Studies on Cardiac Ingredients of Plants. XI.[1] Synthesis of New Bufotoxin Homologues Utilizing Scillarenin (the Genuine Aglycone of Proscillaridin), and Their Biological Activities

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New bufotoxin homologues (3) with various lengths of alkyl chain including longer ones than a suberoyl group at C-3 of the steroid nucleus were prepared from proscillaridin (1) *via* its genuine aglycone, scillarenin (2), in excellent yield. In the course of preparation, we established conditions for efficient enzymatic glycolysis of 1 to give 2 quantitatively. The pharmacological activities of the resulting bufotoxin homologues (3) were evaluated by measurement of the effect on smooth muscle using the mesenteric artery from spontaneously hypertensive rats, inhibitory effect on Na^+, K^+-adenosine triphosphatase (ATPase) prepared from dog kidney, and positive inotropic effect (PIE) on isolated guinea-pig papillary muscle preparations. The bufotoxin homologues (3) showed only slight contraction of vascular muscle followed by a small relaxation, in addition to the high Na^+, K^+-ATPase inhibitory activity and PIE comparable to those of clinically used ouabain, digoxin, and digitoxin. Those bufotoxin homologues (3) may be useful as cardiac agents with a minimal vascular contractile effect.

Keywords proscillaridin; scillarenin; bufotoxin homologue; Na^+, K^+-ATPase inhibition; positive inotropic effect; arterial relaxing effect

Bufotoxins from toad venom possess the suberoylarginine residue at the 3-hydroxyl group in a steroid molecule and show digitalis-like cardiac effects. In a classical investigation of toad venom constituents, vulgarobufotoxin was isolated from the European toad, *Bufo vulgaris* (*Bufo bufo bufo* LINNE)[3)] in 1922, and a tentative structure was proposed in 1941.[4)] The correct structures of bufotoxins were established by means of isolation of resibufotoxin and cinobufagin-3-*O*-suberate in 1968.[5)] Since then, various bufotoxins have been isolated and their structures determined. For example, Shimada *et al.* obtained bufotoxin homologues with succinyl-, adipoyl-, and pimeloylarginine residues in place of suberoylarginine from *Bufo vulgaris formosus* BOULENGER[6)] and the homologue with glutarylarginine from *Bufo bufo gargaizans* CANTOR.[7)] From *Bufo americanus* and *Bufo melanostictus* SCHNEIDER, other bufotoxin derivatives were found which possessed glutamine,[8)] histidine, 3-methylhistidine, or 1-methylhistidine[9)] residues in place of arginine at the end of the suberoyl group. In addition, cardenolide-type bufotoxin analogs were isolated from Ch'an Su[10)] and toad venom.[6b)] On the other hand, the first partial synthesis of a bufotoxin homologue was achieved by Pettit and Kamano in 1972.[11)] They prepared vulgarobufotoxin from bufotalin. Shimada and Nambara prepared cardenolide-3-*O*-suberoylarginine.[12)] They also prepared bufotoxin homologues with succinyl, glutaryl, adipoyl, and pimeloyl groups in place of the suberoyl group of bufotoxin and investigated the structure–activity relationships. Several investigations of the preparation and activity of semi-synthetic bufotoxins and their derivatives have been undertaken,[11,13)] but bufotoxin homologues with longer chains than the suberoyl group have never been prepared, except for digitoxigenin derivatives.[13c)]

The guanidyl group of arginine has been reported to generate nitric oxide (NO) *in vivo* which acts as the endothelium-derived relaxing factor (EDRF).[14)] Although the cardiac glycosides cause the contraction of vascular smooth muscle, which sometimes induces serious arrhythmia, we have reported that proscillaridin nitrate showed a vascular relaxing effect as well as a positive inotropic effect (PIE) in the course of our studies on the chemical modification of proscillaridin (**1**).[1,15)] However, the activity of bufotoxin homologues on the vascular smooth muscle is unknown. These facts prompted us to prepare bufotoxin homologues (**3**) with various dicarboxylates including longer ones than suberate between the arginine residue and genuine aglycone of **1**, scillarenin (**2**), in the hope of finding cardiac agents without smooth muscle contraction activity. Scillarenin (**2**) was not obtained by usual acid hydrolysis of **1**, because of the formation of scillaridin (Chart 1). So we attempted enzymatic glycolysis of **1** to get a sufficient amount of **2**. We now report the synthesis of bufotoxin homologues (**3**) from proscillaridin (**1**) *via* scillarenin (**2**), and elucidation of their cardiotonic activity and effects on smooth muscle.

Chemistry

First, we investigated the optimal conditions for enzymatic glycolysis of **1** to **2**. We used commercially available naringinase, which is known to have α-rhamnosidase activity. Since the combination of organic solvent and water was required in order to obtain sufficient solubility of both naringinase and proscillaridin (**1**), we examined the enzymatic hydrolysis in the three solvent systems containing 25% organic solvent (EtOH, MeCN,

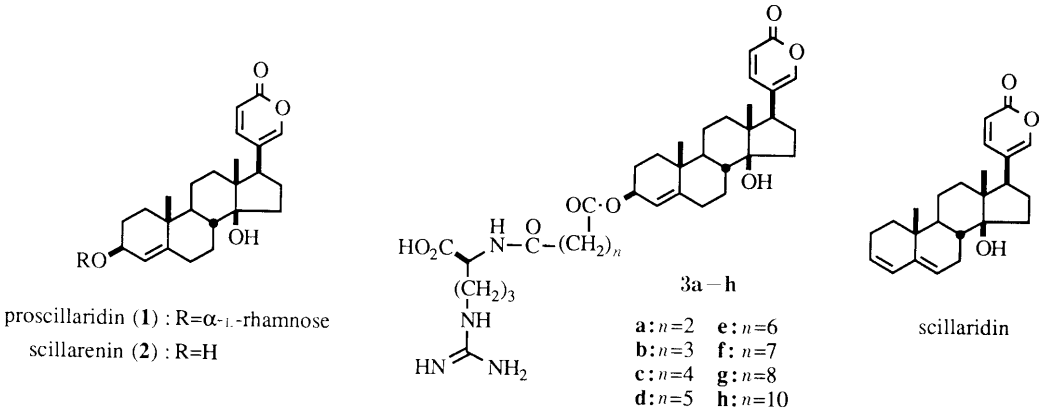

Chart 1

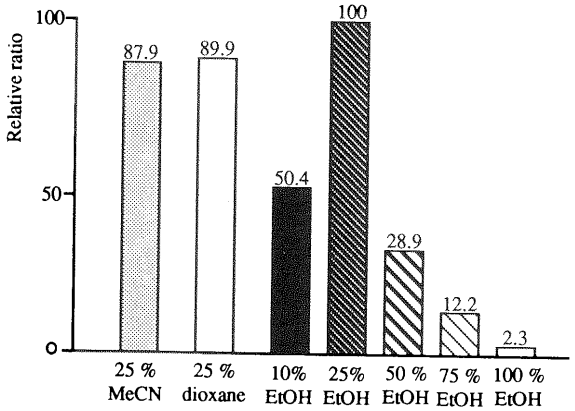

Fig. 1. Effect of Solvent on the α-Rhamnosidase Activity of Naringinase

The activity of each solvent system is indicated by the relative ratio to the maximum activity activity that was obtained in 25% EtOH (v/v) at 40 °C. In this condition, 55.6% of **2** was converted from **1** within 2 h.

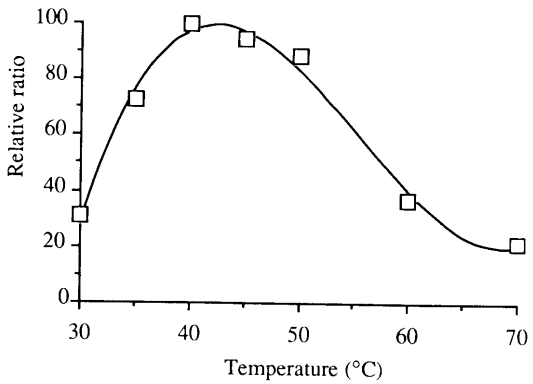

Fig. 2. Effect of Temperature on the α-Rhamnosidase Activity of Naringinase

The activity at each temperature was indicated by the relative ratio to the maximum activity that was obtained in 25% EtOH (v/v) at 40 °C. In this condition, 55.6% conversion of **1** to **2** was achieved in 2 h.

and dioxane). In every case, the hydrolysis gave a single product together with unreacted **1**. The ^{1}H- and ^{13}C-NMR spectra of the product indicated retention of the α-pyrone moiety [δ 8.24 (1H, dd, J=2.3, 9.9 Hz, 22-H), δ 7.47 (1H, d, J=2.3 Hz, 21-H), δ 6.36 (1H, d, J=9.9 Hz, 23-H)] and a trisubstituted olefin at C4–C5 [δ 5.75 (1H, s, 4-H), δ_C 126.4 (C-4), δ_C 145.0 (C-5)] through the enzymatic hydrolysis, while no signals of rhamnose were observed and the signal of 3-H was shifted +0.12 ppm in comparison with that of **1**. The C-3 signal of the product appeared at upper field by 5.5 ppm from that of **1**, and this finding is consistent with a glycosidation shift.[16] Thus, the enzymatic reaction product was concluded to be scillarenin (**2**), the genuine aglycone of **1**.[17]

Among these three solvent systems, the reaction in 25% EtOH showed the highest α-rhamnosidase activity, as judged from the amount of **2** liberated in 2 h (Fig. 1). The amount of **2** was estimated from the peak area of **2** on the HPLC chromatogram by using a calibration line obtained with known amounts of **2**. An increase in the ratio of EtOH in the solvent decreased the enzyme activity, probably due to the low solubility of naringinase.

Then, we tested the effect of temperature and found that the maximum activity was attained at 40 °C (Fig. 2). Finally, in order to evaluate the optimal pH of the

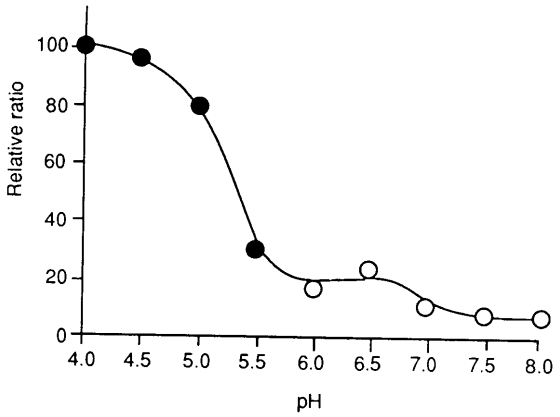

Fig. 3. Effect of pH on the α-Rhamnosidase Activity of Naringinase

Acetate buffer, ●; phosphate buffer, ○. The activity at each pH was indicated by the relative ratio to the maximum activity that was obtained in 25% (v/v) EtOH–acetate buffer (pH 4.0) at 40 °C. In this condition, 70.1% conversion of **1** to **2** was achieved in 2 h.

enzymatic glycolysis, the reaction was carried out at various pH values from 4 to 8 by use of two buffer solution systems (acetate and phosphate buffer) to cover the range. As indicated in Fig. 3, the reaction rate was the highest at pH 4.0 and decreased with increase of the pH. At lower pH than 4.0, scillaridin was formed as a by-product. The reactions using citrate and succinate buffers at pH 4.0

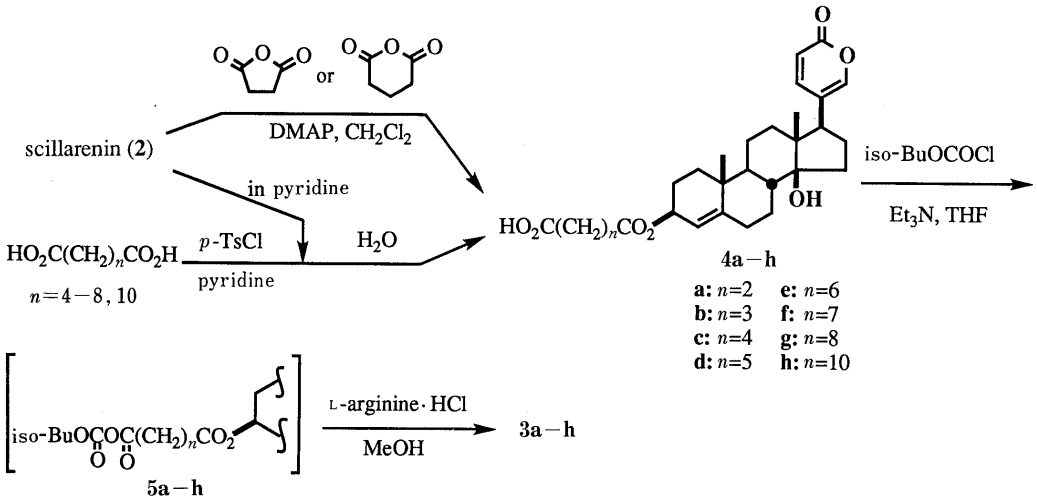

Chart 2

TABLE I. Biological Activities of **4a—h**, **3a—h**, Scillarenin (**2**), and Proscillaridin (**1**)

Compound	pIC_{50}[a]	pD_2[a]	Compound	pIC_{50}[a]	pD_2[a]
4a	7.31±0.001	6.55±0.02	**3a**	7.39±0.04	6.52±0.05
4b	7.33±0.01	6.42±0.04	**3b**	7.40±0.01	6.56±0.08
4c	7.29±0.02	6.37±0.09	**3c**	7.32±0.03	6.66±0.09
4d	7.32±0.01	6.39±0.05	**3d**	7.31±0.02	6.46±0.02
4e	7.35±0.03	6.39±0.04	**3e**	7.29±0.02	6.43±0.07
4f	7.35±0.03	6.59±0.08	**3f**	7.38±0.01	6.56±0.03
4g	7.37±0.02	6.67±0.06	**3g**	7.41±0.02	6.52±0.08
4h	7.30±0.01	6.54±0.11	**3h**	7.42±0.04	6.41±0.05
Scillarenin (**2**)	7.38±0.02	6.83±0.06	Proscillaridin (**1**)	7.44±0.02	7.41±0.14

a) Mean±S.E. (n=3—5).

proceeded more slowly than in acetate buffer. Thus, the optimal conditions were: in 25% EtOH–acetate buffer at pH 4.0 and 40 °C. Large-scale reaction was found to afford **2** quantitatively after 6.5 h.

The synthesis of bufotoxin homologues (**3**) from **2** is summarized in Chart 2. Scillarenin (**2**) was treated with commercially available succinic anhydride as described before to give scillarenin-3-*O*-hemisuccinate (**4a**) in 96% yield.[11] The IR spectrum of **4a** exhibited a broad absorption at 3400 cm^{-1} due to the carboxyl group. In the ^{1}H-NMR spectrum, the signal of 3-H was shifted to lower field, from δ 4.54 (1H, dd, J=6.3, 8.9 Hz) to 5.26 ppm. FAB-MS and elemental analysis also supported the structure. Compound **4b** was similarly obtained from **2**. Compound **4c** was obtained by condensation of adipic acid with **2**, because dicarboxylic acid anhydrides larger than glutaric anhydride are not commercially available and are troublesome to prepare. Adipic acid was activated with *p*-toluenesulfonyl chloride (*p*-TsCl) in pyridine at 0 °C followed by dropwise addition of a pyridine solution of **2** to the reaction mixture to give **4c** quantitatively. Hemicarboxylates (**4d—h**) were similarly obtained by the condensation of corresponding dicarboxylic acids with **2**. Then, the carboxyl group of **4a** was activated as a mixed anhydride (**5a**) in the presence of isobutyl chloroformate and triethylamine followed by addition of L-arginine monohydrochloride in MeOH to give desired bufotoxin homologue (**3a**) in good yield (88%). The compound (**3a**) gave a positive Sakaguchi test[18] and a negative ninhydrin test, which indicated that **4a** had readily condensed with the α-amino group of L-arginine. The ^{1}H-NMR spectrum of **3a** showed the methine proton signal of the L-arginine residue at δ 4.16 ppm. The structure was also confirmed by FAB-MS and elemental analysis. Compounds **3b—h** were similarly obtained in this one-pot reaction from **4b—h**.

Biological Activities

The cardiotonic activities (pIC_{50} and pD_2 values) of scillarenin (**2**), **3a—h**, and the hemicarboxylates (**4a—h**) were examined by means of measurements of the inhibitory activity on dog kidney Na^+, K^+-ATPase[19] and on PIE in isolated guinea-pig papillary muscle. As summarized in Table I, the enzyme inhibitory activities (pIC_{50}) of **3a—h** and **4a—h** were all comparable to that of the parent compound (**1**). The pD_2 values of **3a—h** and **4a—h** were also almost the same in spite of the different lengths of alkyl chain, and were smaller than that of **1**. These data imply that the arginine and dicarboxylate moieties, as well as the length of the dicarboxylate, have little effect on the inhibitory activity towards Na^+, K^+-ATPase, though these large or hydrophilic moieties probably make it difficult for the molecules to approach the active site of the enzyme in the heart muscle cell membrane. In spite of the smaller pD_2 values than **1**, the values of **3a—h** and **4a—h** were still larger than those of clinical agents such as ouabain,

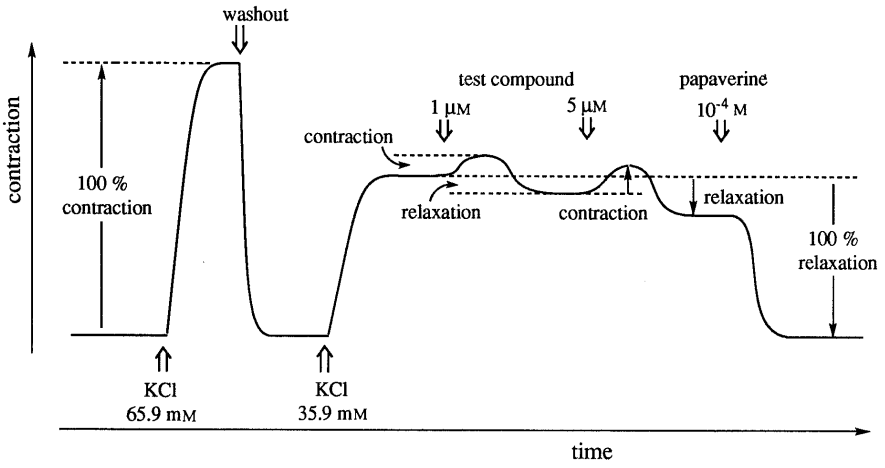

Fig. 4. Illustration of the Contratile and Relaxant Responses of the Test Compounds

TABLE II. Contractile and Relaxant Activities of the Test Compounds in 35.9 mM KCl-Contracted Strips of the Mesenteric Artery Isolated from 13-Week-Old SHR

Compound	1 μM		5 μM	
	Contraction (%)[a]	Relaxation (%)[a]	Contraction (%)[a,b]	Relaxation (%)[a]
3a	3.8±1.7	—	1.0±1.0	5.3±2.0
3b	3.0±1.3	0.2±0.2	1.9±1.2	3.7±2.5
3c	5.6±1.2	—	4.5±1.8	2.3±1.4
3d	4.9±2.7	—	1.3±1.3	4.1±1.9
3e	6.4±1.0	—	9.7±2.3	—
3f	4.0±2.3	0.3±0.3	5.7±2.0	1.3±1.3
3g	3.7±3.0	—	5.5±4.0	2.0±1.4
3h	—	2.4±1.9	—	8.6±3.4
DMSO[c] (control)	—	0.3±0.2	—	3.0±0.9

a) Mean±S.E. (n=4—6). —: not detected. *b*) Contraction caused by ad ditional 4 μM test compounds. *c*) DMSO: dimethyl sulfoxide.

digoxin, and digitoxin.

Finally, we measured the activity of **3a—h** on arterial vascular muscle using strips of mesenteric artery isolated from 13-week-old spontaneously hypertensive rat (SHR). The compounds (**3a—h**) showed only slight effects on the strips at 1 μM. At 5μM, they showed clear but still small contraction followed by relaxation as shown in Table II and Fig. 4, except for **3e, h**. Compound **3e** induced only contraction, while **3h** caused only relaxation. These phenomena suggested that compounds **3a—h** induced both contraction and relaxation and, overall, exhibited only a slight effect on the smooth muscle. Contraction was probably caused by elevation of intracellular Na^+ ion concentration (an inherent activity of cardiac steroids), and the successive relaxation by generation of NO from guanidyl groups. The lag in the appearance of the relaxation might reflect the time required for both metabolism of the guanidyl groups and diffusion of generated NO gas.

In conclusion, we have established an efficient enzymatic glycolysis of proscillaridin (**1**) to obtain large amounts of scillarenin (**2**) quantitatively, then prepared bufotoxin homologues (**3a—h**) with various lengths of alkyl chain from **2** in excellent yields. The compounds (**3a—h**) induced mild contraction followed by slight relaxation of vascular smooth muscle, in addition to ehibiting cardiotonic activity comparable to that of clinical agents.

Experimental

All melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. The UV spectra were recorded with a Shimadzu UV-2100 spectrometer, and electron impact (EI)- and FAB-MS was measured with a JEOL JMS DX-300 mass spectrometer. The IR spectra were obtained with a JASCO IRA-2 spectrometer. The ^{1}H- and ^{13}C-NMR spectra were measured with a JEOL EX-270 spectrometer using tetramethylsilane as an internal standard. The following abbreviations are used; s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Optical rotations were measured on a JASCO DIP-140 digital polarimeter. HPLC was performed using a JASCO 880-PU pump and 870-UV UV detector. TLC was carried out on precoated plates (Kieselgel 60F_{254}, 0.25 mm thick, Merck no. 5715), and spots were detected by illumination with an UV lamp or by spraying 1% $Ce(SO_4)_2$–10% H_2SO_4 followed by heating. Column chromatography was performed on Silica gel BW-200 (Fuji Davison Chemicals Co., Ltd.).

Material Naringinase from *Penicillium decumbens* was purchased from Sigma Co., Ltd. (St. Louis, U.S.A.). All reagents for preparing various buffer solution were obtained from Nakarai Tesque Co., Ltd. (Kyoto, Japan). Solvents for enzymatic hydrolysis were redistilled before use.

General Procedure for Measurement of Enzyme Activity Proscillaridin (**1**, 6.6 mg) in 0.5 ml of solvent was incubated in the presence of naringinase (2.3 mg). After 2 h, an aliquot (50 μl) was withdrawn and was added to EtOH (200 μl). The mixture was concentrated under reduced pressure to furnish a residue, which was subjected to reversed-phase HPLC [Chemcosorb 10C_{18} column (4.6 mm i.d. × 250 mm, Chemco Scientific Co., Ltd.), CH_3CN–H_2O (2 : 3), flow rate 1 ml/min, detector UV at 300 nm]. The enzymatic activity was estimated from the amount of **2** which was obtained from the peak area of **2** on the chromatogram by using a calibration line constructed with known amounts of **2**.

Preparation of Scillarenin (2) by Enzymatic Hydrolysis Using Naringinase A solution (partial suspension) of **1** (66.3 mg, 0.125 mmol) and naringinase (23.2 mg) in EtOH (1.25 ml)–0.02 M acetate buffer (pH 4.0, 3.75 ml) was incubated at 40 °C for 6.5 h. After addition of EtOH (30 ml), the whole mixture was concentrated under reduced pressure. The resulting residue was purified by column chromatography (SiO_2, 10g, *n*-hexane–EtOAc (1 : 1)) to furnish **2** (48 mg, quant.). **2**[20]: Colorless prisms. mp 243—245 °C (iso-Pr_2O–MeOH). $[\alpha]_D^{25}$ −20.1° (c=0.7, MeOH), [lit.[17] $[\alpha]_D^{25}$ −16.5° (c=1.3, MeOH)]. IR (ν_{max}^{KBr} cm^{-1}) 3450 (OH), 1710 (CO). UV (λ_{max}, MeOH) nm (ε): 298.9 (5.5×10^3). EI-MS m/z (%): 384 (M^+, 37.5). ^{1}H-NMR (270 MHz, pyridine-d_5) δ: 0.93 (3H, s, 13-CH_3), 0.98 (3H, s, 10-CH_3), 2.46 (1H, dd, J=6.9, 9.5 Hz, 17-H), 4.54 (1H, dd, J=6.3, 8.9 Hz, 3-H), 5.75 (1H, s, 4-H), 6.36 (1H, d, J=9.9 Hz, 23-H), 7.47 (1H, d, J=2.3 Hz, 21-H), 8.24 (1H, dd, J=2.3,

TABLE III. Yield and Physical Properties of **4a—h**

Compound	Yield (%)	mp (°C)[a] (iso-Pr_2O–MeOH)	$[\alpha]_D^{25}$ (°) (*c*, MeOH)	IR (ν_{max}^{KBr} cm^{-1})	FAB-MS *m/z* (%)	Formula	*Anal.* (%) Calcd (Found) C	H
4a	96	182—184	−39.6 (0.5)	3450 (OH, CO_2H) 1705—1725 (CO)	485 $(M+H)^+$	$C_{28}H_{36}O_7$	69.39 (69.41	7.49 7.47)
4b	98	113—115	−40.8 (0.6)	3500 (OH, CO_2H) 1710—1730 (CO)	499 $(M+H)^+$	$C_{29}H_{38}O_7$	69.84 (69.90	7.69 7.62)
4c	100	119—121	−50.9 (0.5)	3460 (OH, CO_2H) 1705—1720 (CO)	513 $(M+H)^+$	$C_{30}H_{40}O_7$	70.29 (70.33	7.87 7.82)
4d	100	153—155	−42.2 (0.5)	3460 (OH, CO_2H) 1700—1730 (CO)	527 $(M+H)^+$	$C_{31}H_{42}O_7$	70.68 (70.72	8.04 8.00)
4e	100	133—135	−33.1 (0.7)	3440 (OH, CO_2H) 1700—1725 (CO)	563 $(M+Na)^+$	$C_{32}H_{44}O_7$	71.07 (71.00	8.21 8.40)
4f	100	141—143	−37.5 (0.5)	3465 (OH, CO_2H) 1700—1730 (CO)	577 $(M+Na)^+$	$C_{33}H_{46}O_7$	71.44 (71.51	8.36 8.29)
4g	100	152—154	−34.2 (0.59	3450 (OH, CO_2H) 1710—1725 (CO)	591 $(M+Na)^+$	$C_{34}H_{48}O_7$	71.79 (71.77	8.51 8.79)
4h	96	181—183	−52.1 (0.6)	3460 (OH, CO_2H) 1705—1730 (CO)	619 $(M+Na)^+$	$C_{36}H_{52}O_7$	72.44 (72.51	8.79 8.69)

a) Colorless crystalline powders.

9.9 Hz, 22-Hz). ^{13}C-NMR (67.8 MHz, pyridine-d_5) δ_C: 17.2 (C-18), 19.0 (C-19), 21.7 (C-11), 29.4 (C-16), 29.4 (C-7), 30.3 (C-2), 32.8 (C-15), 33.0 (C-1), 36.3 (C-6), 37.7 (C-10), 40.7 (C-12), 42.7 (C-8), 48.7 (C-13), 50.6 (C-9), 51.4 (C-17), 67.3 (C-3), 84.2 (C-14), 115.3 (C-23), 123.4 (C-20), 126.4 (C-4), 145.0 (C-5), 147.5 (C-22), 149.5 (C-21), 162.1 (C-24). *Anal.* Calcd for $C_{24}H_{32}O_4$: C, 74.97; H, 8.39. Found: C, 74.81; H, 8.66.

Hemiesterification of Scillarenin (2) 4a, b: *N*,*N*-Dimethylaminopyridine (DMAP; 203 mg, 1.67 mmol) and succinic anhydride (166 mg, 1.67 mmol) were added to a CH_2Cl_2 solution of **2** (200 mg, 0.520 mmol) and the mixture was stirred at room temperature for 2 h, poured into water and extracted with EtOAc three times. The extract was washed with 5% HCl and brine, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by column chromatography (SiO_2, $CHCl_3$–MeOH (25 : 1)) to give **4a** (242 mg, 85%). Compound **4b** was similarly obtained from **2** and glutaric anhydride.

4c—h: A solution of adipic acid (266 mg, 1.82 mmol) in pyridine (10 ml) was treated with p-TsCl (634 mg, 1.66 mmol) and the mixture was stirred at 0 °C for 30 min, followed by dropwise addition of pyridine solution (6 ml) of **2** (200 mg, 0.520 mmol). The reaction mixture was stirred at room temperature for 4 h, poured into water and extracted with EtOAc three times. The organic phase was washed with 5% HCl and brine, dried over $MgSO_4$, and evaporated under reduced pressure. The residue was purified by column chromatography (SiO_2, $CHCl_3$–MeOH–H_2O (7 : 3 : 1), lower phase) to give **4c** quantitatively. Compounds **4d—h** were also quantitatively prepared from **2** and corresponding dicarboxylic acids in the same manner. The physical properties of **4a—h** are listed in Table III.

Scillarenin 3-(Hydrogen Succinate) (**4a**): ^{1}H-NMR ($CDCl_3+CD_3OD$) δ: 0.73 (3H, s, 13-CH_3), 1.06 (3H, s, 10-CH_3), 2.64 (4H, m, $(CH_2)_2$), 5.26 (2H, m, 3-H, 4-H), 6.28 (1H, dd, J=0.9, 9.6 Hz, 23-H), 7.25 (1H, dd, J=0.9, 2.3 Hz, 21-H), 7.85 (1H, dd, J=2.3, 9.6 Hz, 22-H).

Scillarenin 3-(Hydrogen Glutarate) (**4b**): ^{1}H-NMR ($CDCl_3$) δ: 0.73 (3H, s, 13-CH_3), 1.06 (3H, s, 10-CH_3), 1.97 (2H, t, J=7.2 Hz, $CH_2\underline{CH_2}CH_2$), 2.41 (2H, m, $\underline{CH_2}CH_2\underline{CH_2}$), 2.47(1H, dd, J=6.2, 9.5 Hz, 17-H), 5.25 (2H, m, 3-H, 4-H), 6.27 (1H, dd, J=0.7, 9.8 Hz, 23-H), 7.23 (1H, dd, J=0.7, 2.5 Hz, 21-H), 7.85 (1H, dd, J=2.5, 9.8 Hz, 22-H).

Scillarenin 3-(Hydrogen Adipate) (**4c**) ^{1}H-NMR ($CDCl_3$) δ: ($CDCl_3$): 0.73 (3H, s, 13-CH_3), 1.06 (3H, s, 10-CH_3), 2.31—2.39 (4H, m, $COCH_2$), 2.47 (1H, dd, J=6.2, 9.5 Hz, 17-H), 5.25 (2H, m, 3-H, 4-H), 6.27 (1H, dd, J=0.7, 9.6 Hz, 23-H), 7.24 (1H, dd, J=0.7, 2.6 Hz, 21-H), 7.83 (1H, dd, J=2.6, 9.6 Hz, 22-H).

Scillarenin 3-(Hydrogen Pimelate) (**4d**): ^{1}H-NMR ($CDCl_3$) δ: 0.73 (3H, s, 13-CH_3), 1.06 (3H, s, 10-CH_3), 2.31—2.39 (4H, m, $COCH_2$), 2.47 (1H, dd, J=6.4, 9.5 Hz, 17-H), 5.25 (2H, m, 3-H, 4-H), 6.27 (1H, dd, J=1.0, 9.9 Hz, 23-H), 7.23 (1H, dd, J=1.0, 2.3 Hz, 21-H), 7.82 (1H, dd, J=2.3, 9.9 Hz, 22-H).

Scillarenin 3-(Hydrogen Suberate) (**4e**): ^{1}H-NMR ($CDCl_3$) δ: 0.73 (3H, s, 13-CH_3), 1.06 (3H, s, 10-CH_3), 2.27—2.38 (4H, m, $COCH_2$), 2.47 (1H, dd, J=6.4, 9.5 Hz, 17-H), 5.25 (2H, m, 3-H, 4-H), 6.27 (1H, d, J=9.7 Hz, 23-H), 7.25 (1H, d, J=2.5 Hz, 21-H), 7.83 (1H, dd, J=2.5, 9.7 Hz, 22-H).

Scillarenin 3-(Hydrogen Azelate) (**4f**) ^{1}H-NMR ($CDCl_3$) δ: 0.73 (3H, s, 13-CH_3), 1.05 (3H, s, 10-CH_3), 2.26—2.37 (4H, m, $COCH_2$), 2.46 (1H, dd, J=6.5, 9.5 Hz, 17-H), 5.25 (2H, m, 3-H, 4-H), 6.27 (1H, dd, J=0.9, 9.6 Hz, 23-H), 7.23 (1H, dd, J=0.9, 2.6 Hz, 21-H), 7.82 (1H, dd, J=2.6, 9.6 Hz, 22-H).

Scillarenin 3-(Hydrogen Sebacate) (**4g**): ^{1}H-NMR ($CDCl_3$) δ: 0.71 (3H, s, 13-CH_3), 1.03 (3H, s, 10-CH_3), 2.20—2.34 (4H, m, $COCH_2$), 2.45 (1H, dd, J=6.4, 9.3 Hz, 17-H), 5.20 (2H, m, 3-H, 4-H), 6.26 (1H, d, J=9.9 Hz, 23-H), 7.22 (1H, dd, J=2.5 Hz, 21-H), 7.82 (1H, dd, J=2.5, 9.9 Hz, 22-H).

Scillarenin 3-(Hydrogen 1,10-Decanedicarboxylate) (**4h**): ^{1}H-NMR ($CDCl_3$) δ: 0.73 (3H, s, 13-CH_3), 1.06 (3H, s, 10-CH_3), 2.25—2.38 (4H, m, $COCH_2$), 2.46 (1H, dd, J=6.5, 9.5 Hz, 17-H), 5.25 (2H, m, 3-H, 4-H), 6.28 (1H, d, J=9.7 Hz, 23-H), 7.24 (1H, d, J=1.5 Hz, 21-H), 7.85 (1H, dd, J=1.5, 9.7 Hz, 22-H).

Condensation of Hemiesters (4a—h) with L-Arginine Compound **4a** (200 mg, 0.39 mmol) in tetrahydrofuran (THF, 18 ml) was stirred with triethylamine (0.30 ml, 2.11 mmol) at −15 °C for 15 min, followed by dropwise addition of isobutyl chloroformate (0.10 ml, 0.78 mmol). After 30 min, L-arginine monohydrochloride (270 mg, 1.76 mmol) in MeOH (2.7 ml) was added dropwise and the reaction mixture was stirred at 0 °C for 2 h. The solvent was removed under reduced pressure, MeOH was added to the residue and the solution was filtered through to remove the excess L-arginine. The filtrate was evaporated *in vacuo*, and the residue was chromatographed on silica gel ($CHCl_3$–MeOH–H_2O (7 : 3 : 1), lower phase) to give **3a** (524 mg, 88%). Compounds **3b—h** were prepared in the same manner from corresponding hemiesters (**4b—h**) and L-arginine monohydrochloride in 82—95% yields. The physical properties of **3a—h** were are listed in Table IV.

Scillarenin 3-Succinyl-L-arginine Ester (**3a**): ^{1}H-NMR ($CDCl_3+CD_3OD$) δ: 0.73 (3H, s, 13-CH_3), 1.06 (3H, s, 10-CH_3), 2.47 (1H, dd, J=6.5, 9.2 Hz, 17-H), 4.32 (1H, m, CH in Arg), 5.22 (2H, m, 3-H, 4-H), 6.28 (1H, d, J=9.5 Hz, 23-H), 7.26 (1H, dd, J=1.6 Hz, 21-H), 7.90 (1H, dd, J=1.6, 9.5 Hz, 22-H).

Scillarenin 3-Glutaryl-L-arginine Ester (**3b**): ^{1}H-NMR ($CDCl_3+CD_3OD$) δ: 0.73 (3H, s, 13-CH_3), 1.05 (3H, s, 10-CH_3), 2.47 (1H, dd, J=6.8, 8.9 Hz, 17-H), 4.20 (1H, m, CH in Arg), 5.22 (2H, m, 3-H, 4-H), 6.28 (1H, d, J=9.6 Hz, 23-H), 7.26 (1H, d, J=2.5 Hz, 21-H), 7.88 (1H, dd, J=2.5, 9.6 Hz, 22-H).

Scillarenin 3-Adipoyl-L-arginine Ester (**3c**): ^{1}H-NMR ($CDCl_3+CD_3OD$) δ: 0.73 (3H, s, 13-CH_3), 1.05 (3H, s, 10-CH_3), 2.47 (1H, dd, J=6.9, 9.2 Hz, 17-H), 4.17 (1H, m, CH in Arg), 5.22 (2H, m, 3-H, 4-H), 6.26 (1H, d, J=9.6 Hz, 23-H), 7.25 (1H, dd, J=2.3 Hz, 21-H), 7.87 (1H,

TABLE IV. Yields and Physical Properties of **3a—h**

Compound	Yield (%)	mp (°C)[a] (iso-Pr_2O–MeOH)	$[\alpha]_D^{25}$ (°) (*c*, MeOH)	IR (ν_{max}^{KBr} cm^{-1})	FAB-MS *m/z* (%)	Formula	*Anal.* (%) Calcd (Found) C	H	N
3a	88	211—213	−19.4 (0.7)	3400 (OH, CO_2H, NH_2) 1700—1725 (CO)	663 $(M+Na)^+$	$C_{34}H_{38}N_4O_8 \cdot H_2O$	62.94 (62.88)	6.22 (6.30)	8.64 (8.59)
3b	82	208—210	−18.1 (0.6)	3405 (OH, CO_2H, NH_2) 1700—1730 (CO)	677 $(M+Na)^+$	$C_{35}H_{40}N_4O_8 \cdot H_2O$	63.42 (63.47)	6.39 (6.41)	8.46 (8.45)
3c	90	215—217	−22.6 (0.5)	3410 (OH, CO_2H, NH_2) 1705—1730 (CO)	691 $(M+Na)^+$	$C_{36}H_{42}N_4O_8 \cdot H_2O$	63.88 (63.92)	6.56 (6.50)	8.28 (8.27)
3d	90	220—222	−23.5 (0.5)	3385 (OH, CO_2H, NH_2) 1705—1730 (CO)	705 $(M+Na)^+$	$C_{37}H_{44}N_4O_8 \cdot H_2O$	64.32 (64.33)	6.72 (6.72)	8.11 (8.14)
3e	95	218—220	−31.0 (0.5)	3380 (OH, CO_2H, NH_2) 1700—1725 (CO)	719 $(M+Na)^+$	$C_{38}H_{46}N_4O_8 \cdot H_2O$	64.74 (64.77)	6.87 (6.82)	7.95 (7.92)
3f	90	230—232	−33.0 (0.5)	3380 (OH, CO_2H, NH_2) 1695—1725 (CO)	733 $(M+Na)^+$	$C_{39}H_{48}N_4O_8 \cdot H_2O$	65.15 (65.10)	7.01 (7.10)	7.80 (7.78)
3g	88	239—241	−29.4 (0.6)	3385 (OH, CO_2H, NH_2) 1700—1730 (CO)	747 $(M+Na)^+$	$C_{40}H_{50}N_4O_8 \cdot H_2O$	65.54 (65.51)	7.16 (7.18)	7.65 (7.64)
3h	86	245—247	−19.9 (0.5)	3400 (OH, CO_2H, NH_2) 1700—1730 (CO)	775 $(M+Na)^+$	$C_{42}H_{54}N_4O_8 \cdot H_2O$	66.28 (66.30)	7.42 (7.40)	7.37 (7.38)

a) Colorless crystalline powders.

dd, J=2.3, 9.6 Hz, 22-H).

Scillarenin 3-Pimeloyl-L-arginine Ester (**3d**): ^{1}H-NMR ($CDCl_3$+CD_3OD) δ: 0.73 (3H, s, 13-CH_3), 1.06 (3H, s, 10-CH_3), 2.47 (1H, dd, J=6.6, 9.2 Hz, 17-H), 4.19 (1H, m, CH in Arg), 5.23 (2H, m, 3-H, 4-H), 6.28 (1H, dd, J=9.9 Hz, 23-H), 7.26 (1H, d, J=1.7 Hz, 21-H), 7.89 (1H, dd, J=1.7, 9.9 Hz, 22-H).

Scillarenin 3-Suberoyl-L-arginine Ester (**3e**): ^{1}H-NMR ($CDCl_3$+CD_3OD) δ: 0.73 (3H, s, 13-CH_3), 1.05 (3H, s, 10-CH_3), 2.47 (1H, dd, J=6.8, 9.2 Hz, 17-H), 4.17 (1H, m, CH in Arg), 5.22 (2H, m, 3-H, 4-H), 6.22 (1H, dd, J=0.7, 9.7 Hz, 23-H), 7.26 (1H, dd, J=0.7, 1.5 Hz, 21-H), 7.88 (1H, dd, J=1.5, 9.7 Hz, 22-H).

Scillarenin 3-Azelaoyl-L-arginine Ester (**3f**): ^{1}H-NMR ($CDCl_3$+CD_3OD) δ: 0.73 (3H, s, 13-CH_3), 1.05 (3H, s, 10-CH_3), 2.47 (1H, dd, J=6.6, 8.6 Hz, 17-H), 4.16 (1H, m, CH in Arg), 5.22 (2H, m, 3-H, 4-H), 6.27 (1H, d, J=9.6 Hz, 23-H), 7.22 (1H, dd, J=2.3 Hz, 21-H), 7.99 (1H, dd, J=2.3, 9.6 Hz, 22-H).

Scillarenin 3-Sebacoyl-L-arginine Ester (**3g**): ^{1}H-NMR ($CDCl_3$+CD_3OD) δ: 0.73 (3H, s, 13-CH_3), 1.05 (3H, s, 10-CH_3), 2.47 (1H, dd, J=6.4, 9.4 Hz, 17-H), 4.30 (1H, m, CH in Arg), 5.23 (2H, m, 3-H, 4-H), 6.27 (1H, d, J=9.6 Hz, 23-H), 7.26 (1H, dd, J=2.6 Hz, 21-H), 7.89 (1H, dd, J=2.6, 9.6 Hz, 22-H).

Scillarenin 3-(1,10-Decanedicarboxyl)-L-arginine Ester (**3h**): ^{1}H-NMR ($CDCl_3$+CD_3OD) δ: 0.73 (3H, s, 13-CH_3), 1.06 (3H, s, 10-CH_3), 2.47 (1H, dd, J=6.3, 9.6 Hz, 17-H), 4.16 (1H, m, CH in Arg), 5.24 (2H, m, 3-H, 4-H), 6.27 (1H, d, J=9.6 Hz, 23-H), 7.26 (1H, d, J=1.6 Hz, 21-H), 7.87 (1H, dd, J=1.6, 9.6 Hz, 22-H).

Measurement of Cardiotonic Activities The cardiotonic activities (pIC_{50} and pD_2 values) of test compounds were examined by use of a dog kidney Na^+, K^+-ATPase preparation (Sigma Co., Ltd.) and isolated guinea-pig papillary muscle preparations, respectively. The measurements were performed as described before.[1,21]

Measurement of the Activity on Smooth Muscle The effects on smooth muscle were examined by essentially the same method as described before.[1] Briefly, helical strips (0.7 mm in width and 6 mm in length) of mesenteric artery isolated from 13-week-old SHR were prepared according to the method described by Asano *et al.*[22] The strips were mounted for isometric recording of tension in a water-jacketed (37±0.5 °C) tissue bath containing 20 ml of oxygenated Krebs' bicarbonate solution. The strips were maximally activated by 65.9 mM KCl, and after washout, the strips were contracted with 35.9 mM KCl. After the contraction had reached a plateau, one of the test compounds was added. The arterial contraction induced by the compound is expressed as a percentage of the maximum contraction developed by 65.9 mM KCl and the relaxation is expressed as a percentage of the difference between the relaxation developed by 10^{-4} M papaverine and the contraction developed by 35.9 mM KCl, as illustrated in Fig. 4.

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