 1 d. The solution was concentrated and the residue was dissolved in 80% AcOH (10 ml). After 30 min, the usual work-up followed by HPLC on a C-18 silica gel using a gradient of MeOH–H_2O (1 : 1 (v/v) 0—70%) in 0.05 M ammonium phosphate (pH 7.0) over 32 min at the flow rate of 2 ml/min (Fig. 3) gave **11** (39%, 1320 A_{254}). The product **11** was hydrolyzed by using snake venom phosphodiesterase to A, pA, and pBrA in a ratio of 1.00 : 0.97 : 1.01. *Rf* 0.88 (to pA) solvent C, *Rm* 0.55 (to pA). UV $\lambda_{max}^{H_2O}$ (pH 7.0) nm: 256.

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