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SYNTHESIS AND BIOLOGICAL ACTIVITIES OF SOME *N*-ACYL-2,6-DIAMINOPYRIDINES AND RELATED LINKER MODE IDENTICAL TWIN DRUGS

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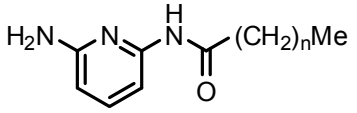
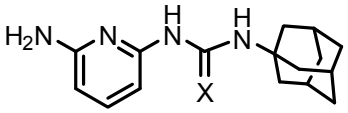
Abstract – In connection with our studies on biologically active compounds in the class of *N*-acyl-2,6-diaminopyridines, some molecular modifications were attempted. All of the synthesized compounds were evaluated for biological activity with herpes virus type 1 (HSV-1) by a plaque reduction assay. We observed that most of the synthesized derivatives showed no significant anti-HSV-1 activity, but we found that compounds **5** and **6** with a branched long alkyl chain showed high cytotoxicity to Vero cells.

In our synthetic studies on symmetrical 2,6-diaminopyridine (DAP) derivatives[1,2,3] to search for antiviral compounds, we have found that some *N*-mono- or *N,N'*-di-substituted DAP derivatives showed considerably high levels of antiviral activity (EC_{50}=20.5～60.7 μM) against HSV-1 (Figure 1). Among the synthesized compounds, some DAP derivatives such as **Aa–c**[2] and **B**[3] seemed to be biologically active leads for new antiviral candidates. We became interested in molecular modification of this simple structure attracted our attention for finding new biologically active compounds, and we therefore carried out further synthetic investigation and biological evaluation of this class of compounds.





Aa: n=8, EC_{50}= 60.7 μM
Ab: n=10, EC_{50}= 52.5 μM
Ac: n=12, EC_{50}= 57.9 μM

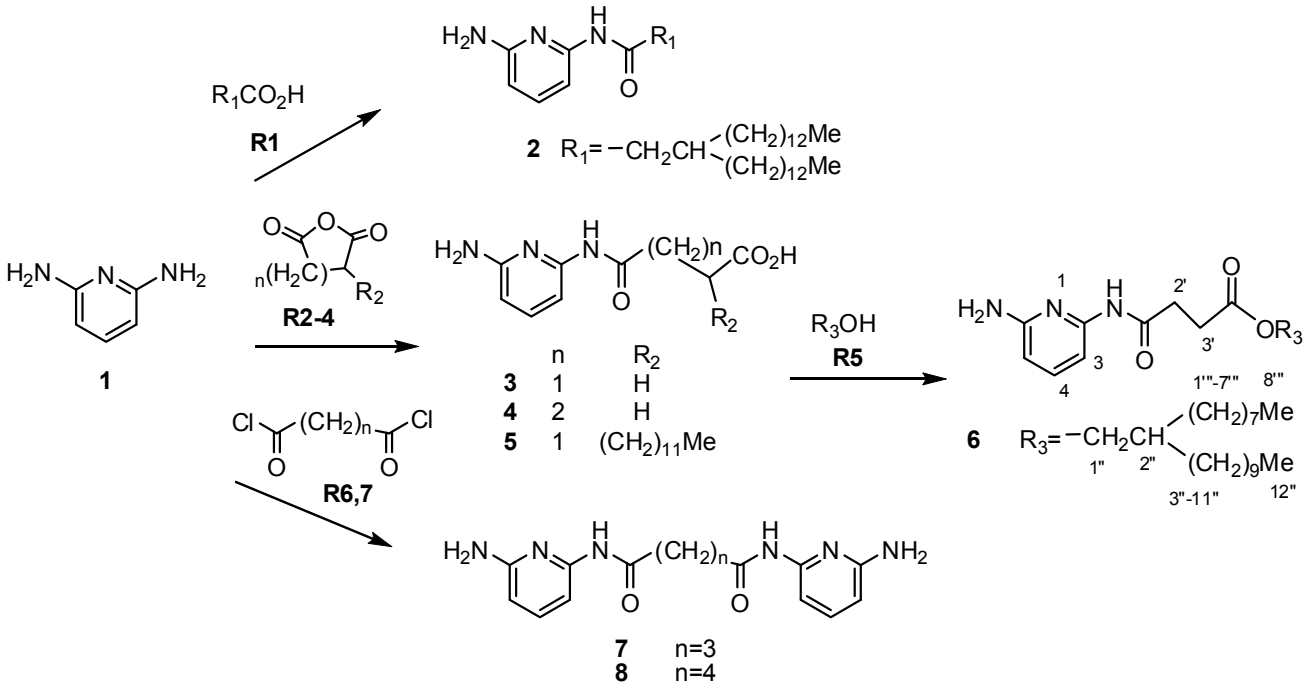
Ba: X=O, EC_{50}= 59.0 μM
Bb: X=S, EC_{50}= 20.5 μM

Figure 1. Anti-HSV-1 active compounds

In this paper, we describe the molecular modification of *N*-acyl functionalities of DAP as well as an identical linker mode twin-drug approach of *N*-acyl DAP. Results of biological evaluation of the synthesized DAP derivatives by plaque reduction assays are also described.

Chemistry. The overall reaction stages for target compounds **2–8** are shown in Scheme 1. Synthesis of compound **2** could be achieved by condensation reaction of DAP (**1**) and the corresponding carboxylic acid (**R1**, 3-tridecylhexadecanoic acid) in 45% yield. Compounds **3**,[4] **4**, and **5** were prepared by acylation of DAP by corresponding carboxylic anhydrides (**R2**, succinic anhydride; **R3**, glutaric anhydride; **R4**, *n*-dodecylsuccinic anhydride) in 58%, 89%, and 18% yields, respectively. The formation of another isomer[5] together with *N,N'*-diacyl derivatives caused the low yield of compound **5** (as a racemic form). Compound **6** could be obtained from condensation reaction of carboxylic acid **3** and a branched alcohol (**R5**, 2-octyl-1-dodecanol). A few esterification methods attempted for preparation of compound **6** resulted in quite low yields because the starting compound **3** is easily dehydrated to give a cyclized imide derivative. The condensation method using TsOH as a catalyst was effective, though in low yield, for the preparation without protection of an amino group on C-6 in the pyridine ring. By this procedure, *N*-acyl DAP derivative **6** with a branched long alkyl chain could be isolated in 9% yield (see Experimental). Synthesis of identical linker mode twin-drug type molecules **7**[6] and **8**[4] could be easily achieved by acylation of DAP with two corresponding diacylchlorides (**R6**, glutaryl dichloride; **R7**, adipoyl dichloride) in 50% and 73% yield, respectively.

All of the structures of the target compounds synthesized as described above were easily confirmed by spectroscopic and elemental analyses.



Scheme 1. Synthesis of *N*-acyl 2,6-diaminopyridines

Evaluation of biological activity and discussion. The anti-HSV-1 activities of synthesized *N*-acyl DAP derivatives were evaluated by plaque reduction assays[7] with Vero cells. The results for compounds **2**–**8** are summarized in Table 1.

Table 1. Antiviral activity (EC_{50}) and cytotoxicity (IC_{50}) against HSV-1

Compound	EC_{50} (μM)[b)]	IC_{50} (μM)
Aa[a)]	60.7	147
Ab[a)]	52.5	172
Ac[a)]	57.9	117
2	>75.5	> 604
3	> 382	> 382
4	>87.0	348
5	>51.5	77.8
6	>81.6	84.3
7·HCl	>189	>189
8·HCl	>181	>181

a) Data of compounds **Aa**–**c** were taken from ref. 2.

b) All compounds synthesized in this study showed no distinct inhibitory concentration (EC_{50}) values less than 50 μM. Among these compounds, compound **5** (as a racemic form) showed the highest level of activity against HSV-1 (EC_{25}=51.5 μM); however, its correct EC_{50} value could not be determined because cytotoxicity appeared at the 50% inhibitory concentration (EC_{50}). Twin-drug type compounds **7** and **8** also showed no significant antiviral activities against HSV-1 virus.

In our preliminary hypothesis to design this class of compounds, we assumed that the functional group 2-aminopyridyl moiety plays an important role in molecular recognition. Furthermore, *N*-long-chain acyl groups may enhance the affinity of the molecules as an anchor to the biological membranes of lipid bilayers.

The results for our newly modified DAP derivatives described in this article provide useful information regarding the structural features for biological activities. Twin-drug type molecules (such as compounds **7** and **8**) including two identical 2-aminopyridyl moieties are C_2 symmetrical, and *N*-monoacyl derivatives (such as compounds **3** and **4**) containing a hydrophilic –COOH as an acyl functionality are unsymmetrical single drug type molecules. Both of these compounds with introduced hydrophilic groups showed no significant antiviral activity. This fact may indicate that an *N*-long chain lipophilic anchor is required to elicit significant antiviral activity for *N*-monoacyl DAPs as a single drug.

It is noteworthy that the identical linker mode of twin-drugs[8] (compounds **7** and **8**) that have a C_2 symmetry axis did not show higher levels of anti-HSV activity than those of the single drug type of unsymmetrical *N*-monoacyl 2,6-diamopyridines (**Aa**–**c**) obtained by the introduction of a lipophilic long-chain acyl group.

In addition, we confirmed that the introduction of lipophilic branched long-chain acyl groups (e.g., compounds **5** and **6**) resulted in enhancement of cytotoxicity (IC_{50}=77.8~84.3 μM) to Vero cells with maintenance of a similar level of antiviral activity (EC_{50}=51.5~81.6 μM). The results obtained for compounds **5** and **6** suggest that the introduced lipophilic groups may play an important role in the cytotoxicity to host cells. Compounds **5** and **6** with a lipophilic branched long alkyl chain showed high levels of cytotoxicity (IC_{50}=77.8 and 84.3 μM, respectively), while compound **2**, which has a branched long alkyl chain of similar length, showed a low level of cytotoxicity (IC_{50}>604 μM). Compounds **3** and **4** also showed low levels of cytotoxicity (IC_{50}>382 and IC_{50}=348 μM, respectively). These observations suggested that both the factors of bulkiness and lipophilicity of the introduced alkyl chain may play a crucial role in the cytotoxicity of this class of compounds.

Based on these findings, we are studying further synthetic molecular modifications for C_2-symmetrical *N,N'*-diacyl DAPs or unsymmetrical single type *N*-monoacyl DAPs by introduction of different lipophilic linkers or anchors into the DAP template in research for biologically cytotoxic compounds as a new class of anticancer agents.

EXPERIMENTAL

Melting points were determined using a micro melting point apparatus (Yanagimoto MP-S3) without correction. IR spectra were measured by a Shimadzu FTIR-8100 IR spectrophotometer. Low- and high-resolution mass spectra (LR-MS and HR-MS) were obtained by a JEOL JMS HX-110 double-focusing model equipped with an FAB ion source interfaced with a JEOL JMA-DA 7000 data system. ^{1}H- and ^{13}C-NMR spectra were obtained by JEOL JNM A-500. Chemical shifts were expressed in δ ppm downfield from an internal TMS signal for ^{1}H-NMR and the carbon signal of the corresponding

solvent [$CDCl_3$ (77.00 ppm), CD_3OD (49.00 ppm), and DMSO-d_6 (39.50 ppm)] for ^{13}C-NMR. In the case of D_2O solvent, chemical shifts were expressed in δ ppm downfield from an internal sodium trimethylsilylpropionate-2,2,3,3-d_4 (TSP) signal. Microanalyses were performed with a Yanaco MT-6 CHN corder. Routine monitoring of reactions was carried out using precoated Kieselgel 60F_{254} plates (E. Merck). Centrifugal or flash column chromatography was performed on silica gel (Able-Biott or Fuji Silysia FL40D, respectively) with a UV detector. Commercially available starting materials were used without further purification.

***N*-(6-Amino-2-pyridinyl)-3-tridecylhexadecanamide (2).** To a stirred solution of 3-tridecylhexadecanoic acid (**R1**, 462 mg, 1.0 mmol, 100 mol%) in dry benzene (1 mL) and a drop of dry DMF was added thionyl chloride (0.11 mL, 1.5 mmol, 150 mol%) and the resulting solution was refluxed for 3 h under N_2 atmosphere. After evaporation of the solvent, the residue was dissolved in dry THF (2 mL). To this solution was added dropwise a solution of DAP (**1**, 111 mg, 10 mmol, 100 mol%) and Et_3N (0.14 mL, 1.0 mmol, 100 mol%) in dry THF (1 mL) with stirring at 0 ºC under N_2 atmosphere. The resulting mixture was allowed to stand at room temperature with stirring for 1 d. After removal of Et_3N·HCl salt by filtration, evaporation of the solvent afforded a viscous oil. Purification by centrifugal chromatography (SiO_2, 30% AcOEt in *n*-hexane) gave product **2** (0.240 g, 0.45 mmol, 45% yield) as a white solid.

4-[(6-Amino-2-pyridinyl)amino]-4-oxobutanoic acid[4] (3). This compound was prepared from the reaction of succinic anhydride (**R2**, 816 mg, 8.2 mmol, 100 mol%) and DAP (**1**, 960 mg, 8.8 mmol, 107 mol%) in dry dioxane (30 mL) with reflux for 3 h according to the procedure described by Bernstein *et al.*[4] The crude product **3** was obtained by filtration (970 mg, 4.6 mmol, 58% yield) and was recrystallized from EtOH to give an analytically pure sample **3**.

5-[(6-Amino-2-pyridinyl)amino]-5-oxopentanoic acid (4). This compound was also prepared from the reaction of glutaric anhydride (**R3**, 1.164 g, 10.2 mmol, 100 mol%) and DAP (**1**, 1.169 g, 10.7 mmol, 105 mol%) in dry dioxane (30 mL) with reflux for 1 h in a manner similar to that described by Bernstein *et al*.[4] The crude product **4** (1.990 g, 8.9 mmol, 89% yield) was obtained. Recrystallization from EtOH gave an analytically pure sample **4** as colorless crystals.

4-[(6-Amino-2-pyridinyl)amino]-2-dodecyl-4-oxobutanoic acid (5). To a solution of *n*-dodecylsuccinic anhydride (**R4**, 510 mg, 1.9 mmol, 100 mol%) in dry THF (10 mL) was slowly added a solution of DAP (**1**, 150 mg, 1.4 mmol, 140 mol%) in dry THF (2 mL), and the resulting solution was refluxed for 18 h. After evaporation of the solvent, purification by centrifugal chromatography (SiO_2, CH_2Cl_2/95% EtOH/28% NH_4OH, 80 : 19 : 1 to 70 : 29 : 1, v/v) gave pure product **5** (123 mg, 0.33 mmol, 18%) and byproduct **5'** (36 mg, 0.095 mmol, 5%).[5]

Table 2. Physical data of *N*-acyl 2,6-diaminopyridine derivatives (**2–8**)

Compd.	mp (℃) (Recryst solvent) Appearance	Formula	Analysis (%) Calcd (Found)			Formula, HR-MS *m/z*	IR (cm^{-1})
			C	H	N	Calcd (Found)	(KBr)
2	53—55	$C_{34}H_{63}N_3O$	77.07	11.98	7.93	$C_{34}H_{64}N_3O$ $(M+H)^+$	3335 (NH),
	—[a]		(77.13	12.00	7.68)	530.5049	1665 (CONH)
	white solid					(530.5048)	
3	183—187	$C_9H_{11}N_3O_3$	51.67	5.30	20.09	$C_9H_{12}N_3O_3$ $(M+H)^+$	3345 (CONH, COOH),
	(EtOH)		(51.53	5.36	20.17)	210.0879	1710 (COOH), 1645 (CONH)
	pale brown powder					(210.0881)	1580 (amide)
4	145—147	$C_{10}H_{13}N_3O_3$	52.12	6.04	18.24	$C_{10}H_{14}N_3O_3$ $(M+H)^+$	3475 (NH_2), 3325, 3210 (CONH,
	(EtOH)	·$0.4H_2O$	(52.13	6.00	18.15)	224.1035	COOH), 1770, 1685 (COOH), 1655,
	colorless needles					(224.1034)	1640 (CONH), 1560 (amide)
5	103—105	$C_{21}H_{35}N_3O_3$	64.95	9.40	10.82	$C_{21}H_{36}N_3O_3$ $(M+H)^+$	3350, 3215 (CONH,NH_2),
	—[a]	·$0.6H_2O$	(64.87	9.31	10.92)	378.2757	3000 (COOH), 1685 (COOH),
	colorless amorphous					(378.2758)	1655, 1635 (CONH)
6	—[b]	$C_{29}H_{51}N_3O_3$	71.12	10.50	8.58	$C_{29}H_{52}N_3O_3$ $(M+H)^+$	3470, 3365, 3230 (CONH,NH_2),
			(71.02	10.60	8.41)	490.4009	1730, 1295, 1160 (ester)
	pale yellow oil					(490.4011)	1670, 1620, 1540 (amide)
7	71—74[c]	$C_{15}H_{18}N_6O_2$	56.19	5.88	26.21	$C_{15}H_{18}N_6O_2(M+H)^+$	3355 (NH_2), 1685 (CONH),
	(EtOH-H_2O)		(56.20	5.91	26.26)	315.1569	1620 (CONH),1540 (amide)
	colorless fine needles					(315.1560)	
8	223.5—234.0[d]	$C_{16}H_{20}N_6O_2$	56.96	6.27	24.91	$C_{16}H_{20}N_6O_2$ $(M+H)^+$	3450,3280 (CONH,NH_2),
	—[a]		(57.13	6.19	24.70)	329.1726	1675 (CONH),
	colorless amorphous					(329.1739)	1300(C-NH)
7	198—202	$C_{15}H_{18}N_6O_2$	42.56	5.71	19.58	$C_{15}H_{19}N_6O_2$ $(M+H)^+$	3315 (CONH, NH_3^+, H_2O)
·2HCl	(95%EtOH)	·2HCl	(42.61	5.68	19.94)	315.1569	1655 (COOH)
	hygroscopic colorless powder	·$2H_2O$				(315.1577)	1620 (CONH)
8	226—229	$C_{16}H_{20}N_6O_2$	43.41	6.06	18.98	$C_{16}H_{20}N_6O_2$ $(M+H)^+$	3100 br (CONH, NH_3^+, H_2O)
·2HCl	(MeOH)	·2HCl	(43.46	5.96	18.73)	329.1726	1635 (CONH)
	white powder	·$2.3H_2O$				(329.1728)	

a) This compound was purified by chromatography. b) This compound was obtained as an oil. c) Mp 77.5-79 ºC as a pale brown solid was reported in ref. 6. d) Mp 174-175 ºC (dec.) from 75% MeOH was reported in ref. 4.

4-[(6-Amino-2-pyridinyl)amino]-4-oxobutanoic acid 2-octyldodecanyl ester (6). The reaction mixture of compound **3** (420 mg, 2.0 mmol, 100 mol%), 2-octyl-1-dodecanol (**R5**, 2.87 g, 9.6 mmol, 480 mol%),

Table 3. ^{1}H-NMR data (*J* in Hz) of *N*-acyl 2,6-diaminopyridine derivatives (**2**–**8**)

H No.	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**7**·2HCl	**8**·2HCl
Py ring H-3	7.55 d (7.9)	7.19 dd (7.9, 0.6)	7.22d (7.9),	7.19 br d (7.6)	7.48 br d (7.9)	7.25 d (7.9)	7.23 d (7.9)	6.59 d (8.5)	6.62 d (8.9)
H-4	7.43 t (7.9)	7.31 t (7.9)	7.32 t (7.9),	7.39 t (8.1)	7.42 t (7.9),	7.33 t (7.9)	7.33 t (7.9)	7.76 dd (8.5, 7.9)	7.82 t (8.4)
H-5	6.23 d (7.9)	6.15 dd (7.9, 0.6)	6.16 dd (7.9, 0.6)	6.28 dd (7.6, 0.6)	6.22 d (7.9)	6.18 dd (7.9,0.6)	6.18 d (7.9)	6.51 d (7.9)	6.67 d (7.9)
NH_2	4.27 s (2H)	5.65 br s	5.65 s		4.31 br s	5.65 s	5.70 br		8.23 br s
-NHCO-	7.62 s (1H)	9.80 s	9.74 s		7.90 br s	9.76 s	9.76 s		12.17 s
CH_3	0.88 t (6.9)			0.89 t (7.0)	0.88 t (6.9)				
H-2'	2.24 d (7.0)	2.57 t (6.7)	2.36 t (7.3)	2.52 dd (15.3, 5.5)	2.65 or 2.73	2.37 t (7.5)	2.34 br s	2.63 t (7.0)	2.56 m
H-3'	1.93 br s	2.47 t (6.7)	1.77 qui (7.3)	2.86 m	2.73 or 2.65	1.85 qu (7.5)	1.57 br s	2.12 qui (7.0)	1.69 m
H-4'		0.88 t (6.9)	2.24 t (7.3)	1.57 m, 2.86 m				2.63 t (7.0)	
others	1.25—1.33 m (48H, H4'—15')	12.02 br s (COOH)	12.00 br s (COOH)	1.25—1.4 (H5'—14')	1.2—1.35 m (H1'"—8'", H3"—11"), 1.62 m (H2"), 4.00 d (5.8, H1")				

a) **2** and **6** were measured in $CDCl_3$; **3**, **4**, **7**, **8**, and **8**·2HCl were measured in DMSO-d_6; **5** and **7**·2HCl were measured in CD_3OD and D_2O, respectively.

and TsOH monohydrate (415 mg, 2.2 mmol, 110 mol%) in dry benzene (30 mL) was refluxed with a Dean-Stark apparatus for 2 d. After cooling, insoluble starting material **3** was filtrated off and the filtrate was evaporated in vacuo and then the residue was purified by centrifugal chromatography (SiO_2, CH_2Cl_2/EtOH, 98 : 2, v/v) to give pure product **6** (84 mg, 0.17 mmol, 9%) as a pale yellow oil.

***N*1,*N*5-Bis(6-amino-2-pyridinyl)pentanediamide (7)[6] and the HCl salt (7·HCl).** This compound **(7)** was prepared in a manner similar to Nabeshima's method.[6] Thus, to a solution of DAP (**1**, 8.72 g 80 mmol, 533 mol%) and Et_3N (11.1 mL, 80 mmol, 533 mol%) in dry THF (100 mL), a solution of glutaryl dichloride (**R6**, 2.54 g 15 mmol, 100 mol%) in dry THF (10 mL) was added dropwise with stirring at -20 ºC under N_2 atmosphere and stirring was continued for 4.5 h. After cooling, the reaction mixture was separated by filtration into Et_3N·HCl salt and mother liquid layer. The mother liquid was concentrated and purified by flash chromatography (SiO_2, CH_2Cl_2/95% EtOH/28% NH_4OH, 930 : 65 : 5, v/v) to give compound **7** (2.34 g, 7.45 mmol, 50%) as a white solid. Recrystallization from EtOH-H_2O gave an analytically pure sample as fine colorless needles. Compound **7**·HCl salt was prepared by treatment of this free base (**7**) with 1 M HCl in EtOH. Evaporation of the solvent and then recrystallization from 95% EtOH gave **7**·HCl as a colorless hygroscopic powder.

Synthesis of *N*1,*N*6-bis(6-amino-2-pyridinyl)hexanediamide (8)[4] and the HCl salt (8·HCl) These compounds were prepared in a manner similar to that described above for the preparation of compound **7** and **7**·HCl salt from DAP (**1**) and adipoyl dichloride (**R7**). Purification by flash chromatography (SiO_2, CH_2Cl_2/95% EtOH/28% NH_4OH, 930 : 65 : 5 to 900 : 95 : 5, v/v) gave **8** as a colorless amorphous

Table 4. ^{13}C-NMR data of *N*-acyl 2,6-diaminopyridine derivatives (**2**–**8**)

C No.	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**7**·2HCl	**8**·2HCl
Py ring C-2	149.88	150.35	150.35	150.92 or 159.73	149.72	158	150	156.00	143.93
C-3	103.34	100.76	100.9	103.49	103.33	101	101	109.57	105.54
C-4	140.13	138.67	138.69	140.76	140.04	139	139	147.67	144.85
C-5	104.09	103.08	103.16	105.49	104.2	103	103	103.29	98.74
C-6	157.02	158.34	158.34	159.73 or 150.92	157.08	150	158	146.85	152.76
-NHCO-	171.3	170.38	171.08	172.75	169.69	171	172	179.04	174.43
CH_3	14.07			14.39					
C-2'	43.12	30.88	35.13	39.87	29.29, 29.32, or 32.17	35.40	35.9	38.47	35.55
C-3'	35.43	28.68	20.36	43.15	29.29, 29.32, or 32.17	20.9	24.7	22.94	23.71
C-4'	33.85		32.96	33.03	172.82				
C-5'	26.95			28.10					
C-6'	29.34—29.91 (C6'—13')								
others	22.67 (C15'), 29.34—29.91 (C6'—13'), 31.92 (C14')	173.68 (COOH)	173.99 (COOH)	179.68 (COOH)	14.06 (C12", 9'"), 22.65 (C11", 8'"), 26.71 (C4", 2'"), either of 29.29, 29.32 , 29.55, 29.59, 29.62, 29.64, 29.93 (C5"—9", C3'"—6'"), 31.23 (C3", 1'"), 31.88 and 31.90 (C10", 7'"), 37.33 (C2"), 67.76 (C1")				

a) **2** and **6** were measured in $CDCl_3$; **3**, **4**, **7**, **8**, and **8**·2HCl were measured in DMSO-d_6; **5** and **7**·2HCl were measured in CD_3OD and D_2O, respectively.

solid in 73% yield. The compound **8**·HCl salt was prepared from treatment of free base **8** with 1 M HCl in EtOH. Recrystallization from MeOH gave compound **8**·HCl as colorless powder.

All of the physical and spectroscopic (^{1}H- and ^{13}C-NMR) data for the prepared compounds (**2**–**8**) are summarized in Tables 1–4.

Antiviral activity assay and cytotoxicity. The antiviral activities of synthesized compounds were measured by using a plaque reduction assay[7] as described in our previous paper.[1] Results of antiviral activity (EC_{50}) and cytotoxicity (IC_{50}) values with Vero cells are summarized in Table 4.

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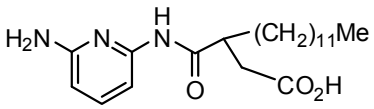
5. The isomer **5'** formed from an alternative way of acylation by acid anhydride **R4**, in which the *n*-dodecyl chain is attached to C2' instead of C3', was obtained as a minor product (isolatable as colorless crystals). This compound was unstable in solution and decomposed during nmr measurement in $CDCl_3$. Elemental analysis was performed by HR positive ion FAB-MS: Calcd for $C_{21}H_{36}O_3N_3$ ($M+H^+$): 378.2757. Found: 328.2755.



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