Bioactive Metabolites of EM574 and EM523, Erythromycin Derivatives Having Strong Gastrointestinal Motor Stimulating Activity

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Two motilides, EM574 (*N*-demethyl-*N*-isopropyl-8,9-anhydroerythromycin A 6,9-hemiacetal) and EM523 (*N*-demethyl-*N*-ethyl-8,9-anhydroerythromycin A 6,9-hemiacetal) have strong gastrointestinal motor stimulating (GMS) activity. When administered orally to dogs, these agents showed strong GMS activity, but their plasma levels were very low and the metabolites which have been determined so far using the radio-labeled compounds show only weak GMS activity. The findings suggested that unknown bioactive metabolites might be responsible for the GMS activity.

From the liver of dogs given EM574 intravenously, two bioactive metabolites, EM574 P1 and P2, were isolated by solvent extraction and chromatography as detected by contractile activity. They both showed the same UV spectra as EM574 and the molecular ion peaks at *m/z* 760 (MH^+) and 602 (MH^+ − cladinose) in the FAB-MS. From 2D-NMR experiments, the structures of EM574 P1 and P2 were unveiled to be the 15- and 14-hydroxyl derivatives of EM574, respectively. EM523 P1 and P2 were also isolated in the same procedure. In order to prepare these bioactive metabolites, EM574 and EM523 were converted enzymatically with dog liver homogenates in the presence of co-enzymes to give the corresponding P1 and P2. The structures of the metabolites are shown in Fig 1. They exhibited stronger contractile activity *in vitro* and GMS activity *in vivo* than the parent compounds.

Recently, erythromycin (EM) has been reported to have GMS activities other than antibacterial activities.[1~4] Firstly EM was found to induce strong migrating contractions, of which pattern was similar to that of naturally occuring interdigestive migrating contractions in dogs.[1,2] As the action of EM was very similar to that of the gastrointestinal hormone motilin, ITOH and ŌMURA[5,6] designated macrolides having motilin-like activities as "motilides." Later studies have provided evidence that EM is an agonist on motilin receptors.[7~9] These evidences suggested that EM derivatives having stronger GMS activity without antimicrobial activities are promising new prokinetic drugs.

In the course of developing EM derivatives without antibacterial activity, EM574 (**1**) and EM523 (**2**) were found to have 248- and 18-fold stronger GMS activity than EM A, respectively, when given intravenously.[10,11] Although both agents showed strong GMS activity upon oral or intraduodenal administration, the plasma levels of the agents were very low. These findings suggested that bioactive metabolites might be responsible for the GMS activity.

In a metabolic experiment using the *N*-[^{14}C]Me derivative of **2**, *N*-demethyl (M-I), *N*-oxide (M-II), 3-*O*-decladinosyl (M-III) and 6,9:9,12-spiroketal[12] (M-VI) derivatives were identified as the metabolites of **2**. On the other hand, **1** and **2** are easily translactonized into pseudo[13]-erythromycins (pseudo-**1** and pseudo-**2**) in neutral or weak alkaline solutions such as diluted sodium bicarbonate as in the case with EM A. However,

M-I, M-II, M-III, M-VI, pseudo-**1** and pseudo-**2** prepared chemically, showed very weak or no GMS activity. None of the proposed metabolites had enough activity to explain the strong GMS activity in dogs. These observations prompted us to search for genuine "bioactive metabolites." The part of this paper has been orally reported.[14]

Results

Detection of Bioactive Metabolites in Dogs

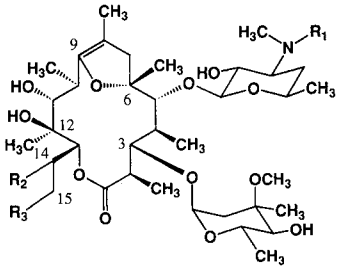
First, to determine whether bioactive metabolites were produced after the administration of **1** and **2** to the dog, we examined the contractile activity *in vitro* using an isolated rabbit duodenum. A solution of **1** lactobionate (**1**L) was injected intravenously *via* a cephalic vein in the foreleg of the dog. After 0, 5, 15 and 30 minutes, a blood sample was removed from the vein. One hour after injection, the dog was killed; blood was collected, and the liver was removed. In another experiment, urine and bile were collected *via* a cannula inserted into the bladder and bile duct, respectively, for 4 hours after injection. The liver was homogenized in methanol and centrifuged.

The contractile activity of these samples was examined to check for the presence of bioactive metabolites. All the samples showed contractile activity, but they were too complex to analyze the quantity of the remaining **1**. As a trial for preliminary purification, the bile was subjected to an adsorptive resin, Diaion HP-20, chromatography eluting with aqueous methanol. Contractile activity was detected in the fractions of 80% methanol in 5 mM hydrochloric acid. The bile was partitioned between ethyl acetate and water (pH 3, 7 and 9). The portions showing contractile activity were the acidic aqueous layer and the organic layer at pH 9. These behavior of the active fraction indicated that the bioactive metabolites have a basic, fat-soluble nature, as in the case with the starting materials, **1** and **2**. On the basis of these results, the other samples were partially purified using a series of HP-20 chromatography and solvent extraction at pH 9 to obtain the crude extracts as shown in Fig. 2. The contractile activity was examined and compared with that of **1**, and the extracts were subjected to analytical HPLC (ODS, 37% acetonitrile-0.02 M phosphate buffer, pH 4.0; solvent A) to quantify the amount of remaining **1**. The difference between the activity estimated as **1** (*b*) and the real amount of **1** (*c*) indicated the existence of bioactive metabolites. These results are shown in Table 1.

The activity observed in blood samples was mainly caused by the unchanged **1**, and the bioactive metabolites existed in extracts from liver, bile and urine.

The crude extract from the bile was subjected to preparative HPLC (ODS), and all the fractions were examined for contractile activity. In HPLC using 50% acetonitrile-0.02 M phosphate buffer (pH 4.0) as a mobile phase, the active fractions eluted within 3.5 minutes, and no activity was observed in the fractions eluted after **1**.

Fig. 1. Structures of EM574, EM523 and their metabolites.



Compound	R_1	R_2	R_3
EM574 (**1**)	i-Pr	H	H
EM523 (**2**)	Et	H	H
EM574 P1 (**3**)	i-Pr	H	OH
EM574 P2 (**4**)	i-Pr	OH	H
EM523 P1 (**5**)	Et	H	OH
EM523 P2 (**6**)	Et	OH	H

Fig. 2. Preparation of crude extracts.

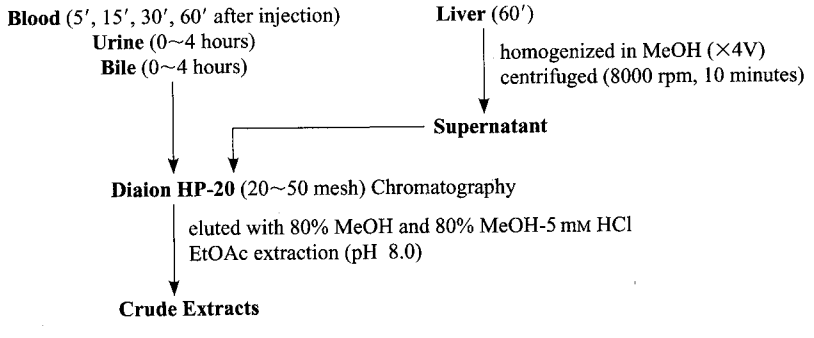


Table 1. Contractile activity of crude extracts.

Sample	Volume (a, ml)	Crude (mg)	Activity (b, μg/ml)	EM574 (c, μg/ml)	$b-c$ (μg/ml)	Active metabolites total (μg)$=a(b-c)$
Blood 5′	30	—	0.61	0.40	0.21	—
Blood 15′	30	—	0.22	0.22	0.0	—
Blood 30′	30	—	0.03	0.16	−0.13	—
Blood 60′	400	1.1	0.23	0.08	0.15	60
Liver 60′	270	14.4	0.99	0.30	0.69	186
Bile (0∼4 hours)	15	2.1	28.8	2.10	26.7	400
Urine (0∼4 hours)	14.5	3.0	144.8	20.7	124.1	1800

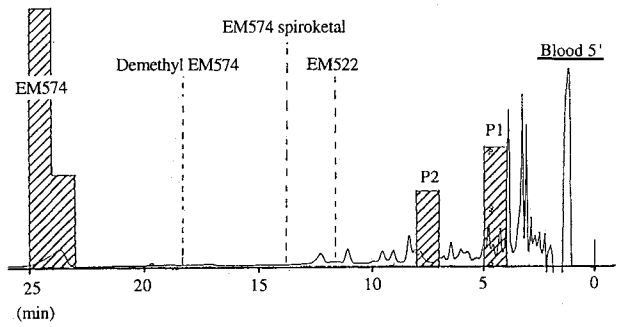
b; Contractile activity *in vitro* expressed as amount of **1**.
c; Estimated by HPLC analysis (solvent A).

Table 2. HPLC(ODS) preparation of crude extracts.

Sample	Solvent	Recovery (%)	Component ratio (%)			
			P1	P2	EM574	Others
Blood 5′	B	11.5	21.7	13.6	64.8	—
Blood 15′	B	12	25.6	12.0	62.4	—
Blood 30′	B	60	36.3	11.7	52.0	—
Liver 60′	C	91	38.6	60.9	—	—
Urine	B	18.4	14	61	26	—
Bile	B	55	28.2	46.1	21.7	3.8

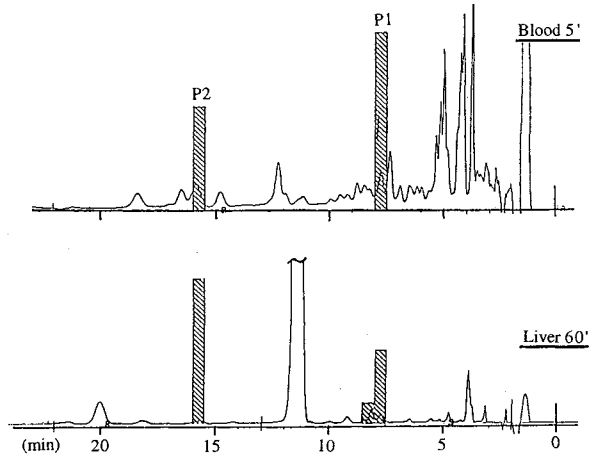
Fig. 3. Behavior of active fraction upon HPLC analysis.

(a) 32% Acetonitrile-0.02 M phosphate buffer (pH 4.0)



EM522; *N*-Demethyl-8,9-anhydroerthromycin A 6,9-hemiacetal

(b) 28% Acetonitrile-0.02 M phosphate buffer (pH 4.0)



Chromatogram; UV abs. 214 nm
Histogram; contractile activity *in vitro*

Next, 32% acetonitrile-0.02 M phosphate buffer (pH 4.0), solvent B, was employed as a mobile phase. The active fractions eluted 4∼5 and 7∼8 minutes after injection while those corresponding to **1** eluted 23∼25 minutes after injection. Other extracts were analyzed in the same manner showing nearly the same HPLC pattern as the bile extract as listed in Table 2. In order to analyze the active fractions in detail, 28% acetonitrile-0.02 M phosphate buffer (pH 4.0), solvent C, was used. Comparing the active fractions and the absorption peaks detected at UV 214 nm, the peaks for retention time 7.8 and 15.8 minutes corresponded to the contractile activity. The bioactive components were designated P1 and P2, respectively. The HPLC patterns of the crude extracts from blood and liver are shown in Fig. 3. The extracts from the liver, bile and urine contained P1 and P2.

Isolation of Bioactive Metabolites

A procedure for isolation of EM574 P1 (**3**) and P2 (**4**) is shown in Fig. 4. Compound **1L** was administered (10 mg/kg, iv) to dogs. After 30 minutes, the dogs were killed and the livers were removed. They were homogenized in methanol and centrifuged. The supernatant was purified according to the method described above using a series of HP-20 chromatography and solvent extraction at pH 8.5. The extracts was subjected to preparative HPLC (ODS) using solvent C as a mobile phase to give the fractions containing P1 and P2. They were extracted with ethyl acetate at pH 8.5 to afford **3** and **4**, respectively. The amount of the metabolite was too small to be weighed; therefore, the weight was estimated by integration of the peak area detected at UV 214 nm on HPLC analysis upon the hypothesis that

Fig. 4. Procedure for isolation of EM574 metabolites.

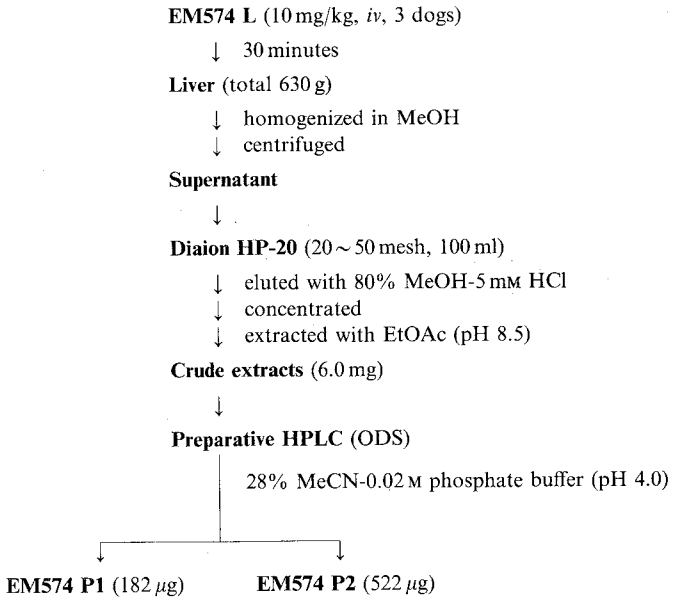
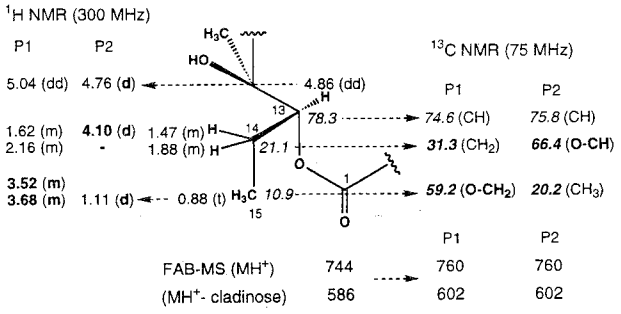


Fig. 5. Spectroscopic comparison of EM574 P1, P2 and EM574.



both metabolites have the same UV absorption pattern.

The extracts from urine were purified by HPLC in the same manner to afford **3** and **4**, respectively.

Structures of Bioactive Metabolites

The bioactive metabolites, **3** and **4**, showed single peaks and the same UV spectra as **1** on three dimensional HPLC analysis. In the FAB-MS analysis, they both showed *m/z* 760 (MH^+) and 602 (MH^+ −cladinose). These data indicate that the metabolites are oxidized derivatives of **1**. When the ^1H NMR spectra of **3** and **4** were compared with that of **1**, major differences were observed only in the signals corresponding to the ethyl group (C13, 14 and 15). In the ^1H-^1H COSY analysis, **3** had no 15-methyl group but had methylene protons (δ 3.5~3.8 ppm), which were regarded as those neighboring to *O*-atom. The metabolite **4** showed a doublet 15-methyl signal instead of the triplet methyl signal, and the 13-methine proton was observed as a doublet signal. A comparison of the spectral data for **3**, **4** and **1** is presented in Fig. 5. These results indicated that **3** and **4** are the oxidized derivatives of **1** at position 15 and 14, respectively.

The lactobionate of **2** (**2L**) was treated in the same manner to obtain the corresponding EM523 P1 (**5**) and EM523 P2 (**6**). Their structures were also elucidated by spectral analysis and found to be the 15- and 14-hydroxyl derivatives of **2**, respectively, as shown in Fig. 1. The complete assignment of ^{13}C NMR signals is shown the accompanying paper.[15]

Preparation of the Bioactive Metabolites with Dog Liver Homogenates

The amounts of the bioactive metabolites obtained were too small to investigate the biological properties and the physico-chemical data were not sufficient to confirm the structures. Therefore, oxidation of **1** and **2** with mammalian liver homogenates was examined. In the first trial, **1** was incubated in dog liver homogenate for 1 hour, but it remained unchanged in the homogenate.

In general, enzymatic oxidation with mammalian liver homogenate requires NADPH as the co-enzyme along with enzyme, substrate and inorganic salts. Therefore, NADP and D-glucose-6-phosphate (G6P) with G6P dehydrogenase (for NADP reduction) were employed as the *in situ* NADPH supplying system.[16]

Table 3. Conversion with dog liver homogenate.

Substrate	Conversion rate (%)	Yield (mg)		
		P1	P2	Substrate recovery**
EM574 (150 mg)	54	5.8 (3.6%)	6.2* (3.0%)	69.0 (46.0%)
(560 mg)	70	51.0 (13.0%)	27.0 (7.0%)	110.0 (30.0%)
EM523 (67 mg)	70	2.8 (3.8%)	4.7 (7.0%)	19.9 (30.0%)
(65 mg)	70	2.8 (4.2%)	5.0 (7.5%)	20.0 (30.0%)

*Purity 73%, **not isolated, estimated by HPLC (solvent A).

Table 4. Pharmacological activities of EM574, EM523 and their metabolites.

Compound	Contractile activity (EC_{50}, nM)	GMS activity (ED_{200} μg/kg, *iv*)
EM574 (**1**)	1.6	0.96
EM523 (**2**)	3.6	6.1
EM574 P1 (**3**)	2.6	0.38
EM574 P2 (**4**)	1.3	0.40
EM523 P1 (**5**)	2.7	4.0
EM523 P2 (**6**)	3.6	2.1

The liver homogenates were prepared as follows. The liver was homogenized in phosphate buffer (pH 7.4) with a Potter-Elvehjem homogenizer. The homogenate itself could be used as the enzyme fluid, but in order to avoid any contamination, the homogenate was centrifuged, and the supernatant was used for the enzymatic reaction.

In the preliminary experiment, the substrate (**1**L) concentration was 100 μg/ml, and the reaction temperature was 37°C. The reaction process was monitored by HPLC analysis to detect the remaining substrate in the reaction mixture. As the result, 84% of **1** was consumed during the first hour of the reaction. The procedure for isolation of the metabolites from dog liver was applicable to this enzymatic conversion. The reaction mixture was purified by HP-20 chromatography and preparative HPLC (ODS) to afford **3** and **4**. Compounds **5** and **6** were prepared in the same manner from **2**. The conversion yields of P1 and P2 are summarized in Table 3.

Biological Activity

The contractile activity in isolated rabbit duodenum (*in vitro*) and GMS activity (*in vivo*) of the obtained metabolites are summarized in Table 4.

Discussion

Here we have described the isolation, structure determination and the preparation of 15- and 14-hydroxyl derivatives of EM574 (**1**) and EM523 (**2**) as the genuine bioactive metabolites. All the metabolites showed activity comparable to that of the parent compound in the *in vitro* assay. On the other hand, they exhibited 2- to 3-fold stronger GMS activity than **1** and **2** *in vivo*. In the metabolic experiments, bioactive metabolites **3** and **4** were detected in the plasma after oral administration of [^{14}C]-labeled **1** to dogs;[14] therefore, the metabolites **3** and **4** are designated as M-IV and M-V, respectively. These observations indicate that these metabolites, rather than the parent compound, are responsible for the majority of the GMS activity. Actually, **1** is undergoing clinical trials for treatment of chronic gastritis and the metabolites M-IV and -V are detected in blood of human treated. The pharmacokinetical study of these metabolites is in progress.

Experimental

Contractile Activity Using Isolated Rabbit Duodenum *In Vitro*[17]

Male New Zealand white rabbits weighing about 3 kg were killed by injecting air into a vein, and the duodenum was removed. A preparation of about 2 cm was mounted along the longitudal axis in an organ bath containing 20 ml of tyrode solution which was kept at 37°C and aerated continuously with a gas of 5% CO_2 in oxygen. Each preparation was loaded with a tension of 1.0 g, and isotonic contractions were recorded on a recorder (Recti-Horiz-8K, NEC San-ei, Japan) by means of an isotonic transducer and amplifier (ME-4012, NEC San-ei). After a stabilization period, each compound was added cumulatively to the organ bath. Test compounds were dissolved in EtOH, and lactobionic acid (1 mg/mg of test compound) was added. The resulting solution was diluted with saline. Contractions were expressed as a percentage of that induced by 10^{-4} M acetylcholine, and the EC_{50} values for the test compounds were obtained.

GMS Activity *In Vivo*[18]

Male beagle dogs weighing about 10 kg were subjected to laparotomy under pentobarbital anesthesia, and a strain gauge force transducer (F-12 IS-60, Star Medical, Japan) was sutured onto the serosa of the antrum. A

Silastic tube (Dow Corning) was inserted into the superior vena cava through a branch vein of the right external jugular vein, and the outer end was sutured onto the adjacent skin. This tube was used as a route for intravenous injection of test compounds. The dogs were used for the experiment more than 2 weeks after the operation. A lead wire of the strain gauge force transducer was connected to a recorder through an amplifier. The gastric motor activity was recorded on a polygraph (FWR3701, Graphtech, Japan). Solubilized test materials solution prepared as described above was administered intravenously 15 minutes after the spontaneous interdigestive migrating contractions (IMC) in the stomach terminated. To measure the motility quantitatively, the signals from the stomach were put into a signal processor (7T18, NEC San-ei) every 100 msec. The motor index of contractions induced by the test compounds was calculated according to the procedures described previously.[18] The area of contractions induced by the test compounds was calculated and expressed as a percentage of the area assuming that the maximum contraction of IMC lasted for 1 minute. The dose inducing a contraction area of 200% was determined from dose-response curves. GMS activity was expressed as ED_{200} (μg/kg).

Measurement of Physico-chemical Data

The UV spectra were measured at 23~28°C in methanol. The FAB-MS spectra were measured on a Shimadzu 9020-DF spectrometer equipped with a FAB ion source and a computer system. The δ-values in the NMR spectra were recorded in ppm downfield from tetramethylsilane (TMS) using a Bruker AC-300 spectrometer.

Isolation of Bioactive Metabolites from Dog Liver

Compound **1L** (10 mg/ml, solution in saline) was injected intravenously into three dogs (weight, 7.0~8.0 kg) in a dose of 10 mg/kg. After 30 minutes, they were killed and exsanguinated. The livers (weight, 200~220 g, total 630 g) were then immediately excised and homogenized in MeOH (2.5 liters) at ice-cooled temperature with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 8000 rpm for 10 minutes, and the supernatant was concentrated to remove the solvents. The remainder (400 ml) was adsorbed on Diaion HP-20 (20~50 mesh, 100 ml, Mitsubishi Chem. Ind., Japan), washed with water, 50% MeOH aq., 80% MeOH aq. (500 ml each) and eluted with 80% MeOH in 5 mM HCl (400 ml). The eluate was concentrated to about 10 ml, adjusted to pH 8.5 and extracted with EtOAc (10 ml) three times. The organic layer (30 ml) was washed with water, dried over anhydrous sodium sulfate and evaporated to give a crude powder (6.0 mg). This powder was subjected to preparative HPLC (ODS; YMC-Pack AM-324, YMC, Japan, solvent C) to give fractions containing **3** and **4**. These were independently concentrated to about 10 ml, adjusted to pH 8.5 and extracted with EtOAc (10 ml) two times. The organic layers were washed with saline, dried over anhydrous sodium sulfate and evaporated to give powders of **3** (182 μg) and **4** (522 μg).

FAB-MS: **3**, **4**; 760 (MH^+), 602 (MH^+ − cladinose).

Isolation of Bioactive Metabolites from Dog Urine

Compound **1L** (10 mg/ml, solution in saline) was injected intravenously into a dog (weight, 9.6 kg) in a dose of 10 mg/kg. Urine was then collected for 4 hours after injection. The urine (14.5 ml) was purified with HP-20 chromatography and preparative HPLC in the same manner as described above to give powders of **3** (45 μg) and **4** (71 μg).

FAB-MS: **3**, **4**; 760 (MH^+), 602 (MH^+ − cladinose).

Preparation of Dog Liver Homogenate

A beagle dog (weight, 8.0 kg) was killed and exsanguinated. The liver (weight, 216 g) was immediately excised, cut into small pieces (*ca.* 1 cm^3, cubes) and washed with saline at ice-cooled temperature. The cubes (*ca.* 10 g) were homogenized in 10 mM potassium-sodium phosphate buffer (pH 7.4, 90 ml) containing 1.15% potassium chloride with a Potter-Elvehjem homogenizer (cylinder volume, 100 ml). The homogenate (2.1 liters) was centrifuged at 8000 rpm for 10 minutes, and the supernatant (2.0 liters) was used for the enzymatic reaction.

Preparation of **3** and **4** with Dog Liver Homogenates (run 1)

The supernatant (1.0 liter) of the dog liver homogenate prepared as described above was dispensed in 333-ml portions into 1-liter Erlenmeyer flasks. Under ice-cooling, nicotinamide (1.0 M aq., 1.5 ml), $MgCl_2$ (1.0 M aq., 0.75 ml), G6P (255 mg), NADP (34 mg), G6P dehydrogenase (G6P-DH, 100 unit/ml, 150 μl) and **1L** (10 mg/ml in water, 7.5 ml) were added in turn to each of the flask and mixed gently. The flasks were stoppered with urethane plugs and shaken at 37°C for 2 hours. The reaction mixtures were combined, adjusted to pH 5.4 and washed with EtOAc-hexane (2:1, 900 ml). The aqueous layer was mixed with NaCl (100 g), adjusted to pH 8.5 and extracted with EtOAc (500 ml) three times. The EtOAc layers were combined, washed with 10% NaCl aq. (500 ml), dried over anhydrous sodium sulfate and evaporated to give a crude syrup (204 mg). This was dissolved in MeOH (1.0 ml) and subjected to preparative HPLC (YMC-Pack D-ODS-5, solvent C, flow rate 10 ml/minute) to give fractions containing **3** and **4**. These were independently adjusted to pH 7.4, concentrated to about 10 ml, mixed with NaCl (2.0 g), adjusted to pH 8.5 and extracted with EtOAc (8 ml) three times. The organic layers were washed with saline, dried over anhydrous sodium sulfate and evaporated to give powders of **3** (5.8 mg) and **4** (6.2 mg, total yield, 7.8%).

UV: **3**, λ_{max} 210 nm (ε 7600), **4**, λ_{max} 210 nm (ε 8000),
IR: **3**, 3430, 2970, 2940, 1730, 1640, 1460, 1380, 1200,

1170, 1050, 1010, **4**, 3440, 2970, 2930, 1730, 1640, 1460, 1380, 1200, 1170, 1050, 1010 cm^{-1}.

Preparation of **3** and **4** with Dog Liver Homogenates (run 2)

To the supernatant (2.15 liters) of the dog liver homogenate prepared as described above were added nicotinamide (1.0 M aq., 10.8 ml), $MgCl_2$ (1.0 M aq., 5.4 ml), G6P (1.83 g), NADP (0.48 g), G6P-DH (100 unit/ml, 1.08 ml) and **1L** (560 mg) in turn under ice-cooling. The mixture was mixed gently and dispensed in about 350-ml portions into 1-liter Erlenmeyer flasks. The flasks were stoppered with urethane plugs and shaken at 37°C for 2 hours. The reaction mixtures were combined, adjusted to pH 6.9, adsorbed onto HP-20 (20~50 mesh, 360 ml), washed with water and 50% and 80% MeOH aq. (1.5 liters, each) and eluted with 80% MeOH in 5 mM HCl aq. (3.0 liters). The eluate was neutralized, concentrated to *ca.* 200 ml, adjusted to pH 8.5 and extracted with EtOAc (100 ml) three times. The EtOAc layers were combined, washed with 10% NaCl aq. (100 ml), dried over anhydrous sodium sulfate and evaporated to give a crude syrup (350 mg). This was treated in the same manner as described above to give a crude powder of **3** (110 mg) and a pure powder of **4** (27 mg, yield 7.0%). The crude powder (110 mg) was purified by repeating preparative HPLC (D-ODS-5, mobile phase: 23%, 25% acetonitrile and 50% MeOH-0.02 M phosphate buffer; pH 4.0) to give powders of **3** (28 mg, purity 85% and 30 mg, purity 60%, yield of **3**, 13.0%).

Preparation of **5** and **6** with Dog Liver Homogenates

The supernatant (400 ml) of the dog liver homogenate prepared as described above was dispensed in 200-ml portions into two 1-liter Erlenmeyer flasks. Under ice-cooling, nicotinamide (1.0 M aq., 1.0 ml), $MgCl_2$ (1.0 M aq., 0.50 ml), G6P (170 mg), NADP (23 mg), G6P-DH (100 unit/ml, 100 ml) and **2L** (20 mg/ml in water, 2.5 ml) were added in turn to each flask and mixed gently. The flasks were stoppered with urethane plugs and shaken at 37°C for 2.5 hours. The reaction mixtures were treated in the same manner as described above to give a crude syrup (112 mg). This was dissolved in MeOH (1.3 ml) and subjected to preparative HPLC (D-ODS-5, solvent C, flow rate 10 ml/minute) to give fractions containing **5** and **6**. The former was adjusted to pH 7.4, concentrated to about 10 ml, mixed with NaCl (2.0 g), adjusted to pH 8.5 and extracted with EtOAc (8 ml) three times. The organic layers were washed with saline, dried over anhydrous sodium sulfate and evaporated to give a powder of **5** (2.8 mg). The latter was adjusted to pH 7.4, concentrated to about 10 ml, washed with EtOAc-hexane (2:1, 8 ml), mixed with NaCl (2.0 g), adjusted to pH 8.5 and extracted with EtOAc (8 ml) three times. The organic layers were combined, washed with saline, dried over anhydrous sodium sulfate and evaporated to give a powder of **6** (4.7 mg, **5** and **6**, total yield, 12.0%).

FAB-MS: **5**, **6**; 746 (MH^+), 588 (MH^+ − cladinose), IR: 5, 3430, 2970, 2930, 1730, 1630, 1450, 1380, 1200, 1170, 1050, 1010, **6**, 3430, 2970, 2930, 1730, 1640, 1460, 1380, 1170, 1050, 1010 cm^{-1}.

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