Tetrahedron Vol. 46, No. 4, pp. 1181-1190, 1990
Printed in Great Britain

0040-4020/90 $3.00+.00
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SYNTHESIS OF CYTIDYL(3'-5')ADENOSINE BEARING 2'(3')-O-LEUCYL ESTER VIA A PHOSPHOROTHIOATE TRIESTER INTERMEDIATE

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(*Received in Japan* 13 *July* 1989)

Abstract: 2'(3')-O-(Leucyl)CpA was synthesized in the phosphotriester method. The phenylthio group was used as a protecting group of the internucleotidic bond. The P-S bond of the triester intermediate was selectively cleaved by using $(Bu_3Sn)_2O$ under neutral conditions without cleavage of the leucyl ester bond.

INTRODUCTION

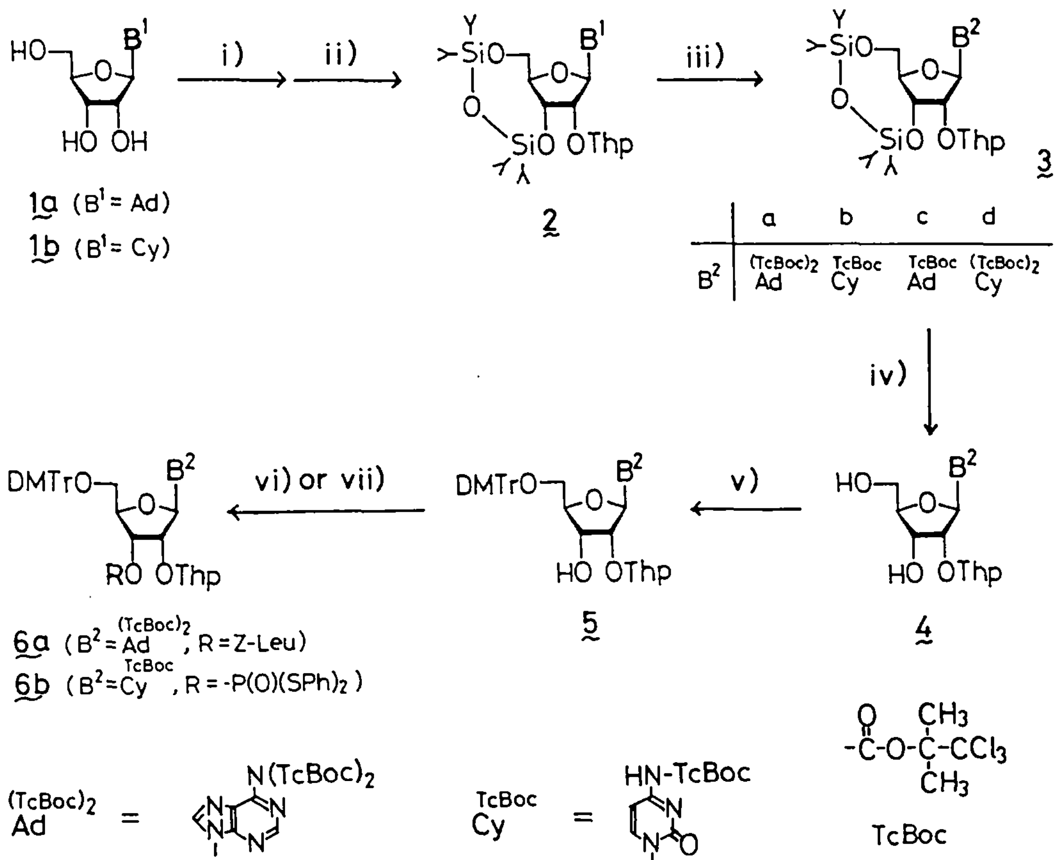
It has been well established that each tRNA has a uniform CCA sequence at the 3'-terminus and that the 2'- or 3'-hydroxyl group of the 3'-terminal adenosine is enzymatically esterified by the cognate amino acid. Therefore, 2'(3')-O-(aminoacyl)oligoribonucleotides (aa-RNA) have been used as powerful tools to study the mechanism of the protein biosynthesis.[1,2] In contrast to the recent progress in the RNA synthesis, the published procedures for the synthesis of aa-RNA still remain essential problem. Since the aminoacyl ester bond of aa-RNA is very labile under alkaline conditions,[3] the usual strategy used in the conventional RNA synthesis must be modified for the synthesis of aa-RNA.

The synthesis of aa-RNA in the phosphotriester method, which enabled us to isolate the fully protected intermediate easily by using silica gel column chromatography, was first accomplished by Chládek's group.[4] Recently, Chládek has reported an improved method using the 2-chlorophenyl and [(9-fluorenyl)oxy]carbonyl (Fmoc) groups as protecting groups for the internucleotidic phosphodiester bond and the amino function of the nucleoside base, respectively.[5] At the deprotection step, these protecting groups were concurrently removed by using N^1,N^1,N^3,N^3-tetramethylguanidine (TMG) and o-nitrobenzaldoxime in dry acetonitrile without the cleavage of the aminoacyl ester bond. However, we judged that the use of strong bases like TMG, which had a possibility to promote the racemization of amino acids, should be prevented. In practice the Fmoc group was removed by the basicity of TMG in a manner of β-elimination. Thus, we have planned to synthesize 2'(3')-O-(leucyl)CpA by using a phenylthio group, which could be removed by

$(Bu_3Sn)_2O$ under neutral conditions,[6] as a protecting group of the inter-nucleotidic phosphodiester bond.

RESULTS AND DISCUSSION

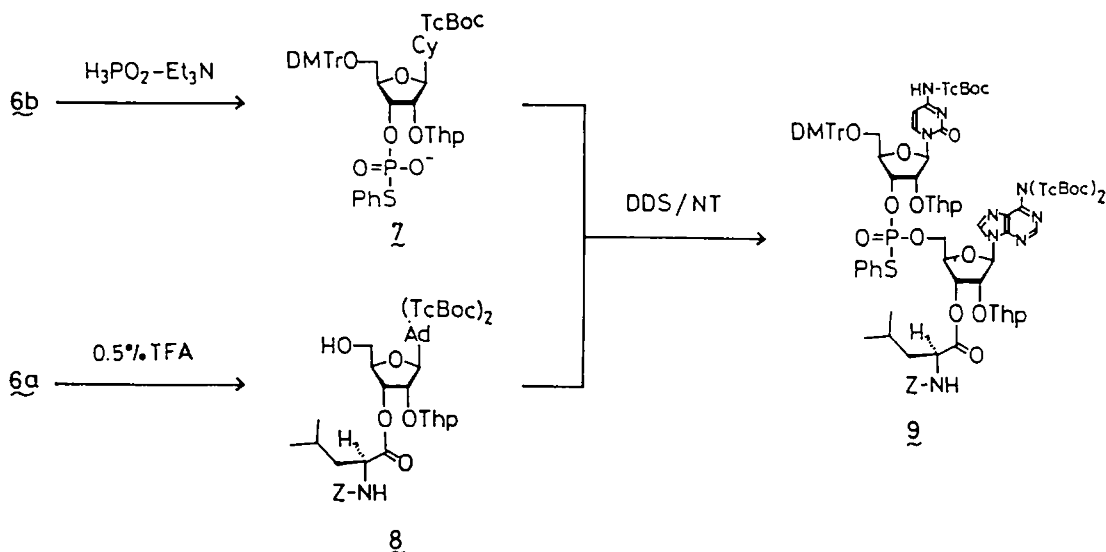
First, the intermediates **2a**,**2b** were synthesized from adenosine (**1a**) or cytidine (**1b**) by a two step procedure.[7] The amino functions of the nucleoside bases were further protected by the trichloro-t-butoxycarbonyl (TcBoc) group which could be reductively removed by zinc-acetylacetone under neutral conditions. It was found that the N^6-monosubstituted derivative of adenosine (**3c**), which was reported by Ugi[8] and Chattopadhyaya,[9] was further oxycarbonylated to afford the N^6,N^6-disubstituted derivative **3a** in 95% yield by the reaction of **2a** (B^1 = Ad) with 2 equiv of TcBocCl in pyridine. In



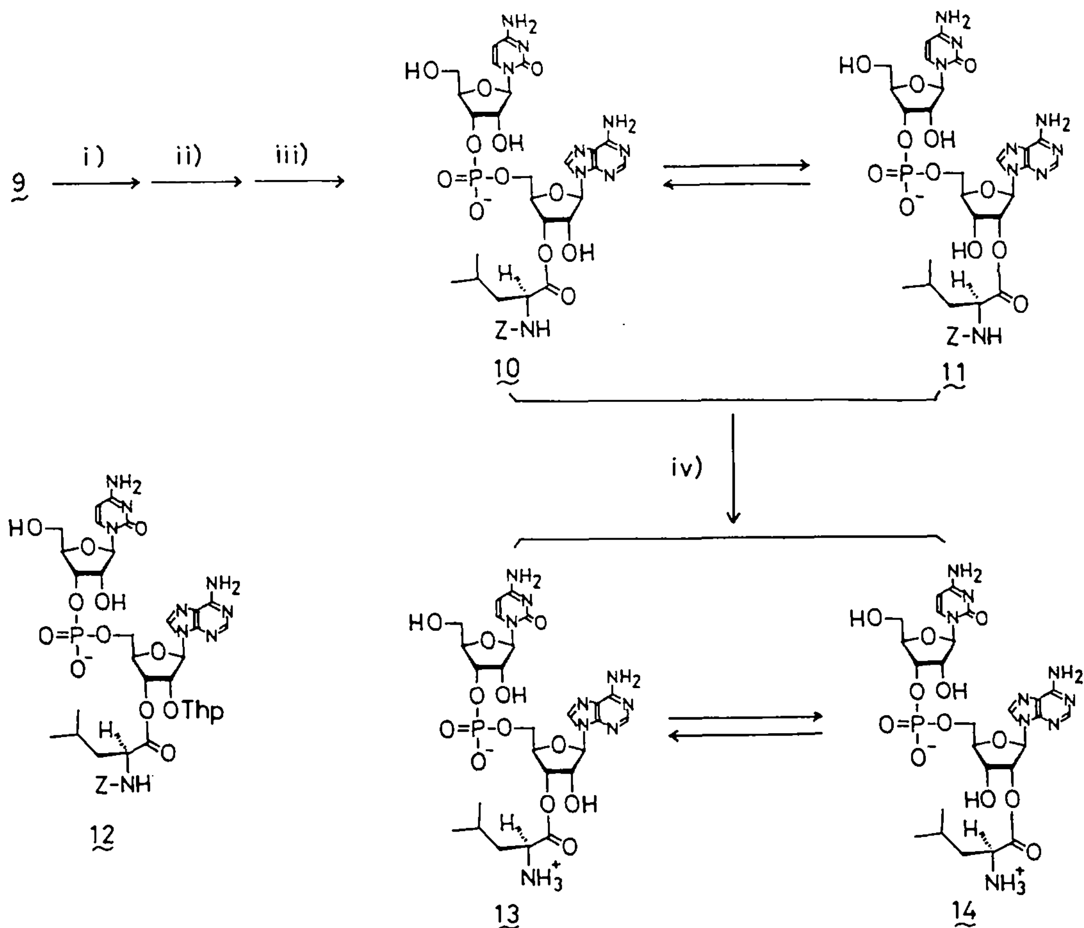
i) 1,3-dichlorotetraisopropyldisiloxane ii) dihydropyrane / TFA iii) TcBocCl iv) KF / Et_4NBr / H_2O v) dimethoxytrityl chloride vi) Z-Leu / DCC / DMAP vii) $(PhS)_2P(O)O^-$ / isodurenedisulfonyl dichloride / 1H-tetrazole

contrast to the above result, the N^4-monosubstituted derivative of cytidine (**3b**) hardly reacted with excess oxycarbonylating agent in pyridine. The corresponding N^4,N^4-disubstituted derivative (**3d**) could be obtained in 88% yield when compound **2b** (B^1 = Cy) was allowed to react with 2.1 equiv of TcBocCl in pyridine especially in the presence of 2.1 equiv of triethylamine. Thus, compound **2b** reacted with 1.2 equiv of TcBocCl in pyridine to give **3b** in 99% yield. The disubstituted derivative of adenosine (**3a**) and the monosubstituted derivative of cytidine (**3b**) were chosen for the synthesis of diribonucleotides. Compounds **3a** and **3b** were desilylated by treatment with KF/Et_4NBr/H_2O and the resulting diols (**4a** and **4b**) were selectively dimethoxytritylated at the 5'-positions to give the 3'-hydroxyl components (**5a** and **5b**). The 3'-hydroxyl group of **5a** was further aminoacylated by N-benzyloxycarbonyl-(L)-leucine (Z-Leu)[10] using N,N'-dicyclohexylcarbodiimide (DCC) in the presence of a catalytic amount of 4-dimethylaminopyridine (DMAP) in dichloromethane to give **6a** in 60% yield. On the other hand, compound **5b** was phosphorylated by S,S-diphenyl phosphorodithioate according to the published procedure[11] to give **6b** in 93% yield.

One of the two phenylthio groups of **6b** was selectively removed by H_3PO_2-Et_3N[12-15] to give the corresponding phosphodiester **7**. Compound **6a** was detritylated by using 0.5% trifluoroacetic acid (TFA) in chloroform at 0°C for 6 min. Compound **8** was obtained in 93% yield. The condensation of **8** with **7** using isodurenedisulfonyl dichloride (DDS) and 3-nitro-1,2,4-triazole (NT) in pyridine giving rise to the fully protected 3'-O-(leucyl)CpA (**9**) in 70% yield.



Deprotection of 9 was first performed as follows; (i) 15 equiv of $(Bu_3Sn)_2O$ in pyridine (2 h) to remove the phenylthio group, (ii) 45 equiv of Zn-acetylacetone[16] in pyridine (1 h) to remove the TcBoc groups, (iii) 0.01 M HCl in aqueous dioxane (pH 2) (40 h) to remove the dimethoxytrityl (DMTr) and tetrahydropyranyl (Thp) groups. After workup, the resulting mixture was analyzed by reversed phase HPLC (0.01 M NH_4OAc, pH 4.5, 0-50% CH_3CN/50 min) and the peaks separated by HPLC were numbered as shown in Figure 1. It was easily identified that the peak 4 was a partially deprotected intermediate still bearing a TcBoc group (UV spectrum; λ_{max} = 268 nm, pH = 4.5). Further, peak 1 and peak 2 were separately subjected to a HPLC column again and both experiments gave the mixture of peak 1 and peak 2 in almost same



i) $(Bu_3Sn)_2O$ ii) Zn / acetylacetone iii) 0.01 N HCl in aqueous dioxane
iv) H_2 / 5% Pd-$BaSO_4$ / 80% AcOH, 0° C

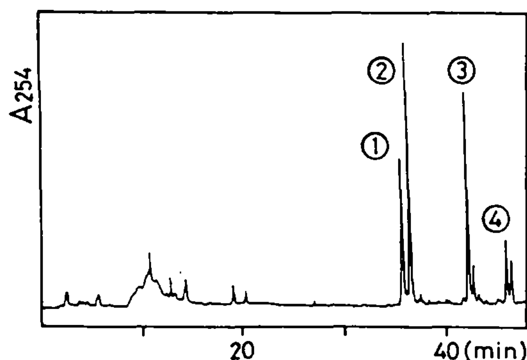


Figure 1. HPLC profile of the crude mixture.

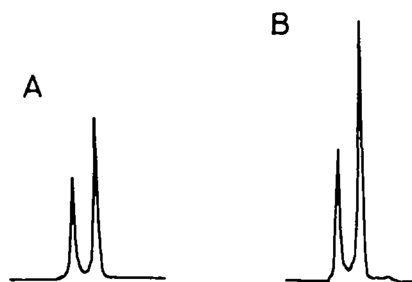


Figure 2. HPLC analysis of the peak 1 (A) and the peak 2 (B).

proportion(Figure 2). This observation indicated that peak 1 and peak 2 corresponded to the 2'- and 3'-isomer of the desired 2'(3')-O-(Z-leucyl)CpA (**10** and **11**) which coexisted at equilibrium. The mixture of peak 1 and peak 2 was briefly treated with 0.1 M NaOH to hydrolyze the leucyl ester linkage and the single product of CpA was obtained as expected. Peak 3 was treated with 0.01 M HCl (pH 2) for 48 h and the product was analyzed by HPLC. Peak 3 disappeared and the mixture of peak 1 and peak 2 was observed. Taking into account the above result and Reese's study,[17] peak 3 was assigned to the intermediate **12** bearing a Thp group at the 2'-hydroxyl group neighboring to the leucyl ester linkage.

Based on the above facts, we tackled the deprotection of **9** once more. Removal of the phenylthio group and TcBoc groups was performed by the same conditions as described above except for the treatment with Zn-acetylacetone

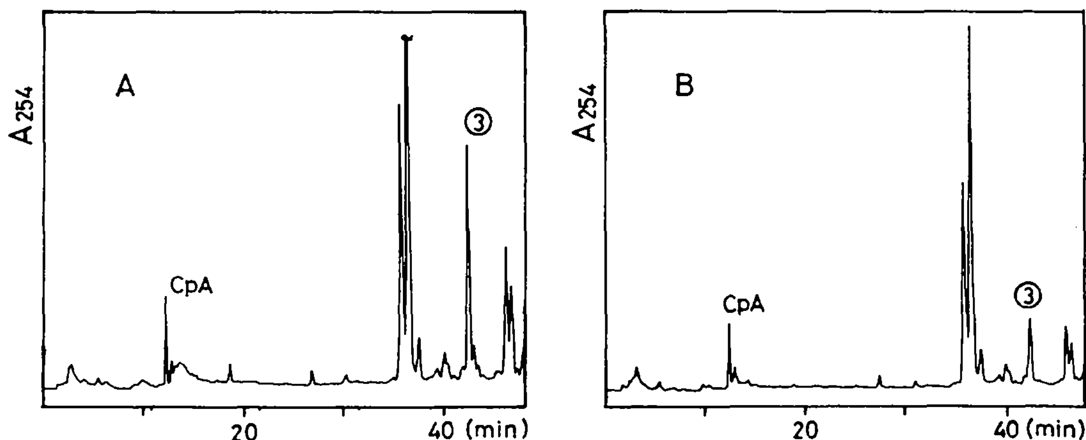


Figure 3. HPLC profile after acidic treatment for 63 h (A) or for 120 h (B).

for 2 h. In this experiment, removal of the Thp groups at pH 2 was analyzed after 63 h and 120 h by HPLC (Figure 3). It was found that the Thp group neighboring to the leucyl ester linkage still partially remained intact even after 120 h. A negligible amount of CpA was formed by the deaminoacylation of **10** or **11**. After 120 h the mixture of **10** and **11** was isolated by repeated subjection to the HPLC in 15% yield from **9**.

Finally, removal of the Z group was performed by hydrogenolysis under the Khorana's condition[18] to give the 2'(3')-O-(leucyl)CpA (**13** and **14**) in 91% yield. The structure of the product was confirmed by both the dansylation of leucine and the digestion by nuclease P1 or snake venom phosphodiesterase after hydrolysis of the leucyl ester under alkaline conditions.

CONCLUSION

The 2'(3')-O-(leucyl)CpA was obtained in susceptible yield. The phenylthio group of **9** was selectively removed without significant loss of the leucyl ester by using $(Bu_3Sn)_2O$. In the case of some 2'(3')-O-aminoacylated adenosine,[19-21] it was reported that 3'-O-aminoacylated adenosine (3'-ester) appeared to be a major product in equilibium with 2'-O-aminoacylated adenosine (2'-ester) and that 3'-ester was eluted slower than 2'-ester by reversed-phase HPLC. Based on the result in Figure 2, it seems likely that peak 1 corresponds to **11** and that peak 2 corresponds to **10**. However, no other evidence was given for the above concept. It was found that the Thp group neighboring to the leucyl ester bond was relatively resistant to the hydrolysis at pH 2. Some modification should be made to remove the protecting group at the 2'-position more easily.

EXPERIMENTAL

Reagent grade pyridine was distilled after being refluxed over p-toluenesulfonyl chloride for several hours, redistilled over calcium hydride after being refluxed for several hours, and stored over molecular sieves 4A. Elemental analysis was performed at the Microanalytical Laboratory, Tokyo Institute of Technology at Nagatsuta. ^{1}H-NMR spectra (60 MHz) were recorded on Hitachi R-24B. Thin layer chromatography was performed on precoated TLC plates (silica gel 60 F-254 Merck, Art. No. 5715) and developed by the solvent system, CH_2Cl_2-MeOH (12:1, v/v). Column chromatography was carried out using Wako gel C-200. Polyamide layer sheet (Chen Chin Trading Co. Ltd.) was purchased from Wako Co. Ltd. Reversed phase HPLC was performed on μ Bondapak C-18 using 0.1 M ammonium acetate buffer (pH 4.5) as eluent with a linear gradient of CH_3CN (1% of CH_3CN/min).

2'-O-(Tetrahydropyran-2-yl)-3',5'-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-N^6,N^6-bis(trichloro-t-butoxycarbonyl)adenosine (**3a**): Compound **2a** (594 mg, 1 mmol) was rendered anhydrous by repeated coevaporation with pyridine and dissolved in pyridine (10 mL). TcBocCl (480 mg, 2 mmol) was added and the mixture was stirred at room temperature. After 2 h, the reaction was quenched with ice (1 g) and the mixture was transferred to a separatory funnel with CH_2Cl_2 (50 mL). The organic layer was washed three times with 5% $NaHCO_3$ (30 mL x 3) and the combined aqueous layer was back-extracted with

CH_2Cl_2 (50 mL). The latter organic layer was further washed with 5% $NaHCO_3$ (30 mL), combined with the former organic layer, and dried over Na_2SO_4. The solvent was removed under reduced pressure and the last traces of pyridine was completely removed by coevaporation with toluene. The residue was subjected to a silica gel column (20 g) and elution was performed with 1% pyridine-CH_2Cl_2 to give **3a** (953 mg); yield 95%. Anal. Calcd for $C_{37}H_{57}O_{10}N_5Cl_6Si_2$: C, 44.41; H, 5.74; N, 7.00; Cl, 21.26. Found: C, 44.11; H, 5.43; N, 6.73; Cl, 21.76.

2'-O-(Tetrahydropyran-2-yl)-3',5'-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-N[4]-(trichloro-t-butoxycarbonyl)cytidine (**3b**): Compound **2b** (718.5 mg, 1.26 mmol) was rendered anhydrous by repeated coevaporation with pyridine and dissolved in pyridine (12 mL). TcBocCl (332.7 mg, 1.39 mmol) was added and the mixture was stirred at room temperature. After 30 min, the reaction was quenched and followed by the same workup as described in the preparation of **3a** to give **3b** (962.1 mg); yield 99%.

2'-O-(Tetrahydropyran-2-yl)-3',5'-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-N[4],N[4]-bis(trichloro-t-butoxycarbonyl)cytidine (**3d**): Compound **2b** (1.14 g, 2 mmol) was rendered anhydrous by repeated coevaporation with pyridine and dissolved in pyridine (20 mL). TcBocCl (500 mg, 2.1 mmol) was added and the mixture was stirred at room temperature. After 30 min, **3b** was detected on TLC as a main spot. After 1 h, TcBocCl (500 mg, 2.1 mmol) was further added to the above mixture and stirred for 1 h. However, no change was observed on TLC. Thus, triethylamine (0.584 mL, 4.2 mmol) was added and stirred for 1 h to give new main spot having higher Rf value than **3b** on TLC. The reaction was quenched and followed by the same workup as described in the preparation of **3a**. Silica gel column chromatography (50 g) was performed by elution with n-hexane-ether (2:1, v/v) containing 1% pyridine to give **3d** (1.726 g): yield 88%.

2'-O-(Tetrahydropyran-2-yl)-N[6],N[6]-bis(trichloro-t-butoxycarbonyl)adenosine (**4a**): Compound **3a** (600 mg, 0.6 mmol) was dissolved in CH_3CN (12 mL). KF (209 mg, 3.6 mmol), Et_4NBr (756 mg, 3.6 mmol) and water (0.5 mL) were added and the mixture was stirred at 50°C. After 1 h, the solvent was removed under reduced pressure and the residue was transferred to a separatory funnel with CH_2Cl_2 (50 mL). The organic layer was washed twice with water (50 mL x 2) and the combined aqueous layer was back-extracted with CH_2Cl_2 (50 mL). The latter organic layer was further washed with water (50 mL), combined with the former organic layer, and dried over Na_2SO_4. The solvent was removed under reduced pressure and the residue was subjected to a silica gel column (50 g). Elution was performed with 1-2% MeOH-CH_2Cl_2 to give **4a** (360 mg, crude 81%; the residue of the disiloxane was contaminated in the diastereomer having the higher Rf value).

^{1}H-NMR chemical shifts (ppm, $CDCl_3$)				acetal	TcBoc
Rf	8-H	2-H	1'-H	CH	CH_3
high	8.78	8.17	5.98 (d, J=7.6 Hz)	4.78 (m)	1.92
low	8.78	8.20	6.10 (d, J=6.0 Hz)	5.02 (t, J=5.4 Hz)	1.92

2'-O-(Tetrahydropyran-2-yl)-5'-O-(4,4'-dimethoxytrityl)-N[6],N[6]-bis(trichloro-t-butoxycarbonyl)adenosine (**5a**): Compound **4a** (crude 360 mg, 0.485 mmol) was rendered anhydrous by repeated coevaporation with pyridine and dissolved in pyridine (5 mL). DMTrCl (246 mg, 0.725 mmol) was added and the mixture was stirred at room temperature. After 5 h, the reaction was quenched with water (0.5 mL) and the mixture was transferred to a separatory funnel with CH_2Cl_2 (50 mL). The organic layer was washed three times with 5% $NaHCO_3$ (50 mL x 3) and the combined aqueous layer was back-extracted with CH_2Cl_2 (50 mL). The latter organic layer was further washed with 5% $NaHCO_3$ (50 mL), combined with the former organic layer, and dried over Na_2SO_4. The solvent was removed under reduced pressure and the residue was subjected to a silica

gel column (40 g). Elution was performed with 0.5-1% MeOH-CH_2Cl_2 to give **5a** (506.6 mg); yield 87%: ^1H-NMR ($CDCl_3$); δ 8.67(s, 1H, 8H); 8.51(s, 1H, 2H); 7.55-6.60(m, 13H, Ph); 6.15(d, J=5.6 Hz, 1H, 1'H); 3.73(s, 6H, CH_3O); 1.90(s, 12H, CH_3 of TcBoc).

<u>2'-O-(Tetrahydropyran-2-yl)-5'-O-(4,4'-dimethoxytrityl)-N^4-(trichloro-t-butoxycarbonyl)cytidine (5b)</u>: Compound **3b** (962.1 mg, 1.25 mmol) was desilylated by the same procedure described in the preparation of **4a**. After extraction of **4b**, the crude mixture was dimethoxytritylated by the same procedure as described in the preparation of **5a** to give **5b** (799.5 mg); yield 77%: ^1H-NMR ($CDCl_3$); δ 3.76(s, 6H, CH_3O); 1.96(s, 6H, CH_3 of TcBoc).

<u>Preparation of **6a** by the aminoacylation of **5a**</u>: Compound **5a** (104 mg, 0.1 mmol) was mixed with N-benzyloxycarbonyl-(L)-leucine stored in 0.7 M toluene solution (0.214 mL, 0.15 mmol). The solvent was removed under reduced pressure and the residue was dissolved in CH_2Cl_2 (1 mL). DCC (41 mg, 0.2 mmol) and DMAP (2.4 mg, 0.02 mmol) were added and the mixture was stirred at room temperature. After 90 min, the mixture was transferred to a separatory funnel with CH_2Cl_2 (50 mL), washed twice with 5% $NaHCO_3$ (50 mL x 2), and dried over Na_2SO_4. The solvent was removed under reduced pressure and the residue was subjected to a preparative TLC plate which was developed with CH_2Cl_2-MeOH (40:1, v/v) to give **6a** (77.4 mg); yield 60%.

<u>Preparation of **6b** by the phosphorylation of **5b**</u>: Compound **5b** (799.5 mg, 0.96 mmol) and cyclohexylammonium S,S-diphenyl phosphorodithioate (549 mg, 1.44 mmol) were mixed, rendered anhydrous by repeated coevaporation with pyridine, and dissolved in pyridine (10 mL). DDS (636 mg, 1.92 mmol) and 1H-tetrazole (Tet)(134 mg, 1.92 mmol) were added and the mixture was stirred at room temperature. After 30 min, the reaction was quenched with water (1 mL) and the mixture was transferred to a separatory funnel with CH_2Cl_2 (50 mL). The organic layer was washed three times with 5% $NaHCO_3$ (50 mL x 3) and the combined aqueous layer was back-extracted with CH_2Cl_2 (50 mL). The latter organic layer was further washed with 5% $NaHCO_3$ (50 mL), combined with the former organic layer, and dried over Na_2SO_4. The solvent was removed under reduced pressure and the residue was subjected to a silica gel column (30 g). Elution was performed with n-hexane-CH_2Cl_2 (1:5, v/v) containing 1% pyridine to give **6b** (981.4 mg); yield 93%: ^1H-NMR ($CDCl_3$); δ 3.73(s, 6H, CH_3O); 1.95(s, 6H, CH_3 of TcBoc).

<u>Preparation of **8** by the detritylation of **6a**</u>: Compound **6a** (77.4 mg, 0.0559 mmol) was dissolved in $CHCl_3$ (6 mL) and stirred at 0°C. TFA (30 μL) was added to the above solution. After 6 min the reaction was quenched with pyridine (1 mL). The mixture was transferred to a separatory funnel with CH_2Cl_2 (50 mL) and washed three times with 5% $NaHCO_3$ (50 mL x 3). The combined aqueous layer was back-extracted with CH_2Cl_2 (50 mL). The latter organic layer was further washed with 5% $NaHCO_3$ (50 mL), combined with the former organic layer, and dried over Na_2SO_4. The solvent was removed under reduced pressure and the residue was subjected to a preparative TLC plate which was developed with CH_2Cl_2-MeOH (20:1, v/v) to give **8** (55.2 mg); yield 93%.

<u>Preparation of the fully protected 3'-O-(leucyl)CpA (**9**)</u>: 5 M aqueous H_3PO_2 (547 mg) was rendered anhydrous by repeated coevaporation with pyridine and dissolved in pyridine (684 μL). Then triethylamine (319 μL) was added to the above solution. Compound **6b** (91 mg, 0.0835 mmol) was separately rendered anhydrous by repeated coevaporation with pyridine. It was mixed with the above solution and stirred at 35°C. After 1 h, the mixture was transferred to a separatory funnel with pyridine-water (1:1, v/v, 50 mL) and washed three times with n-hexane (50 mL x 3). The phosphodiester **7** was extracted three times from the aqueous layer to CH_2Cl_2 (50 mL x 3). The combined organic layer was further washed twice with 0.5 M triethylammonium

bicarbonate (pH 7, 100 mL x 2) and dried over Na_2SO_4. The solvent was removed under reduced pressure and the residue was mixed with **8** (55.2 mg, 0.0557 mmol) and NT (19 mg, 0.167 mmol). The mixture was rendered anhydrous by repeated coevaporation with pyridine and dissolved in pyridine (1 mL). DDS (37 mg, 0.111 mmol) was added to the above solution and the mixture was stirred at room temperature. After 2 h, the mixture was transferred to a separatory funnel with CH_2Cl_2 (50 mL) and washed three times with 5% $NaHCO_3$ (50 mL x 3). The combined aqueous layer was back-extracted with CH_2Cl_2 (50 mL). The latter organic layer was further washed with 5% $NaHCO_3$ (50 mL), combined with the former organic layer, and dried over Na_2SO_4. The solvent was removed under reduced pressure and the residue was subjected to a preparative TLC plate which was developed with CH_2Cl_2-MeOH (30:1, v/v) to give **9** (76.7 mg); yield 70%.

Preparation of 2'(3')-O-(Z-leucyl)CpA (**10** and **11**): Compound **9** (28.1 mg, 14.2 μ mol) was rendered anhydrous by repeated coevaporation with pyridine and dissolved in pyridine (450 μ L). $(Bu_3Sn)_2O$ (109 μ L, 0.213 mmol) was added and the mixture was stirred at room temperature. After 2 h, chlorotrimethylsilane (68 μ L, 0.533 mmol) was added and stirred for 2 min. The mixture was transferred to a separatory funnel with pyridine-water (1:1, v/v, 50 mL) and washed three times with n-hexane (50 mL x 3). The phosphodiester intermediate was extracted three times from the aqueous layer to $CHCl_3$ (50 mL x 3). The combined organic layer was further washed twice with 0.5 M TEAB (pH 7, 100 mL x 2) and dried over Na_2SO_4. The solvent was removed under reduced pressure. The residue was rendered anhydrous by repeated coevaporation with pyridine and dissolved in pyridine (0.5 mL). Zinc powder (42 mg, 0.639 mmol) and acetylacetone (66 μ L, 0.639 mmol) were added and the mixture was stirred at room temperature. After 1 h, the mixture was subjected to a short column of Dowex 50Wx8 (pyridinium form, 2 mL) followed by elution with pyridine-water (1:1, v/v, 30 mL). The solvent was removed under reduced pressure and the last traces of pyridine was completely removed by coevaporation with toluene. The residue was dissolved in 0.01 M HCl (dioxane-water, 1:1, v/v, pH 2, 50 mL) and stirred at room temperature. After 120 h, the mixture was condensed under reduced pressure with occasional addition of 50 mM NH_4OAc (pH 4.5, 10 mL). The resulting solution (ca. 1 mL) was repeatedly subjected to a HPLC column (μ Bondapak C18, 50 mM NH_4OAc, pH 4.5, 0-50% CH_3CN/50 min) to give the mixture of **10** and **11** (A_{280} = 17.8 OD, 0.01 M HCl); yield 15% considering the 10% loss for monitoring removal of Thp group (Figure 3).

Preparation of 2'(3')-O-(leucyl)CpA (**13** and **14**) by the hydrogenolysis of **10** and **11**: A lyophilized mixture of **10** and **11** was dissolved in 80% AcOH (1 mL) and stirred at 0°C. 5%Pd-$BaSO_4$ (ca. 50 mg) was added and the mixture was stirred under H_2 atmosphere. After 30 min, 5%Pd-$BaSO_4$ was removed by filtration and washed with 50mM AcOH. The filtrate was condensed under reduced pressure with occasional addition of 50 mM AcOH (5 mL). The resulting solution (ca. 1 mL) was repeatedly subjected to a HPLC column (μ Bodapak C18, 50 mM NH_4OAc, pH 4.5, 0-50% CH_3CN/50 min) to give the mixture of **13** and **14** (Rt = 20-21 min, A_{280} = 5.68 OD, pH 4.5); yield 91%.

Dansylation of 2'(3')-O-(leucyl)CpA (**13** and **14**): A lyophilized sample of 2'(3')-O-(leucyl)CpA (**13** and **14**)(0.067 OD, 3.16 nmol) was dissolved in 2 M $NaHCO_3$ (10 μ L) and lyopholized. The residue was dissolved in water (10 μ L) and a solution of dansyl chloride (10 μ L, 2.5 mg of DnsCl in 1 mL of acetone) was added. The mixture was allowed to stand at 37°C for 1 h. The leucyl ester linkages of **13** and **14** were cleaved under these conditions. After the lyophilization of the mixture, the residue was dissolved in 95% ethanol (10 μ L) and an aliquot of the solution (ca. 1 μ L) was subjected to a two dimensional TLC on polyamide layer sheet [15 x 15 cm, 1.5% formic acid for the 1st dimension, benzene-AcOH (9:1, v/v) for the 2nd dimension]. The dansylated leucine was detected as a phosphor at the same position as an

authentic sample under UV lamp.

Deaminoacylation of the products (**10** and **11**): A lyophilized sample of 2'(3')-O-(Z-leucyl)CpA (**10** and **11**)(ca. 2 OD) was dissolved in water (100 μL) and 0.2 M NaOH (100 μL) was added. After 2 min, the mixture was neutralized by addition of 0.2 M AcOH (100 μL). The HPLC analysis of the above solution gave a single peak corresponding to CpA (Rt = 13.5 min), which was collected by repeated subjection to the HPLC. The sample of 2'(3')-O-(leucyl)CpA (**13** and **14**) was also deaminoacylated by the same procedure to give a single peak corresponding to CpA.

Nuclease P1 digestion: A lyophilized CpA (ca. 1 OD) was dissolved in 50 mM NH_4OAc (pH 5.4, 50 μL) and the solution of nuclease P1 (10 mg/5 mL, 5 μL) was added. The solution was allowed to stand at 37°C for 6 h and the enzyme was inactivated in boiling water for 2 min. After lyophilization, the residue was dissolved in 50 mM Tris-HCl (pH 8.0, 50 μL) and bacterial alkaline phosphatase (1 unit/μL, 5 μL) was added. The solution was allowed to stand at 37°C for 10 h and the enzyme was inactivated by the same procedure as described above. The resulting mixture was analyzed by HPLC to give two peaks corresponding to C and A (ca. 1:1).

Snake venom phosphodiesterase digestion: A lyophilized CpA (ca. 1 OD) was dissolved in 50 mM Tris-HCl (pH 8.0, 50 μL) and the solution of snake venom phosphodiesterase (1 mg/mL, 5 μL) was added. The mixture was allowed to stand at 37°C for 4 h followed by the dephosphorylation by bacterial alkaline phosphatase as descrived above to give C and A (ca. 1:1).

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