Synthesis and Biological Activity of Aminoacyl Analogs of Ascamycin

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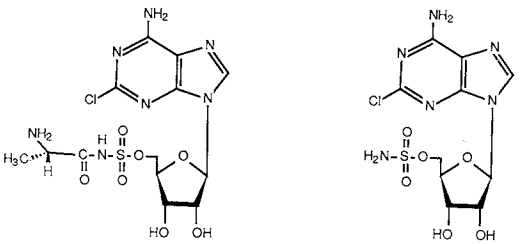
L-Prolyl and other aminoacyl derivatives of ascamycin (**1**) were synthesized by a condensation reaction of N^6-*t*-butyloxycarbonyl-2-chloro-9-(2′,3′-*O*-isopropylidene-5′-*O*-sulfamoyl-β-D-ribofuranosyl)adenine (**9**) with the corresponding *t*-butyloxycarbonylaminoacylimidazole in the presence of Cs_2CO_3 in DMF as the key step. The L-prolyl derivative (**2**) and L-phenylalanyl derivative (**3**) as well as **1** showed selective toxicity against *Xanthomonas citri*. The L-prolyl-L-prolyl derivative (**5**) as well as dealanylascamycin (**6**) showed broad toxicity against various Gram-negative and Gram-positive bacteria. The D-alanyl derivative (**4**) lost its antibacterial activity. **2** was the best substrate (15.3 M/min per mg of protein) for an Xc-aminopeptidase (an ascamycin-dealanylating enzyme from *X. citri* cells) among these analogs. This enzyme scarcely hydrolyzed **4** (0.2 M/min per mg of protein). The substrate specificity of the enzyme accounts for antibacterial activity of the analogs. **2** showed greater selective toxicity against Kirsten sarcoma virus transformed Balb3T3 (KN-3T3) cells than against nontransformed cells (Balb 3T3).

The nucleoside antibiotic ascamycin (**1**)[1] shows selective toxicity to plant pathogenic *Xanthomonas* species, whereas dealanylascamycin (**6**) shows broad antibacterial activities. Both **1** and **6** strongly inhibit protein synthesis *in vitro*, but the antibacterial activity of **1** is masked by the alanyl group, which interferes with the membrane transport in bacteria. *Xanthomonas citri* is susceptible to **1** by virtue of an ascamycin-dealanylating enzyme (Xc-aminopeptidase),[2] which is a proline iminopeptidase existing on the cell surface. Other bacteria which lack the enzyme are not susceptible to **1**.[3] Immunological studies have shown that all the mammalian cells tested possess closely related enzyme(s) on their cell membranes. The ascamycin dealanylating activities of transformed cells are higher than those of nontransformed cells.[4] To obtain additional support for these findings, we synthesized four aminoacyl analogs. As the Xc-aminopeptidase is most specific to the prolyl peptide, we were especially interested in the prolyl derivative of ascamycin.



1 **6**

FIG. 1. Structures of Ascamycin (**1**) and Dealanylascamycin (**6**).

RESULTS

Syntheses of **1**[5] and other aminoacyl analogs were achieved by a condensation reaction of N^6-*t*-butyloxycarbonyl-2-chloro-9-(2′,3′-*O*-

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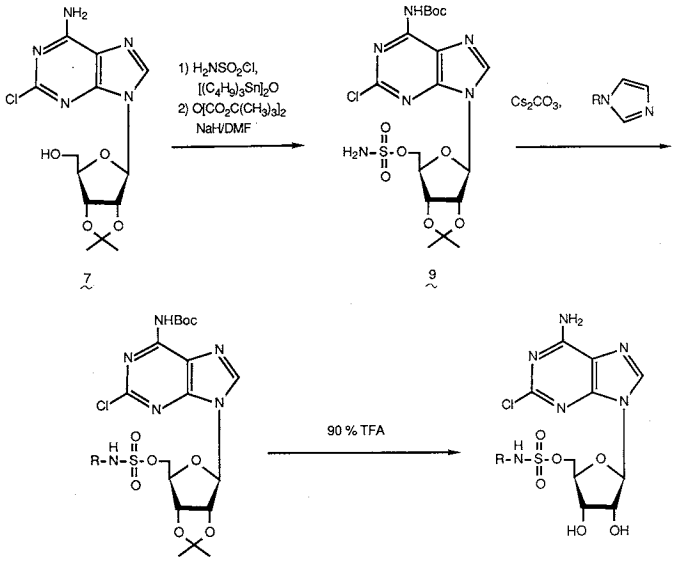

10a: R = L-alanyl, **10b**: R = L-prolyl **1**: R = L-alanyl, **2**: R = L-prolyl
10c: R = L-phenylalanyl, **10d**: R = D-alanyl **3**: R = L-phenylalanyl, **4**: R = D-alanyl
10e: R = L-prolyl-L-prolyl **5**: R = L-prolyl-L-prolyl

FIG. 2. Syntheses of Ascamycin and Its Aminoacyl Analogs.

isopropylidene-5′-*O*-sulfamoyl-β-D-ribofuranosyl)adenine (**9**) with *t*-butyloxycarbonylaminoacylimidazole in the presence of cesium carbonate in *N*,*N*-dimethylformamide as the key step (Fig. 2).

2-Chloroadenosine was prepared from 2,6-dichloropurine and 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose by the previously described methods.[6] A 2-hr refluxing treatment of the 2′,3′-*O*-isopropylidene acetal (**7**) of 2-chloroadenosine with 8 eq. of sulfamoyl chloride in the presence of 3.25 eq. of bis(tri-*n*-butyltin)-oxide in 1,4-dioxane gave 2-chloro-9-(2′,3′-*O*-isopropylidene-5′-*O*-sulfamoyl-β-D-ribofuranosyl)adenine (**8**) in an 82% yield.

The acid-labile protective groups were chosen because of the instability of ascamycin during prolonged storing in alkaline, acidic and even neutral media at room temperature. Treatment of **8** (2 hr, −20∼0°C) with 1.1 eq. of di-*t*-butyldicarbonate in the presence of 5.5 eq. of sodium hydride gave **9** in an 85% yield. Condensation of **9** with 1.5 eq. of *t*-butyloxycarbonyl-L-prolylimidazole in the presence of 1 eq. of cesium carbonate gave **10b** in a 59% yield. Deprotection of **10b** with 90% trifluoroacetic acid (1 hr, 0°C∼room temp.) gave **2** in a 76% yield after purification by HPLC (Senshu Pak ODS-H column, 30% MeOH solvent).

Limited racemization[7] of the aminoacyl moiety (L-alanine–D-alanine = 94 : 6) was checked by the hydrolysis of synthetic ascamycin (**1**) and subsequent HPLC analysis (Sumipax OA-1000 column, hexane–dichloromethane–EtOH solvent = 20 : 2 : 3) of the methyl *N*-3,5-dinitrobenzoylalanylate.

Biological activity

The L-prolyl derivative (**2**) as well as **1** showed selective toxicity against *Xanthomonas citri*, whereas **4** was inactive against the bacteria tested, as shown in Table I. The L-phenylalanyl derivative (**3**) also showed selective toxicity against *X. citri*. The L-prolyl-L-prolyl derivative (**5**) as well as **6** showed strong activity against the various Gram-negative and Gram-positive bacteria tested.

The effect of **2** and other analogs on the uptake of [^{14}C]valine by *X. citri* and *E. coli* is shown in Table II. The L-prolyl derivative (**2**) showed remarkable selective inhibition of the

TABLE I. ANTIMICROBIAL SPECTRA OF ASCAMYCIN ANALOGS

The susceptibility of bacteria to ascamycin and its analogs was determined by the conventional paper disc method using potato-sucrose agar medium. The paper discs contained 8 nmol of each antibiotic.

Organism	Diameter of inhibition zone (mm)					
	1	**2**	**3**	**4**	**5**	**6**
Staphylococcus aureus	–	–	–	–	17	20
Bacillus subtilis	–	–	–	–	±	11
Escherichia coli	–	–	–	–	11	22
Salmonella typhimurium	–	–	–	–	11	14
Pseudomonas aeruginosa	–	–	–	–	12	19
Xanthomonas citri	42	43	40	–	41	45

TABLE II. EFFECTS OF ASCAMYCIN ANALOGS ON THE PROTEIN SYNTHESIS OF *X. citri* AND *E. coli* BE 1186

E. coli BE 1186 and *X. citri* IFO 3781 were grown aerobically at 30°C, using the methods previously described,[3] except that [^{14}C]valine (0.5 μCi) instead of [^{14}C]phenylalanine (1 μCi) was used.

Compound	ID_{50}	
	X. citri (μM)	*E. coli* BE (μM)
1	0.15	>100
2	0.06	>100
3	0.095	90
4	3	>100
5	0.14	28

uptake of [^{14}C]valine into *X. citri*.

The specificity of Xc aminopeptidase to **2** was the greatest (15.3 M/min per mg of protein) among these analogs, and the specificity of this enzyme to **4** was extremely low (0.2 M/min per mg of protein). The L-phenylalanyl analog (**3**) was also a substrate for this enzyme as shown in Table III. Kirsten murine sarcoma virus transformed Balb 3T3 (KN-3T3) cells were more sensitive to **2** than were nontransformed cells (Balb 3T3) as shown in Fig. 3.

TABLE III. SPECIFICITY OF Xc-AMINOPEPTIDASE TO ASCAMYCIN ANALOGS

Xc-aminopeptidase was prepared as previously described.[2] In a cuvette, 5 μl of 1 mM antibiotic (dissolved in 20 mM Tris–HCl at pH 7.8) was placed and permitted to equilibrate at 37°C. At zero time, 5 μl of the test enzyme solution was added to the cuvette and mixed. The changes in the HPLC pattern were recorded (ODS-H column, 263 nm UV, 0.1 range, 0.03% TFA–CH_3CN solvent = 85 : 15), after adding 5 mM $CdCl_2$ (5 μl). A relative specificity of unity is defined as the value at which 2.9 M of ascamycin was hydrolyzed per minute per mg of protein.

Substrate (0.5 mM)	Relative specificity
1	1
2	5.3
3	0.7
4	0.07
5	2.5

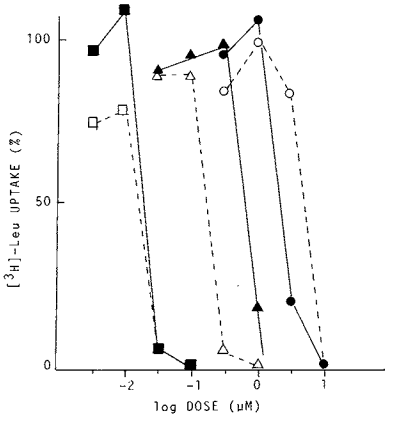

FIG. 3. Inhibition of Leucine Uptake in Transformed and Nontransformed Cells by Ascamycin Analogs.

The inhibition of [^{3}H]leucine uptake into the acid-insoluble fraction was measured in the presence of an antibiotic. The cell lines used in the experiment included mouse-derived Balb 3T3 clone A 31 and Kirsten murine sarcoma virus transformed cell KN-3T3. The experiment was carried as previously described.[4]

Inhibition curves of **1**: ●, Balb 3T3; ○, KN-3T3. Inhibition curves of **2**: ▲, Balb 3T3; △, KN-3T3. Inhibition curves of **6**: ■, Balb 3T3; □, KN-3T3.

DISCUSSION

We have described the synthesis and biological activity of the aminoacyl analogs of **1**. The L-prolyl derivative (**2**) was particularly interesting in its selective antibacterial activity against *X. citri*, highest sensitivity to Xc-aminopeptidase, and remarkably selective in-

hibition of the uptake of [^{14}C]valine into *X. citri*.

The hydrolysis of **5** by Xc-aminopeptidase was followed by HPLC at regular time intervals. We observed that the aminoacyl sulfamoyl bond-cleavage of **5** by Xc-amino peptidase was faster than the common prolyl peptide bond-cleavage by the enzyme. This was also true in the case of **3**, although Xc-aminopeptidase did not hydrolyze L-phenylalanyl-β-naphthylamide, but hydrolyzed **3** (Table III). The substrate specificity of the enzyme accounts for the selective toxicity of **2** and **3** as well as **1** against *X. citri* (Tables I and II). The enzyme existing on the cell surface of *X citri* scarcely hydrolyzed **4**, so that the derivative did not show any activity against the bacteria. The L-prolyl-L-prolyl derivative (**5**), which showed antibacterial activity against all the bacteria used, may possibly have directly permeated the cell membrane through peptide transport.

In mammalian cells, all the analogs except **4** showed toxicity against both Kirsten murine sarcoma virus transformed cells (KN-3T3) and nontransformed cells (Balb 3T3). Preliminary experiments indicated that the order of toxicity against Balb 3T3 and KN-3T3 cells was **6** and **5**>**3**>**2**>**1**≫**4** (data not shown). Only **2** showed higher toxicity against KN-3T3 (Fig. 3).

EXPERIMENTAL

Melting points (mp) were measured on a Yanagimoto micro-melting point apparatus and are uncorrected. UV spectra were measured on a Hitachi 220A spectrophotometer. IR spectra were obtained on a Shimadzu 521 grating infrared spectrometer, and ^{1}H-NMR spectra were recorded on a JEOL FX-400 FT NMR spectrometer. SIMS spectra were taken on a Hitachi M80 spectrometer, and high-resolution mass spectra were acquired using a JEOL JMS-DX303 spectrometer and JMA-DA500 data system. HPLC was carried out on a Hitachi 635A liquid chromatograph (Senshu Pak ODS-H, Sumipax OA-1000).

*2-Chloro-9-(2′,3′-O-isopropylidene-5′-O-sulfamoyl-β-*D*-ribofuranosyl)adenine* (**8**). To a solution of **7** (68.2 mg, 0.21 mmol) in benzene (6 ml) was added bis(tri-*n*-butyltin)oxide (0.33 ml, 3.25 eq.). The solution was heated under reflux, and water was continuously removed by using 4A molecular sieves. After 2 hr, to the reaction mixture was added dropwise sulfamoyl chloride (76.4 mg, 4 eq.) in 1,4-dioxane (3 ml) at 5°C. The mixture was stirred for 3 hr at room temperature and for 10 hr at 5°C. To the reaction mixture was further added dropwise sulfamoyl chloride (76.4 mg, 4 eq.) in 1,4-dioxane (3 ml) at 5°C. After stirring for 22 hr at room temperature, the solution was evaporated to dryness. The residue was washed with hot hexane and diluted with ethyl acetate. The ethyl acetate-soluble fraction was washed with aq. KF solution, and brine, dried over $MgSO_4$ and evaporated. The residue was purified by silica gel TLC, using chloroform–methanol (10 : 1, v/v) as the solvent, to give 72 mg of **8** (82%). This compound (**8**) was identical to that previously prepared.[8]

*N^6-t-Butyloxycarbonyl-2-chloro-9-(2′,3′-O-isopropylidene-5′-O-sulfamoyl-β-*D*-ribofuranosyl)adenine* (**9**). To a suspension of NaH (a 55% dispersion in mineral oil, 20.6 mg, 0.476 mmol) in DMF (0.5 ml) was added dropwise **8** (36 mg, 0.086 mmol) in DMF (0.72 ml) at −20°C. The mixture was stirred for 30 min at room temperature. To this mixture was added dropwise di-*t*-butyl dicarbonate (21.6 μl, 0.094 mmol) in DMF (0.5 ml) at −20°C, the temperature of the bath being gradually raised to 0°C over 2 hr. The reaction mixture was diluted with ethyl acetate, and washed with 10% citric acid and brine. The organic layer was dried over Na_2SO_4 and evaporated to dryness. The residue was purified by silica gel TLC ($CHCl_3$–MeOH = 9 : 1, v/v) to give 38 mg of **9** (85%). SIMS *m/z*: 521 $(M+H)^+$. ^{1}H-NMR δ_{CDCl_3} (TMS): 8.12 (1H, s, C8-H), 6.17 (1H, d, J = 3 Hz, C1′-H), 5.31 (1H, dd, J = 3 Hz, J = 6.7 Hz, C2′-H), 5.07 (1H, dd, J = 4 Hz, J = 6.7 Hz, C3′-H), 4.57 (1H, m, C4′-H), 4.39 (2H, m, C5′-H). UV λ_{max} (MeOH): 255 (ε = 11000 sh), 273 (18200), 280 (16100 sh).

*N^6-t-Butyloxycarbonyl-2-chloro-9-[2′,3′-O-isopropylidene-5′-O-(N-t-butyloxycarbonyl-*L*-alanyl)sulfamoyl-β-*D*-ribofuranosyl]adenine* (**10a**).To a suspension of Cs_2CO_3 (58 mg, 0.135 mmol) and **9** (50 mg, 0.096 mmol) in DMF (1 ml) was added dropwise *t*-butyloxycarbonyl-L-alanylimidazole (prepared from 27 mg of *t*-butyloxycarbonyl-L-alanine and 23 mg of *N,N′*-carbonyldiimidazole in 0.25 ml of DMF; 0.142 mmol) at −20°C. The temperature of the bath was gradually raised to room temperature over 8 hr. The reaction mixture was diluted with ethyl acetate, washed with 10% citric acid and brine, dried over Na_2SO_4, evaporated to dryness, and chromatographed by silica gel TLC ($CHCl_3$–MeOH = 9 : 1, v/v) to give 57 mg of **10a** (86%). This product (**10a**) was used for the next step without further purification. SIMS *m/z*: 714 $(M+Na)^+$. ^{1}H-NMR δ_{CDCl_3} (TMS): 8.54 (1H, s, C8-H), 6.31 (1H, d, J = 3 Hz, C1′-H), 5.28 (1H, dd, J = 3 Hz, J = 6.3 Hz, C2′-H), 5.05 (1H, dd, J = 2 Hz, J = 6.3 Hz, C3′-H), 4.57 (1H, m, C4′-H).

N^6-t-Butyloxycarbonyl-2-chloro-9-[2′,3′-O-isopropylidene-5′-O-(N-t-butyloxycabonyl-L-prolyl)sulfamoyl-β-D-ribofuranosyl]adenine (**10b**). The product (**10b**) was obtained in a 59% yield by the reaction of **9** (40 mg, 0.077 mmol) with 1.5 eq. of *t*-butyloxycabonyl-L-prolylimidazole in the same manner as that for **10a**. This product (**10b**) was used for the next step without further purification. SIMS *m/z*: 718 $(M+H)^+$. ^{1}H-NMR δ_{CDCl_3} (TMS): 6.29 (1H, d, $J=3.4$ Hz, C1′-H), 4.1 (1H, dd, C2′′-H), 3.32 (2H, m, C4′′-H).

N^6-t-Butyloxycarbonyl-2-chloro-9-[2′,3′-O-isopropylidene-5′-O-(N-t-butyloxycarbonyl-L-phenylalanyl)sulfamoyl-β-D-ribofuranosyl]adenine (**10c**). The product (**10c**) was obtained in an 86% yield by the reaction of **9** (416. mg, 0.08 mmol) with 1.5 eq. of *t*-butyloxy-L-phenylalanylimidazole in the same manner as that for **10a**. This product (**10c**) was used for the next step without further purification. SIMS *m/z*: 768 $(M+H)^+$. ^{1}H-NMR δ_{CDCl_3} (TMS): 7.0～7.3 (5H, m, phenyl), 6.24 (1H, d, $J=3$ Hz, C1′-H).

N^6-t-Butyloxycarbonyl-2-chloro-9-[2′,3′-O-isopropylidene-5′-O-(N-t-butyloxycarbonyl-D-alanyl)sulfamoyl-β-D-ribofuranosyl]adenine (**10d**). The product (**10d**) was obtained in a 53% yield by the reaction of **9** (40 mg, 0.076 mmol) with 1.5 eq. of *t*-butyloxy-D-alanylimidazole in the same manner as that for **10a**. This product (**10d**) was used for the next step without further purification. SIMS *m/z*: 692 $(M+H)^+$. ^{1}H-NMR δ_{CDCl_3} (TMS): 8.65 (1H, s, C8-H), 6.31 (1H, d, $J=3$ Hz, C1′-H), 5.30 (1H, dd, $J=3$ Hz, $J=6.7$ Hz, C2′-H).

N^6-t-Butyloxycarbonyl-2-chloro-9-[2′,3′-O-isopropylidene-5′-O-(N-t-butyloxycarbonyl-L-prolyl-L-prolyl)sulfamoyl-β-D-ribofuranosyl]adenine (**10e**). The product (**10e**) was obtained in a 19.8% yield by the reaction of **9** (84 mg, 0.16 mmol) and 1.5 eq. of *t*-butyloxy-L-prolyl-L-prolylimidazole in the same manner as that for **10a**. This product (**10e**) was used for the next step without further purification. SIMS *m/z*: 815 $(M+H)^+$, 837 $(M+Na)^+$. ^{1}H-NMR δ_{CDCl_3} (TMS): 8.64 (1H, s, C8-H), 6.38 (1H, d, $J=2.74$ Hz, C1′-H).

2-Chloro-9-[5′-O-(N-L-alanyl)sulfamoyl-β-D-ribofuranosyl]adenine, ascamycin (**1**). To a solution of 90% trifluoroacetic acid (0.1 ml) was added **10a** (20 mg, 0.029 mmol) at 0°C. The reaction mixture was stirred for 15 min at 0°C and for 1 hr at room temperature. The resulting mixture was diluted with water and lyophilized. The resulting powder was purified by HPLC (Senshu Pak ODS-H, 30% MeOH solvent) to give 10 mg of **1** (79%). $[\alpha]_D^{20}$ −0.95° ($c=1.05$, H_2O). UV λ_{max} (MeOH): 263 nm ($\varepsilon=12270$). ^{1}H-NMR δ_{D_2O} (Dioxane): 6.02 (1H, d, $J=4.8$ Hz, C1′-H), 3.65 (1H, q, $J=7$ Hz, C2′′-H), 1.27 (1H, d, $J=7$ Hz, C3′′-H). FBMS *m/z*: 452.0763 $(M+H)^+$; calcd. for $C_{13}H_{19}O_7N_7SCl$: 452.0771. *Anal.* Found: C, 32.31; H, 4.03; N, 19.59. Calcd. for $C_{13}H_{18}N_7O_7SCl\cdot 2H_2O$: C, 32.00; H, 4.55; N, 20.1.

2-Chloro-9-[5′-O-(N-L-prolyl)sulfamoyl-β-D-ribofuranosyl]adenine (**2**). The product (**2**) was obtained in a 76% yield by deprotecting **10b** (30.5 mg, 0.0425 mmol) in the same manner as that for **1**. $[\alpha]_D^{20}$ −13.5° ($c=0.84$, H_2O). ^{1}H-NMR δ_{D_2O} (Dioxane): 6.04 (1H, d, $J=5$ Hz, C1′-H), 4.21 (1H, dd, $J=6.6$ Hz, $J=9.5$ Hz, C2′′-H), 3.38 (2H, m, C5′′-H). FABMS *m/z*: 478.0899 $(M+H)^+$; calcd. for $C_{15}H_{21}O_7N_7SCl$: 478.08886. *Anal.* Found: C, 32.31; H, 4.18; N, 18.84. Calcd. for $C_{15}H_{20}O_7N_7SCl\cdot 2H_2O$: C, 35.05; H, 4.71; N, 19.08.

2-Chloro-9-[5′-O-(N-L-phenylalanyl)sulfamoyl-β-D-ribofuranosyl]adenine (**3**). The product (**3**) was obtained in a 58% yield by deprotecting **10c** (49.3 mg, 0.064 mmol) in the same manner as that for **1**. $[\alpha]_D^{20}$ −4.47° ($c=0.75$, H_2O–MeOH). ^{1}H-NMR δ_{D_2O} (Dioxane): 5.9 (1H, d, $J=5.4$ Hz, C1′-H), 3.9 (1H, dd, $J=7.8$ Hz, $J=6.3$ Hz, C2′′-H), 7.2 (5H, m, phenyl). FABMS *m/z*: 528.1053 $(M+H)^+$; calcd. for $C_{19}H_{23}O_7N_7SCl$: 528.1038. *Anal.* Found: C, 41.35; H, 4.25; N, 17.75. Calcd. for $C_{19}H_{22}O_7N_7SCl\cdot H_2O$: C, 41.79; H, 4.43; N, 17.96.

2-Chloro-9-[5′-O-(N-D-alanyl)sulfamoyl-β-D-ribofuranosyl]adenine (**4**). The product (**4**) was obatained in a 40.3% yield by deprotecting **10d** (26.6 mg, 0.0385 mmol) in the same manner as that for **1**. $[\alpha]_D^{20}$ −9.63° ($c=0.44$, H_2O). ^{1}H-NMR δ_{D_2O} (Dioxane): 6.03 (1H, d, $J=5$ Hz, C1′-H), 3.66 (1H, q, $J=7$ Hz, C2′′-H), 1.28 (1H, d, $J=7$ Hz, C3′′-H). FABMS *m/z*: 452.0739 $(M+H)^+$; calcd. for $C_{13}H_{19}O_7N_7SCl$: 452.0723. *Anal.* Found: C, 32.84; H, 3.99; N, 19.61. Calcd. for $C_{13}H_{18}O_7SCl\cdot 2H_2O$: C, 32.00; H, 4.55; N, 20.10.

2-Chloro-9-[5′-O-(N-L-prolyl-L-prolyl)sulfamoyl-β-D-ribofuranosyl]adenine (**5**). The product (**5**) was obtained in a 97.2% yield by deprotecting **10e** (14 mg, 0.0172 mmol) in the same manner as that for **1**. $[\alpha]_D^{20}$ −67.6° ($c=0.84$, H_2O). ^{1}H-NMR δ_{D_2O} (Dioxane): 6.01 (1H, d, $J=5.2$ Hz, C1′-H), 4.37 (1H, dd, $J=6.8$ Hz, $J=8.9$ Hz, C2′′-H or C2′′′-H), 3.90 (1H, m, C2′′-H or C2′′′-H). FABMS *m/z*: 575.1432 $(M+H)^+$; calcd. for $C_{20}H_{28}O_8N_8SCl$: 575.1425.

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