SYNTHESIS AND CYTOSTATIC ACTIVITY OF THE ANTITUMOR ANTIBIOTIC CHARTREUSIN DERIVATIVES†

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In order to overcome the rapid biliary excretion of chartreusin, which diminished its activity when administered iv, a series of 3′,4′-*O*-substituted derivatives of chartreusin were synthesized. Exo-type of 3′,4′-*O*-benzylidene-chartreusin was found active both by ip and iv administration. Therefore, this compound was selected for further modification on its 6-phenol to obtain broader spectra and better pharmacokinetic parameters than the original compound. Several 6-*O*-acyl-3′,4′-*O*-exo-benzylidene-chartreusins had high antitumor activity against some murine tumors both by iv and po administration.

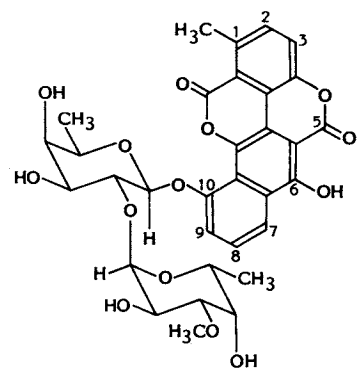
Chartreusin (**1**) produced by *Streptomyces chartreusis* was originally reported in 1953,[2] and its chemical structure (Fig. 1) was fully elucidated in 1964.[3,4] Originally chartreusin was studied because of its antibacterial activity, but latter its significant antitumor effect against murine L1210 and P388 leukemias, as well as against B16 melanoma, were revealed.[5] However, this antitumor activity of chartreusin were observed only when both the tumor cells and the drug were injected ip (ip-ip system). Under these conditions, chartreusin precipitated in the peritoneal cavity resulting in prolonged contact between the drug and the tumor cells. When the drug was administered iv (ip-iv system), it was rapidly eliminated through the bile. Therefore the drug concentration fell below the effective dose quickly, and antitumor activity was not observed by iv route.[5]

Oral (ip-po system) as well as subcutaneous (ip-sc system) administration of chartreusin also failed to observe a positive antitumor response.[5]

Because of these drawbacks, chartreusin was not selected for clinical trials. Its novel structure and high activities against some experimental tumors suggested synthetic modification of chartreusin.

Until recently, no chartreusin derivative with improved antitumor activity has been reported. The replacement of the disaccharide moiety of chartreusin with neutral monosaccharide, as the only attempt to synthesize new derivatives, failed to develop a better compound than the original one.[6]

Fig. 1. Structure of chartreusin.



1

† See ref 1.

Recently the related antibiotic elsamicin, having an amino group on the sugar moiety, was isolated by Bristol-Myers' group.[7] It was more effective than chartreusin against some murine tumors in ip-ip system, and also showed antitumor activity in both ip-iv and sc-iv systems.[8]

To explore the structure-activity relationship of chartreusin derivatives, we have modified the hydroxyl groups of chartreusin.

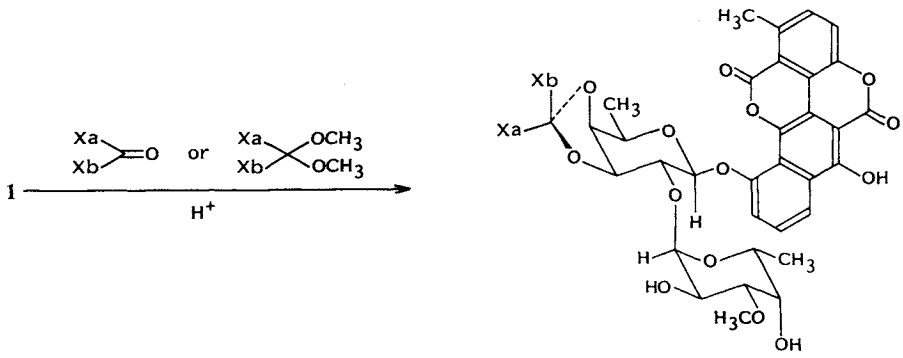
Chemistry

The acetals of chartreusin at 3',4'-dihydroxyl groups (listed in Table 1) were synthesized by the reaction with the corresponding aldehydes (ketones) or the dimethyl acetals in the presence of an acid catalyst as shown in Scheme 1.

In that way, it is possible to obtain a pair of isomers. The reaction with benzaldehyde resulted in a mixture of exo and endo benzylidene derivatives and the ratio ranged from 1 : 1 to 1 : 2. On the contrary, the reaction with an aliphatic aldehyde or ketone yielded nearly a single isomer (endo isomer). From these results, we believe that the endo form is more stable thermodynamically than the exo form.

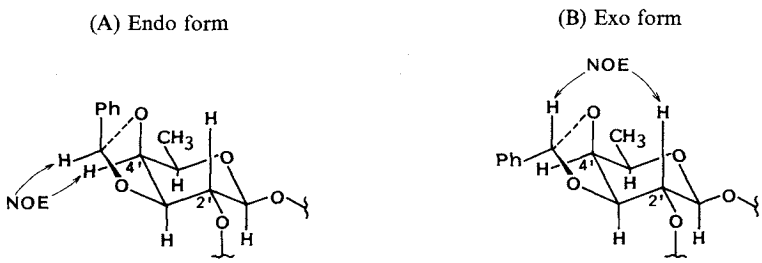
The configuration of these isomers were determined by the measurements of these differential nuclear Overhauser effects (dif-NOE): In the derivative **12**, the irradiation of the benzyl proton resulted in the observation of the dif-NOE not on the 2'-H but on the 4'-H. In the other derivative **13** (the isomer of **12**), the dif-NOE was observed reversely. On the basis of these NOE experiments, the configurations of **12** and **13** were assigned the endo form (Fig. 2(A)) and the exo form (Fig. 2(B)), respectively.

Scheme 1. Synthesis of 3',4'-*O*-substituted chartreusin.

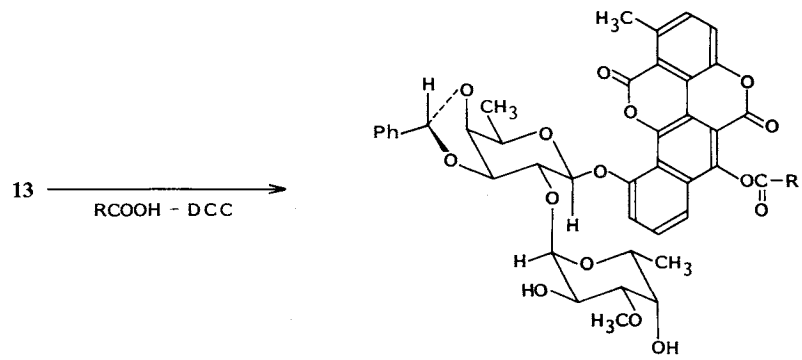


2~13

Fig. 2. Differential NOE's on endo and exo forms.



Scheme 2. Synthesis of the phenol ester of **13** at 6-position.



14~26

In all aliphatic acetal derivatives except compound **9**, the irradiation of the acetal-proton led to be observed the dif-NOE not on the 2'-H but on the 4'-H such as **12**. Therefore, the configuration of these aliphatic derivatives (**2~7**) was determined to be the endo form. In the derivative **9**, the reaction of chartreusin with methyl ethyl ketone resulted in a pair of the acetal isomers, but the evaluation of these isomers was carried out without separation because of the difficulty of separation.

Phenol esters of 3',4'-*O*-exo-benzylidene-chartreusin (**13**) (listed in Tables 3 and 4) were prepared by the condensation of the corresponding carboxylic acids using dicyclohexylcarbodiimide (DCC) as shown in Scheme 2.

In the case of **20**, its intermediate was obtained by the reaction of **13** with *N*-benzyloxycarbonyl-*N*-methyl-*β*-alanine in the same manner as described above, and the resulting compound was reduced with hydrogen in the presence of 5% palladium-carbon to obtain **20**.

Antitumor Activity and Discussion

In order to investigate the antitumor activity of the 3',4'-*O*-substituents of chartreusin, the cytostatic activity of these derivatives has been determined in ip-ip system against B16 melanoma.

Table 1 shows that the activity of the 3',4'-*O*-substituted derivatives was compared with that of chartreusin. In the alkylidene series (**2~10**), the bulky substituent in **7** or **10** extinguished the antitumor activities, and the activities of all derivatives except **8** were lower than that of chartreusin. In case of the cyclohexylidene derivative (**11**), which acetal moiety is larger than that of inactive **10** (the moiety is less bulky than that of **10**), its antitumor activity was the same level as that of **2**, **3** or **9**. On the other hand, the benzylidene derivatives (**12** and **13**) were more potent than other derivatives, but a remarkable differences of the antitumor activity regarding with their stereoisomers also has been observed.

The exo type of 3',4'-*O*-benzylidene-chartreusin (**13**) was the most potent.

Two derivatives (**8** and **13**) were further evaluated in ip-iv system against P388 leukemia, in which system chartreusin is inactive. Table 2 shows that **8** and **13** were active in this system. On the basis of the plasma pharmacokinetic profile,[9] it is thought that the elimination of the drug to the biliary duct might be decreased by the introduction of these substituents.

Compared the two derivatives, **13** was more effective than **8** in both ip-ip and ip-iv systems. Although **13** was the most effective among the 3',4'-*O*-substituted derivatives, its antitumor activity against B16 melanoma (see Table 4) in the ip-po system was not outstanding.

Table 1. Effects of ip-administered 3′,4′-*O*-substituted derivatives of chartreusin on the survival time of mice inoculated ip with B16 melanoma.

Compound No.	Structure		T/C (%)	
	Xa	Xb	20 mg/kg/day	40 mg/kg/day
Chartreusin (**1**)	—	—	163	170
2	H	CH_3	144	167
3	H	CH_2CH_3	142	150
4	H	$(CH_2)_2CH_3$	101	124
5	H	$(CH_2)_3CH_3$	130	147
6	H	$(CH_2)_4CH_3$	119	148
7	H	$(CH_2)_5CH_3$	106	99
8	CH_3	CH_3	155	190
9[a]	CH_3	CH_2CH_3	148	142
10	CH_2CH_3	CH_2CH_3	105	113
11	$-CH_2CH_2CH_2CH_2CH_2-$		140	164
12	H	Phenyl	164	193
13	Phenyl	H	145 (5 mg/kg)	199 (10 mg/kg)

Each derivatives was suspended in 0.9% saline containing about 5% Tween 80, and administered ip once daily for 9 days.

[a] The mixture of isomers (*ca.* 1 : 1).

Table 2. Effects of iv-administered 3′,4′-*O*-substituted derivatives of chartreusin on the survival time of mice inoculated ip with P388 leukemia.

Compound No.	Structure		T/C (%)	
	Xa	Xb	25 mg/kg/day	50 mg/kg/day
Chartreusin (**1**)	—	—	108	108
8	CH_3	CH_3	105	162
13	Phenyl	H	209 (13 mg/kg)	175 (25 mg/kg)

Chartreusin was dissolved in 0.9% saline containing 20% dimethyl formamide; the others were suspended in 0.9% saline containing 10% Tween 80, and administered iv on days 1, 4 and 7.

13 was not metabolized to chartreusin in plasma and tissues, therefore **13** would not be a prodrug of chartreusin. Further modification of compound **13** has been attempted to obtain broader antitumor spectra and a possible candidate for clinical investigation.

Eleven 6-*O*-acyl derivatives of compound **13** were evaluated for antitumor activity against P388 leukemia in the ip-iv system (Table 3).

Both lipophilic and hydrophilic substituent at 6-position led to high activity against P388 leukemia. The acylation of the phenol in **13** did not destroy its activity, when the substituent was relatively small. These results indicated that this modification would be useful to obtain derivatives which would have suitable physiological properties for different route of administration.

The compounds listed in Table 4 were further evaluated for antitumor activities against several murine tumors in ip-po system (lipophilic derivatives) or ip-iv system (hydrophilic derivatives).

Lipophilic derivatives (**15**, **16**, **22** and **24**) did not show any activity against B16 melanoma in ip-po system when the drug was administered on days 1, 5 and 9, although they were active against P388 in ip-iv system (Tables 3 and 4). Interestingly, the newly synthesized **25** had higher activity against B16 melanoma than the original compound (**13**) in ip-po system, and had also high potency against P388 and

Table 3. Effects of iv-administered 6-*O*-acyl-3′,4′-*O*-exo-benzylidene-chartreusin on the survival time of mice inoculated ip with P388 leukemia.

Compound No.	Structure	T/C (%)	
	R (6-*O*-COR)	20 mg/kg/day	40 mg/kg/day
14	$CH_2CH(CH_3)_2$	202	—
15	$CH_2CH_2COCH_3$	203	243
16	CH_2OCOCH_3	185	250
17	$CH(CH_3)CH_2NHCOCF_3$	203	241
18	$(CH_2)_7NHCOCF_3$	135	186
19	$CH_2N(CH_3)_2 \cdot HCl$	207 (18 mg/kg)	250 (35 mg/kg)
20	$CH_2CH_2NHCH_3 \cdot HCl$	180	177
21	CH_2CH_2-pyrrolidino·HCl	165	292
22	2-Furyl	199	217
23	*N*-Methyl-2-pyrrolyl	189	230
24	2-Indolyl	199	255

Each derivative was dissolved (No. **19**, **20**, and **21**) or suspended in 0.9% saline containing 0~5% Tween 80, and administered iv on days 1, 5 and 9.

Table 4. Effects of po- or iv-administered 3′,4′-*O*-exo-benzylidene-chartreusin (**13**) and its 6-*O*-acyl derivatives on the survival time of mice inoculated ip with several murine tumors.

Compound No.	Structure	Tumor	T/C (%)-Dose (mg/kg/day)	
	R (6-*O*-COR)			
13	— (6-O-H)	B16	127 (75)	139 (150)
14	$CH_2CH(CH_3)_2$	B16	139 (75)[a]	145 (150)[a]
15	$CH_2CH_2COCH_3$	B16	117 (75)	133 (150)
16	CH_2OCOCH_3	B16	102 (75)	128 (150)
22	2-Furyl	B16	128 (75)	134 (150)
24	2-Indolyl	B16	112 (75)	103 (150)
25	$CH_2CH_2OCH_2CH_3$	B16	149 (80)	179 (160)
		B16	154 (80)[a]	199 (160)[a]
		L1210	256 (80)	294 (160)
		P388	235 (80)	273 (160)
26	CH_2CH_3	B16	124 (80)[a]	151 (160)[a]
		P388	219 (80)	—
19	$CH_2N(CH_3)_2 \cdot HCl$	B16	122 (35)	156 (70)
		B16	150 (18)[a]	156 (35)[a]
		L1210	378 (35)	>600 (70)
		P388	250 (35)	362 (70)
20	$CH_2CH_2NHCH_3 \cdot HCl$	B16	129 (20)	147 (60)
		L1210	209 (20)	>500 (60)
		P388	180 (20)	214 (60)

25 and **26** were suspended in distilled water containing 0.5% (w/v) sodium carboxymethyl cellulose; **19** and **20** were dissolved in 0.9% saline containing about 2% Tween 80; the others were suspended in distilled water containing 5% dimethylformamide and 10% Tween 80; and administered po (lipophilic derivatives) or iv (hydrophilic derivatives) on days 1, 5 and 9.

[a] Administered on days 1, 5, 9, 13 and 17.

L1210 leukemia. These findings suggested that **25** would have better lipophilicity to be absorbed from gastro-intestinal tract, and also indicated that the bioavailability of the derivatives might be deeply effected by the 6-*O*-substituent.

On the other hand, the hydrophilic derivatives (**19** and **20**) were not so active against B16, but they showed very remarkable antitumor activity against P388 and L1210 leukemia cell lines. Especially, their

excellent activity against L1210 leukemia was detected, and in case of the higher dose, the T/C value was over 500 and several cured mice were observed. Although their potencies were almost the same against B16 melanoma, **19** was superior to **20** not only in its activity against P388 and L1210 leukemia but also in its solubility for the iv injection.

Both **25** and **19** turned out to be a prodrug of **13**, because the ester of 6-*O*-position was rapidly hydrolyzed in plasma within about 30 minutes and also because the *in vitro* activity was disappeared by the blocking of the phenol at 6-position. Consequently, both compounds would be metabolized to the same active compound (**13**) *in vivo* (and *in vitro*), and these acyl substituents would be influenced on the pharmacokinetic parameters. Comparing the lipophilic **25** with the hydrophilic **19**, it seems that a lipophilic derivative of **13** became more active against B16 melanoma and that a hydrophilic one became more active against P388 and L1210 leukemia cell lines than the original compound (**13**). The variation of the 6-*O*-substituent altered the distribution in mice, and it is very likely that this was due to the altered uptake of the derivative into the cells. However, it is unclear whether there is any correlation between the hydrophilicity of the derivatives and their activity against solid tumor (B16 melanoma) or leukemia cell lines (P388 and L1210). There are not enough data available yet to clarify this question. This will be a future subject to be studied.

In conclusion, we found that **13** is a promising new derivative of chartreusin. Two modified **13** such as **25** (with possible oral activity) and **19** (as possible intravenous drug) were obtained by the acylation of the phenol. These two compounds are interesting for further studies.

Experimental

MP's were determined with a Yanagimoto micro melting point apparatus, type MP-S3, and were not corrected. NMR spectra were recorded on a Jeol JNM-PMX-60 spectrometer and a Jeol JNM-GSX-400 spectrometer. Chemical shifts are given in δ values with tetramethylsilane as an internal standard.

Column chromatography was carried out with Silica gel 60 (E. Merck Co.). Preparative TLC was conducted on 20×20 cm glass plates coated in our laboratory with a 0.6~0.7-mm thickness of Silica gel GF_{254} (E. Merck Co.).

Synthesis of 3',4'-*O*-Alkylidene-chartreusin (**2**~**13**)

General Procedure for **2**, **3**, **8** and **11**: The corresponding dimethyl acetal (10~30 mmol) and *p*-toluenesulfonic acid (*ca.* 0.05 mmol) were added to a solution of chartreusin (1.0 mmol) in absolute chloroform (20~30 ml). The mixture was stirred at room temperature for 4~20 hours, then poured into saturated sodium bicarbonate and extracted with chloroform. The extract was washed with saturated brine, dried and concentrated to obtain crude product, which was purified by silica gel column chromatography (eluted with $CHCl_3$-MeOH) to give the desired product in about 80% yield.

3',4'-*O*-Endo-ethylidenechartreusin (**2**): MP 154.0~158.0°C; ^{1}H NMR ($CDCl_3$) δ 1.42 (3H, d, J=6.8 Hz), 1.51 (3H, d, J=6.4 Hz), 1.65 (3H, d, J=4.8 Hz), 2.95 (3H, s), 3.48 (3H, s), 3.81~5.93 (10H, m), 5.33 (1H, q, J=4.8 Hz), 7.43~8.38 (5H, m), 11.77 (1H, s).

Anal Calcd for $C_{34}H_{34}O_{14}$: C 61.26, H 5.14.
Found: C 61.46, H 5.15.

3',4'-*O*-Endo-*n*-propylidenechartreusin (**3**): MP 197.0~206.0°C; ^{1}H NMR ($CDCl_3$) δ 1.08 (3H, t, J=7.2 Hz), 1.35 (3H, d, J=6.8 Hz), 1.44 (3H, d, J=6.4 Hz), 2.86 (3H, s), 3.39 (3H, s), 3.69~5.85 (10H, m), 5.05 (1H, t, J=4.8 Hz), 7.34~8.29 (5H, m), 11.68 (1H, s).

Anal Calcd for $C_{35}H_{36}O_{14}$: C 61.76, H 5.33.
Found: C 61.72, H 5.59.

3′,4′-*O*-Isopropylidenechartreusin (**8**): MP 168.0~170.0°C; ^{1}H NMR ($CDCl_3$) δ 0.91 (3H, d, J=6.8 Hz), 0.96 (3H, s), 0.98 (3H, d, J=6.4 Hz), 1.24 (3H, s), 2.41 (3H, s), 2.95 (3H, s), 3.26~5.43 (10H, m), 6.90~7.84 (5H, m), 11.22 (1H, s).

Anal Calcd for $C_{35}H_{36}O_{14}$: C 61.76, H 5.33.
Found: C 61.42, H 5.40.

3′,4′-*O*-Cyclohexylidenechartreusin (**11**): MP 243.5~253.5°C; ^{1}H NMR ($CDCl_3$) δ 1.43 (3H, d, J=6.4 Hz), 1.51 (3H, d, J=6.4 Hz), 2.91 (3H, s), 3.43 (3H, s), 3.74~5.92 (10H, m), 7.38~8.32 (5H, m), 11.70 (1H, s).

Anal Calcd for $C_{38}H_{40}O_{14}$: C 63.33, H 5.59.
Found: C 63.10, H 5.79.

General Procedure for **4~7** and **9~10**: An appropriate aldehyde or ketone (5~10 mmol), *p*-toluenesulfonic acid (*ca.* 0.05 mmol) and Molecular Sieves 4A or 5A (*ca.* 5 g) (or $CuSO_4$: *ca.* 1 g) were added to a solution of chartreusin (1.0 mmol) in absolute chloroform (20~30 ml). The mixture was stirred at room temperature for 5~24 hours, then filtered into saturated sodium bicarbonate and extracted with chloroform. The extract was washed with saturated brine, dried and concentrated to afford crude product, which was purified by silica gel column chromatography (eluted with $CHCl_3$-MeOH) to give the desired product in 30~70% yield.

3′,4′-*O*-Endo-*n*-butylidenechartreusin (**4**): MP 214.0~220.0°C; ^{1}H NMR ($CDCl_3$) δ 0.99 (3H, t, J=7.2 Hz), 1.35 (3H, d, J=6.8 Hz), 1.43 (3H, d, J=6.8 Hz), 2.87 (3H, s), 3.39 (3H, s), 3.73~5.85 (10H, m), 5.09 (1H, t, J=4.8 Hz), 7.24~8.30 (5H, m), 11.69 (1H, s).

Anal Calcd for $C_{36}H_{38}O_{14}$: C 62.24, H 5.51.
Found: C 62.19, H 5.53.

3′,4′-*O*-Endo-*n*-pentylidenechartreusin (**5**): MP 229.5~237.0°C; ^{1}H NMR ($CDCl_3$) δ 1.01 (3H, t, J=6.6 Hz), 1.43 (3H, d, J=6.4 Hz), 1.53 (3H, d, J=6.8 Hz), 2.94 (3H, s), 3.47 (3H, s), 3.81~5.92 (10H, m), 5.15 (1H, t, J=4.8 Hz), 7.42~8.37 (5H, m), 11.77 (1H, s).

Anal Calcd for $C_{37}H_{40}O_{14}$: C 62.71, H 5.69.
Found: C 62.86, H 5.73.

3′,4′-*O*-Endo-*n*-hexylidenechartreusin (**6**): MP 201.0~204.0°C; ^{1}H NMR ($CDCl_3$) δ 0.97 (3H, t, J=6.8 Hz), 1.42 (3H, d, J=7.2 Hz), 1.51 (3H, d, J=6.4 Hz), 2.94 (3H, s), 3.47 (3H, s), 3.81~5.92 (10H, m), 5.15 (1H, t, J=4.8 Hz), 7.41~8.37 (5H, m), 11.75 (1H, s).

Anal Calcd for $C_{38}H_{42}O_{14}$: C 63.15, H 5.86.
Found: C 63.02, H 5.84.

3′,4′-*O*-Endo-*n*-heptylidenechartreusin (**7**): MP 183.0~185.0°C; ^{1}H NMR ($CDCl_3$) δ 0.95 (3H, t, J=6.8 Hz), 1.42 (3H, d, J=6.4 Hz), 1.51 (3H, d, J=6.4 Hz), 2.94 (3H, s), 3.47 (3H, s), 3.81~5.92 (10H, m), 5.15 (1H, t, J=4.8 Hz), 7.41~8.36 (5H, m), 11.74 (1H, s).

Anal Calcd for $C_{39}H_{44}O_{14}$: C 63.58, H 6.02.
Found: C 63.77, H 6.14.

3′,4′-*O*-Isobutylidenechartreusin (**9**: The Mixture of Endo and Exo Isomers): MP 203.0~208.0°C; ^{1}H NMR ($CDCl_3$) δ 1.23 (3/2H, s), 1.44 (3/2H, s), 1.30 (3H, d, J=6.0 Hz), 1.38 (3H, d, J=6.4 Hz), 2.80 (3H, s), 3.33 (3H, s), 3.69~5.82 (10H, m), 7.27~8.21 (5H, m), 11.58 (1H, s).

Anal Calcd for $C_{36}H_{38}O_{14}$: C 62.24, H 5.51.
Found: C 62.08, H 5.60.

3′,4′-*O*-Isopentylidenechartreusin (**10**): MP 246.0~252.0°C; ^{1}H NMR ($CDCl_3$) δ 0.99 (3H, t, J=6.8 Hz), 1.14 (3H, t, J=6.8 Hz), 1.42 (3H, d, J=6.4 Hz), 1.52 (3H, d, J=6.8 Hz), 2.93 (3H, s), 3.45 (3H, s), 3.76~5.93 (10H, m), 7.39~8.34 (5H, m), 11.73 (1H, s).

Anal Calcd for $C_{37}H_{40}O_{14}$: C 62.71, H 5.69.
Found: C 62.64, H 5.81.

Synthesis of the Endo and Exo Forms of 3',4'-*O*-Benzylidene-chartreusins (**12** and **13**)

Benzaldehyde (30 ml), *p*-toluenesulfonic acid (1.0 g) and Molecular Sieves 4A (50 g) were added to a solution of chartreusin (10.0 g) in absolute chloroform (300 ml). The mixture was stirred at room temperature for 20 hours, then filtered through Celite and the filtrate was concentrated to a volume of about 150 ml. The resulting solution was subjected to several repetitions of silica gel column chromatography to obtain crystals of the endo (**12**) form and the exo (**13**) form of 3',4'-*O*-benzylidenechartreusin. Both isomers were recrystallized from a mixture of chloroform and ethanol, and 4.8 g of the endo form and 2.7 g of crystals of the exo form were obtained.

3',4'-*O*-Endo-benzylidenechartreusin (**12**): MP 262.0~266.5°C; ^{1}H NMR ($CDCl_3$) δ 1.01 (3H, d, J=6.8 Hz, 5''-CH_3), 1.41 (3H, d, J=6.6 Hz, 5'-CH_3), 2.16 (1H, br s, 4''-OH), 2.32 (1H, d, J=8.4 Hz, 2''-OH), 2.79 (3H, s, Ar-CH_3), 3.34 (3H, s, 3''-OCH_3), 3.35 (1H, dd, J=9.8 and 3.2 Hz, 3''-H), 3.75 (1H, ddd, J=9.8, 8.4 and 3.9 Hz, 2''-H), 3.77 (1H, br s, 4''-H), 4.07 (1H, dq, J=2.4 and 6.6 Hz, 5'-H), 4.12 (1H, q, J=6.8 Hz, 5''-H), 4.15 (1H, dd, J=5.8 and 2.4 Hz, 4'-H), 4.25 (1H, dd, J=7.6 and 7.2 Hz, 2'-H), 4.39 (1H, dd, J=7.2 and 5.8 Hz, 3'-H), 5.24 (1H, d, J=7.6 Hz, 1'-H), 5.76 (1H, d, J=3.9 Hz, 1''-H), 6.35 (1H, s, PhCHO-), 7.31~8.19 (5H, m, aromatic protons), 11.53 (1H, s, Ar-OH).

Anal Calcd for $C_{39}H_{36}O_{14}$: C 64.28, H 4.98.
Found: C 64.03, H 5.03.

3',4'-*O*-Exo-benzylidenechartreusin (**13**): MP 165.0~200.0°C; ^{1}H NMR ($CDCl_3$) δ 1.29 (3H, d, J=6.8 Hz, 5''-CH_3), 1.46 (3H, d, J=6.4 Hz, 5'-CH_3), 2.19 (1H, d, J=2.0 Hz, 4''-OH), 2.26 (1H, d, J=8.3 Hz, 2''-OH), 2.87 (3H, s, Ar-CH_3), 3.39 (1H, dd, J=10.3 and 3.4 Hz, 3''-H), 3.40 (3H, s, 3''-OCH_3), 3.81 (1H, ddd, J=10.3, 8.3 and 3.9 Hz, 2''-H), 3.89 (1H, br s, 4''-H), 4.03 (1H, dq, J=2.0 and 6.4 Hz, 5'-H), 4.17 (1H, dd, J=5.4 and 2.0 Hz, 4'-H), 4.31 (1H, q, J=6.8 Hz, 5''-H), 4.41 (1H, dd, J=7.8 and 7.8 Hz, 2'-H), 4.58 (1H, dd, J=7.8 and 5.4 Hz, 3'-H), 5.28 (1H, d, J=7.8 Hz, 1'-H), 5.94 (1H, d, J=3.9 Hz, 1''-H), 6.35 (1H, s, PhCHO-), 7.37~8.28 (5H, m, aromatic protons), 11.64 (1H, s, Ar-OH).

Anal Calcd for $C_{39}H_{36}O_{14}$: C 64.28, H 4.98.
Found: C 64.04, H 4.95.

Measurements of the Differential NOEs (Determination of Structural Isomers)

Differential NOEs spectra were recorded on a Jeol JNM-GSX-400 spectrometer using a pre-settled pulse-sequence mode (NOEDIF): Compound **12** was dissolved in $CDCl_3$ so that a 0.03-M solution was obtained. The benzyl proton of the benzylidene group was observed at 5.93 ppm as a singlet peak. Irradiation at this signal led to a 6.0% enhancement of the 4'-proton (δ 4.15, dd, J=5.8 and 2.4 Hz) absorption. The measurements of the dif-NOEs on **13** and **2**~**7** were done by the same way as that of **12**. These irradiation peaks (acetal-proton) and the enhancements observed were follows: **13**, 6.35 ppm-14.0% (δ 4.41, dd, J=7.8 and 7.8 Hz, 2'-H); **2**, 5.33 ppm-4.5% (δ 4.07, dd, J=5.6 and 2.0 Hz, 4'-H); **3**, 5.05 ppm-7.6% (δ 3.99, dd, J=5.2 and 2.4 Hz, 4'-H); **4**, 5.09 ppm-6.5% (δ 3.98, dd, J=5.6 and 2.4 Hz, 4'-H); **5**, 5.15 ppm-4.5% (δ 4.06, dd, J=5.8 and 2.4 Hz, 4'-H); **6**, 5.15 ppm-6.1% (δ 4.06, dd, J=6.0 and 2.4 Hz, 4'-H); **7**, 5.15 ppm-7.4% (δ 4.06, dd, J=6.0 and 2.4 Hz, 4'-H).

Synthesis of 6-*O*-Acyl-3',4'-*O*-exo-benzylidene-chartreusin (**14**~**18** and **22**~**26**)

General Procedure for **14**~**18** and **22**~**26**: An appropriate carboxylic acid (0.15~0.3 mmol) and dicyclohexylcarbodiimide (0.2~0.3 mmol) were added to a solution of 3',4'-*O*-exo-benzylidenechartreusin (**13**: 0.1 mmol) in absolute chloroform (1.0 ml) and pyridine (1.0 ml), and the resulting mixture was stirred at room temperature for 3~20 hours. After completion of the reaction, a small amount of methanol (*ca.* 0.05~0.1 ml) and ethyl acetate (3~5 ml) were added, and the mixture was stirred for about 0.5 hour (urea was precipitated). The resulting suspension was filtered through Celite, and the filtrate was concentrated under reduced pressure to obtain crude product, which was directly purified by preparative TLC ($CHCl_3$ - MeOH, 30 : 1, multiple development) to give the desired product in 80~95% yield.

6-*O*-(3-Methylbutyryl)-3',4'-*O*-exo-benzylidenechartreusin (**14**): MP 194.0~204.0°C; ^{1}H NMR ($CDCl_3$) δ 1.17 (3H×2, d, J=7 Hz), 1.32 (3H, d, J=7 Hz), 1.50 (3H, d, J=7 Hz), 2.92 (3H, s), 3.46 (3H, s), 5.33 (1H, d, J=8 Hz), 5.96 (1H, d, J=4 Hz), 6.40 (1H, s).

Anal Calcd for $C_{44}H_{44}O_{15}$: C 65.02, H 5.46.
Found: C 65.29, H 5.49.

6-*O*-(3-Acetopropionyl)-3′,4′-*O*-exo-benzylidenechartreusin (**15**): MP 178.0~186.0°C; ^{1}H NMR ($CDCl_3$) δ 1.28 (3H, d, J=7 Hz), 1.46 (3H, d, J=7 Hz), 2.20 (3H, s), 2.81 (3H, s), 3.35 (3H, s), 5.25 (1H, d, J=8 Hz), 5.87 (1H, d, J=4 Hz), 6.29 (1H, s).

Anal Calcd for $C_{44}H_{42}O_{16}$: C 63.92, H 5.12.
Found: C 63.77, H 5.20.

6-*O*-Acetoxyacetyl-3′,4′-*O*-exo-benzylidenechartreusin (**16**): MP 156.0~163.0°C; ^{1}H NMR ($CDCl_3$-CD_3OD) δ 1.30 (3H, d, J=7 Hz), 1.48 (3H, d, J=7 Hz), 2.25 (3H, s), 2.91 (3H, s), 3.40 (3H, s), 5.17 (2H, s), 5.36 (1H, d, J=8 Hz), 5.92 (1H, d, J=4 Hz), 6.37 (1H, s).

Anal Calcd for $C_{43}H_{40}O_{17}$: C 62.32, H 4.86.
Found: C 63.45, H 4.99.

6-*O*-[2-Methyl-3-(trifluoroacetylamino)propionyl]-3′,4′-*O*-exo-benzylidenechartreusin (**17**): MP 163.0~173.0°C; ^{1}H NMR ($CDCl_3$) δ 1.30 (3H, d, J=7 Hz), 1.48 (3H×2, d, J=7 Hz), 2.87 (3H, s), 3.37 (3H, s), 5.26 (1H, d, J=8 Hz), 5.89 (1H, d, J=4 Hz), 6.33 (1H, s).

Anal Calcd for $C_{45}H_{42}NO_{16}F_3$: C 59.41, H 4.65, N 1.54, F 6.26.
Found: C 59.41, H 4.89, N 1.65, F 6.40.

6-*O*-[(8-Trifluoroacetylamino)octanoyl]-3′,4′-*O*-exo-benzylidenechartreusin (**18**): MP 173.0~181.0°C; ^{1}H NMR ($CDCl_3$-CD_3OD) δ 1.12~2.00 (m), 2.87 (3H, s), 3.38 (3H, s), 5.33 (1H, d, J=8 Hz), 5.90 (1H, d, J=4 Hz), 6.33 (1H, s).

Anal Calcd for $C_{49}H_{50}NO_{16}F_3$: C 60.93, H 5.22, N 1.45, F 5.90.
Found: C 60.83, H 5.13, N 1.46, F 6.19.

6-*O*-(2-Furoyl)-3′,4′-*O*-exo-benzylidenechartreusin (**22**): MP 190.0~195.0°C; ^{1}H NMR ($CDCl_3$-CD_3OD) δ 1.28 (3H, d, J=7 Hz), 1.45 (3H, d, J=7 Hz), 2.79 (3H, s), 3.30 (3H, s), 5.24 (1H, d, J=8 Hz), 5.79 (1H, d, J=4 Hz), 6.24 (1H, s).

Anal Calcd for $C_{44}H_{38}O_{16}$: C 64.23, H 4.65.
Found: C 64.12, H 4.65.

6-*O*-(*N*-Methyl-2-pyrroyl)-3′,4′-*O*-exo-benzylidenechartreusin (**23**): MP 181.0~185.0°C; ^{1}H NMR ($CDCl_3$) δ 1.30 (3H, d, J=7 Hz), 1.46 (3H, d, J=7 Hz), 2.85 (3H, s), 3.40 (3H, s), 3.93 (3H, s), 5.33 (1H, d, J=8 Hz), 5.92 (1H, d, J=4 Hz), 6.20 (1H, s).

Anal Calcd for $C_{45}H_{41}NO_{15}$: C 64.67, H 4.94, N 1.68.
Found: C 64.51, H 4.88, N 1.70.

6-*O*-(3-Indolylcarbonyl)-3′,4′-*O*-exo-benzylidenechartreusin (**24**): MP 198.0~204.0°C; ^{1}H NMR ($CDCl_3$) δ 1.27 (3H, d, J=6 Hz), 1.42 (3H, d, J=6 Hz), 2.84 (3H, s), 3.36 (3H, s), 5.19 (1H, d, J=8 Hz), 5.90 (1H, d, J=4 Hz), 6.21 (1H, s).

Anal Calcd for $C_{48}H_{40}NO_{15}$: C 66.20, H 4.63, N 1.61.
Found: C 66.14, H 4.66, N 1.64.

6-*O*-(3-Ethoxypropionyl)-3′,4′-*O*-exo-benzylidenechartreusin (**25**): MP 189.5~192.0°C; ^{1}H NMR ($CDCl_3$) δ 1.29 (3H, t, J=7 Hz), 1.30 (3H, d, J=6 Hz), 1.46 (3H, d, J=6 Hz), 2.89 (3H, s), 3.15 (2H, t, J=6 Hz), 3.41 (3H, s), 3.64 (2H, q, J=7 Hz), 3.95 (2H, t, J=6 Hz), 5.28 (1H, d, J=8 Hz), 5.91 (1H, d, J=4 Hz), 6.36 (1H, s).

Anal Calcd for $C_{44}H_{44}O_{16}$: C 63.76, H 5.35.
Found: C 63.58, H 5.30.

6-*O*-Propionyl-3′,4′-*O*-exo-benzylidenechartreusin (**26**): MP 198.0~202.0°C; ^{1}H NMR ($CDCl_3$) δ 1.37 (3H, d, J=6 Hz), 1.47 (3H, t, J=7 Hz), 1.53 (3H, d, J=7 Hz), 2.96 (3H, s), 3.01 (2H, q, J=7 Hz), 3.48 (3H, s), 5.35 (1H, d, J=8 Hz), 6.01 (1H, d, J=4 Hz), 6.43 (1H, s).

Anal Calcd for $C_{42}H_{40}O_{15}$: C 64.28, H 5.14.
Found: C 64.12, H 5.16.

Synthesis of 6-*O*-(*N*,*N*-Dimethylglycyl)-3',4'-*O*-exo-benzylidenechartreusin Hydrochloride (**19**)

3',4'-*O*-Exo-benzylidenechartreusin (**13**: 3.64 g, 5.00 mmol) was added to a solution of *N*,*N*-dimethylglycine hydrochloride (767 mg, 5.50 mmol), trimethylamine (556 mg, 5.50 mmol) and dicyclohexylcarbodiimide (1.36 g, 6.60 mmol) in absolute chloroform (40 ml) and pyridine (20 ml), and the resulting mixture was stirred at room temperature for 3.5 hours. After completion of the reaction, methanol (*ca.* 0.5 ml) and ethyl acetate (*ca.* 40 ml) were added, and the mixture was stirred for 0.5 hour. The resulting suspension was filtered through Celite, and the filtrate was concentrated under reduced pressure to obtain crude crystals, which were recrystallized from a mixture of chloroform and ethyl acetate to give 6-*O*-(*N*,*N*-dimethylglycyl)-3',4'-*O*-exo-benzylidenechartreusin (2.7 g, **13** and urea was contained). The obtained crystals were further purified by the following partition extract method: The crystals were dissolved in chloroform (300 ml), then the solution was washed with water (100 ml, twice), dil hydrochloric acid (*ca.* 0.005 N: 100 ml), water (100 ml) and brine (100 ml), then dried and concentrated at 5~20°C to obtain crude crystals (pyridine free), which were dissolved in 0.1 N hydrochloric acid (40 ml) and water (260 ml). The resulting solution was filtered through a glass filter to give a clear solution (urea free), which was washed with ethyl acetate (100 ml, three times). After the addition of saturated sodium bicarbonate and brine, the washed solution (**13** free) was extracted with chloroform. The extract was washed with water and brine, dried and concentrated to afford crystals, which were recrystallized from chloroform-ethyl acetate to give pure 6-*O*-(*N*,*N*-dimethylglycyl)-3',4'-*O*-exo-benzylidenechartreusin (2.26 g, 2.78 mmol) in 56% yield: MP 157.0~161.0°C; ^{1}H NMR ($CDCl_3$) δ 1.32 (3H, d, J=6 Hz), 1.50 (3H, d, J=6 Hz), 2.58 (3H×2, s), 2.90 (3H, s), 3.43 (3H, s), 5.30 (1H, d, J=8 Hz), 5.93 (1H, d, J=4 Hz), 6.37 (1H, s).

The above 6-*O*-(*N*,*N*-dimethylglycyl)-3',4'-*O*-exo-benzylidenechartreusin (2.15 g, 2.65 mmol) was dissolved in 0.1 N hydrochloric acid (26.5 ml, 2.65 mmol) and distilled water (*ca.* 50 ml), and the resulting solution was freeze-dried to give 6-*O*-(*N*,*N*-dimethylglycyl)-3',4'-*O*-exo-benzylidenechartreusin hydrochloride (**19**: 2.19 g): MP 152.0~157.0°C.

Anal Calcd for $C_{43}H_{44}NO_{16}Cl$: C 60.74, H 5.22, N 1.65, Cl 4.17.
Found: C 60.47, H 5.48, N 1.59, Cl 3.88.

Synthesis of 6-*O*-(*N*-Methyl-β-alanyl)-3',4'-*O*-exo-benzylidenechartreusin Hydrochloride (**20**)

5% Palladium-carbon (70 mg: 50% wet) was added to a solution of 6-*O*-(*N*-benzyloxycarbonyl-*N*-methyl-β-alanyl)-3',4'-*O*-exo-benzylidenechartreusin (146 mg, 0.15 mmol): MP 175.0~178.0°C; ^{1}H NMR ($CDCl_3$) δ 1.34 (3H, d, J=7 Hz), 1.51 (3H, d, J=7 Hz), 2.92 (3H, s), 3.08 (3H, s), 3.43 (3H, s), 5.22 (2H, s), 5.29 (1H, d, J=8 Hz), 5.95 (1H, d, J=4 Hz), 6.40 (1H, s); which was obtained in the same manner as mentioned in the general procedure for **14**~**18** and **22**~**26**, in 0.1 N hydrochloric acid (1.5 ml) and tetrahydrofuran (3.0 ml). The resulting suspension was stirred under hydrogen atmosphere at 4°C for 2 hours, then filtered into water, and the filtrate was washed with ethyl acetate. The resulting solution was freeze-dried to give 6-*O*-(*N*-methyl-β-alanyl)-3',4'-*O*-exo-benzylidenechartreusin hydrochloride (**20**: 111 mg, 0.13 mmol) in 85% yield: MP 169.0~173.0°C.

Anal Calcd for $C_{43}H_{44}NO_{15}Cl$: C 60.74, H 5.22, N 1.65, Cl 4.17.
Found: C 60.48, H 5.51, N 1.58, Cl 4.35.

Synthesis of 6-*O*-(3-Pyrrolidinopropionyl)-3',4'-*O*-exo-benzylidenechartreusin Hydrochloride (**21**)

3-Pyrrolidinopropionic acid (240 mg, 1.68 mmol) and dicyclohexylcarbodiimide (440 mg, 2.14 mmol) were added to a solution of 3',4'-*O*-exo-benzylidenechartreusin (**13**: 400 mg, 0.55 mmol) in absolute chloroform (2.7 ml) and pyridine (5.5 ml), and the resulting mixture was stirred at room temperature for 7 hours. After completion of the reaction, 6-*O*-(3-pyrrolidinopropionyl)-3',4'-*O*-exo-benzylidenechartreusin hydrochloride (**21**: 232 mg, 0.26 mmol) was obtained in 48% yield by the same manner as the synthetic procedure of **19**: MP 154.5~160.0°C.

Anal Calcd for $C_{46}H_{48}NO_{15}Cl$: C 62.06, H 5.43, N 1.57, Cl 3.98.
Found: C 61.92, H 5.55, N 1.55, Cl 4.11.

Biological Methods

Animals: Inbred DBA/2 mice and C57BL/6j mice, and C57BL/6j×DBA/2F$_1$ (BDF$_1$) mice, 4~5 weeks old, obtained from Charles River Japan, Inc., Kanagawa, Japan, were used throughout the

experiments.

Tumors: P388 Leukemia of DBA/2 mice, L1210 leukemia of DBA/2 mice and B16 melanoma of C57BL/6j mice were supplied by Dr. T. TASHIRO, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research. P388 and L1210 Leukemia cells were maintained as an ascite tumor in male DBA/2 mice by weekly transfer. B16 Melanoma cells were maintained serially by subcutaneous transplantation in male C57BL/6j mice.

Tests: P388 Leukemia cells (10^6) were implanted ip in male BDF_1 mice on day 0. L1210 Leukemia cells (10^5) were implanted ip in male BDF_1 mice on day 0. B16 Melanoma (1 g) was mixed with 10 ml of cold balanced salt solution and homogenized. This tumor homogenate (0.5 ml/mouse) was implanted ip in male BDF_1 mice on day 0. The drug administrations were started on day 1 on an appropriate schedule (six mice/test group). Percent T/C was based on median survival time.

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