Scopadulcic Acid B, a New Tetracyclic Diterpenoid from *Scoparia dulcis* L. Its Structure, H^+, K^+-Adenosine Triphosphatase Inhibitory Activity and Pharmacokinetic Behaviour in Rats

Toshimitsu HAYASHI,*[,a] Kana OKAMURA,[a] Masawo KAKEMI,[a] Shinji ASANO,[a] Motofumi MIZUTANI,[a] Noriaki TAKEGUCHI,[a] Masaru KAWASAKI,[a] Yasuhiro TEZUKA,[b] Tohru KIKUCHI,[b] and Naokata MORITA[a]

Faculty of Pharmaceutical Sciences[a] and Research Institute for Wakanyaku (Oriental Medicines),[b] Toyama Medical and Pharamaceutical University, 2630 Sugitani, Toyama 930-01, Japan. *Received April 4, 1990*

The structure of scopadulcic acid B (2, SDB), a major ingredient of the Paraguayan herb "Typychá kuratũ" (*Scoparia dulcis* L.), was elucidated mainly by comparison of its spectral data with that of scopadulcic acid A (1). SDB inhibited both the K^+-dependent adenosine triphosphatase (ATPase) activity of a hog gastric proton pump (H^+, K^+-ATPase) with a value of 20—30 μM for IC_{50} and proton transport into grastric vesicles. Pharmacokinetic studies of SDB in rats indicated that plasma SDB concentrations after i.v. injection of the sodium salt of SDB (SDB-Na) were described reasonably well by a two-compartment open model with Michaelis–Menten elimination kinetics. Plasma concentrations after oral administration of SDB-Na or SDB showed a much slower decline than what was expected following the i. v. study. It was suggested that the sustained plasma level of SDB after oral administration of SDB-Na or SDB was accounted for by relatively slow but efficient gastro-intestinal absorption in rats.

Keywords scopadulcic acid B; diterpenoid; scopadulan; *Scoparia dulcis* L.; H^+, K^+-ATPase inhibitor; pharmacokinetics; Michaelis–Menten kinetics; noncompartmental analysis

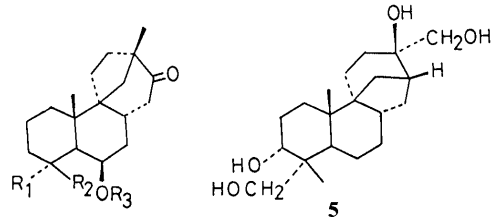
Scoparia dulcis L. is a medicinal plant which has been used in Paraguay to improve digestion and protect the stomach.[1] In Taiwan, the same plant is used as a cure for hypertension,[2] and in India for toothaches, blennorhagia and stomach troubles.[3] Earlier studies of this plant by an Indian worker, Nath, resulted in the isolation of an antidiabetic compound named amellin.[4] Then, C. M. Chen and M. T. Chen in Taiwan reported the isolation of 6-methoxybenzoxazolinone with a hypotensive activity.[5] However, no ingredient exerting a protective activity for the stomach has been isolated from this plant so far.

Previously, we briefly reported the isolation and structure elucidation of diterpenoids with a novel skeleton, scopadulcic acids A (**1**, SDA) and B (**2**, SDB) from Paraguayan *Scoparia dulcis*.[6] The crystal structure of SDA was confirmed by X-ray crystallographic analysis.[7] We report here details of the structure determination of SDB, along with its biological activity and pharmacokinetic behaviour in rats.

Results and Discussion

Structure of SDB (2) SDB was isolated as colorless prisms, mp 228—232 °C, $[\alpha]_D$ −49.6° (MeOH), from the 70% EtOH extract of a Paraguayan herb, "Typychá kuratũ" (*Scoparia dulcis* L.), as previously reported.[6,8] The molecular formula for SDB was determined to be $C_{27}H_{34}O_5$ on the basis of high-resolution mass spectrum (HRMS) and elemental analysis. Its infrared (IR) spectrum showed maximum absorption at 3200, 1730, 1710, 1600 and 1580 cm^{-1}, indicating the presence of hydroxyl, carbonyl and phenyl functions in the molecule. When SDB was treated with diazomethane (CH_2N_2), it produced a methyl ester (**3**), $C_{28}H_{36}O_5$, mp 190—191 °C suggesting the presence of a carboxyl group. The absorption bands at 229 (log ε 3.99), 265 (2.81), 272 (2.83), and 280 (2.74) nm in the ultra violet (UV) spectrum and fragment ion peaks at *m/z* 122, 105 and 77 in the electron impact mass spectrum (EIMS) suggest the presence of a benzoyl group in SDB. This was reinforced by the proton and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectral data [δ_H 7.45 (t, J=7.3 Hz), 7.55 (t, J=7.3 Hz), 8.01 (d, J=7.3 Hz); δ_C 128.5 (2d), 129.6 (2d), 130.5 (s), 133.4 (d), 166.1 (s)]. Furthermore, it was confirmed by alkaline hydrolysis of SDB, which yielded benzoic acid and a debenzoylated product (**4**), $C_{20}H_{30}O_4$, mp 224—228 °C. The ^{1}H-NMR spectrum of SDB showed signals for three tertiary methyls at δ 1.10, 1.36 and 1.55, and an oxygenated methine proton at δ 5.33 which revealed an upfield shifts on debenzoylation. In addition, the ^{13}C-NMR spectrum indicated the signal for a ketone (δ 213.6) along with eight methylene, three methine and four quarternary carbon signals (Table I). These spectral data suggest that SDB has a tetracyclic carbon skeleton with carboxyl, benzoyl and ketone functions. When the ^{1}H- and ^{13}C-NMR spectra of SDB were compared with those of SDA, they were found to be closely related compounds (Table I).[9] Since SDB showed a signal due to an additional tertiary methyl and no signal of hydroxymethylene, its planar structure was estimated to be **2**, which possesses carboxyl and methyl groups at the C-4 position in SDA. This assumption was further supported by the ^{1}H–^{13}C long-range shift correlation (COSY) spectrum of SDB.[10]

The relative stereochemistry of SDB was determined by nuclear Overhauser effect (NOE) difference spectroscopy. Irradiation of 20-methyl enhanced the signal intensity of the 8-, 19- and 2′,6′-protons. Also NOE's were observed between 5- and 6-protons and between 8- and 11-protons.



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1: R_1=CH_2OH, R_2=COOH, R_3=COC_6H_5
2: R_1=COOH, R_2=CH_3, R_3=COC_6H_5
3: R_1=$COOCH_3$, R_2=CH_3, R_3=COC_6H_5
4: R_1=COOH, R_2=CH_3, R_3=H

Chart 1

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TABLE I. ^{13}C- and ^{1}H-NMR Spectral Data of Scopadulcic Acid A (**1**) and B (**2**)

Position	SDA (in acetone-d_6) C (δ ppm)	SDA (in acetone-d_6) H (δ ppm)	SDB (in $CDCl_3$) C (δ ppm)	SDB (in $CDCl_3$) H (δ ppm)
1	35.1 (t)	1.65 (dtd, J=12.5, 3.5, 1.5) 1.70 (td, J=12.5, 4.5)	34.0 (t)	1.62 (m) 1.75 (m)
2	20.1 (t)	1.59 (dqd, J=13.5, 12.5, 4.5, 3.5) 2.37 (tddd, J=13.5, 12.5, 4.5, 3.5)	18.0 (t)	1.68 (m) 1.79 (m)
3	33.5 (t)	1.63 (td, J=13.5, 4.5) 1.93 (tdt, J=13.5, 4.5, 1.5)	39.7 (t)	1.73 (m)
4	48.8 (s)		47.2 (s)	
5	45.0 (d)	2.03 (d, J=2)	44.6 (d)	2.22 (d, J=2)
6	70.4 (d)	5.60 (ddd, J=3, 2.5, 2)	72.9 (d)	5.33 (td, J=3, 2)
7	35.8 (t)	1.73 (ddd, J=15, 12, 3) 1.86 (ddd, J=15, 4.5, 2.5)	35.1 (t)	1.76 (ddd, J=15, 12, 3) 1.88 (ddd, J=15, 4.5, 3)
8	37.0 (d)	2.48 (tddd, J=12, 6.5, 4.5, 1.5)	36.0 (d)	2.49 (tdd, J=12, 6.5, 4.5)
9	54.2 (s)		53.1 (s)	
10	40.0 (s)		38.8 (s)	
11	46.5 (t)	1.47 (br d, J=12) 1.90 (d, J=12)	45.1 (t)	1.54 (br d, J=12.5) 1.83 (d, J=12.5)
12	53.2 (s)		52.3 (s)	
13	212.8 (s)		213.6 (s)	
14	43.5 (t)	2.06 (dd, J=15, 12) 2.12 (dd, J=15, 6.5)	42.5 (t)	2.02 (dd, J=16, 12) 2.25 (dd, J=16, 6.5)
15	37.7 (t)	1.67 (td, J=13, 6) 1.76 (dddd, J=13, 9, 3.5, 1)	36.6 (t)	1.67 (m) 1.81 (m)
16	24.5 (t)	1.85 (dddd, J=15, 9, 6, 1) 2.22 (dddd, J=15, 13, 3.5, 1.5)	23.7 (t)	1.86 (m) 2.21 (m)
17	20.5 (q)	1.02 (s)	19.7 (q)	1.10 (s)
18	68.2 (t)	3.56 (d, J=11.2) 3.79 (d, J=11.2)	184.2 (s)	
19	178.3 (s)		19.3 (q)	1.36 (s)
20	21.6 (q)	1.59 (s)	21.6 (q)	1.55 (s)
21	166.8 (s)		166.1 (s)	
1′	132.5 (s)		130.5 (s)	
2′,6′	130.8 (d)	7.96 (d, J=7.3)	129.6 (d)	8.01 (d, J=7.3)
3′,5′	129 5 (d)	7.43 (t, J=7.3)	128.5 (d)	7.45 (t, J=7.3)
4′	133.7 (d)	7.57 (t, J=7.3)	133.4 (d)	7.55 (t, J=7.3)

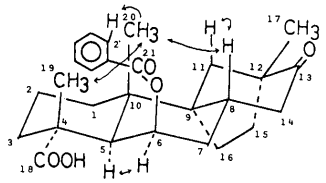

Fig. 1. Relative Configuration of SDB
←→: NOE observed.

From these results, the relative configuration of SDB was elucidated as shown in Fig. 1. The circular dichroism (CD) spectrum of SDB displayed a positive Cotton effect at 297 nm ([θ]+2490) due to the optically active ketone chromophore, testifying to the absolute configuration shown in expression **2**, which is in accord with that of SDA (**1**).

On the basis of the above studies, SDB was characterized as 6β-benzoyl-12-methyl-9(12)$_a$, 9(12)$_b$-dihomo-podocarpane-13-one-18-oic acid (**2**). We propose the name "scopadulan" for the novel skeleton of **1** and **2**. This skeleton is supposedly biosynthesized from labdane via pimarane, as in the case of aphidicolin (**5**) isolated from *Cephalosporium aphidicola* PETCH.[11]

Biological Activity The structural resemblance of SDB to aphidicolin (**5**) prompted us to examine the antiviral activity of SDB.[12] *In vitro* and *in vivo* antiviral activity against herpes simplex virus type 1 (HSV-1) was observed in SDB and the detailed results have already been reported elsewhere.[13]

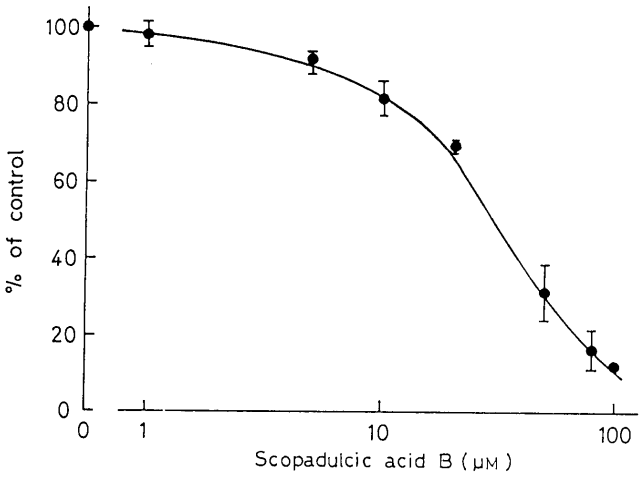

Fig. 2. Effect of SDB on H^+, K^+-ATPase Activity

As *Scoparia dulcis* has been used to protect the stomach, we examined the inhibitory activity of SDB on H^+,K^+-adenosine triphosphatase (ATPase), which is a known proton pump for gastric acid secretion.[14] As shown in Fig. 2, SDB dose-dependently inhibited hog gastric H^+,K^+-ATPase activity. Its half-maximal inhibition concentration (IC_{50}) was 20—30 μM. The inhibition of the H^+,

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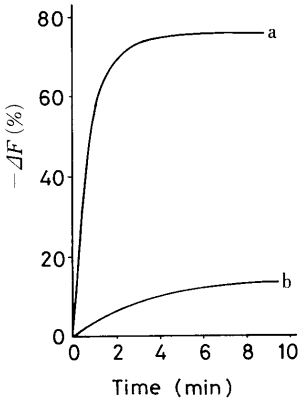

Fig. 3. Effect of SDB on Proton Uptake into Gastric Vesicles in the Presence of 150 mM KCl and 10 μg of Valinomycin

X-Axis shows the time after addition of ATP, and Y-axis the relative change of the fluoresence. Concentrations of SDB were as follows: a, 0; b, 100 μM.

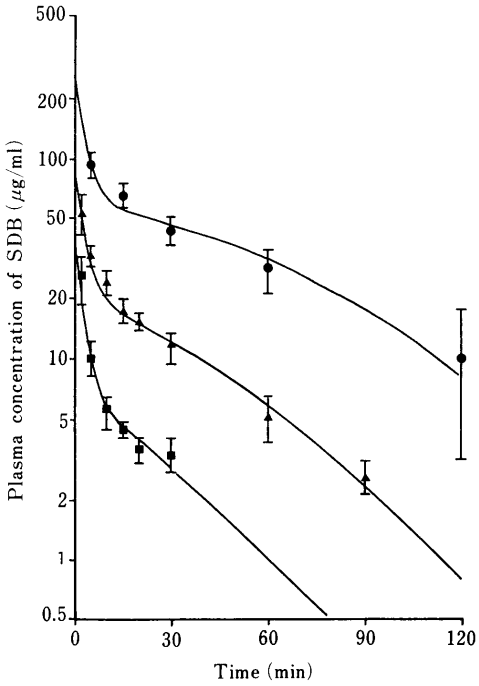

Fig. 4. Plasma Concentrations of SDB after Bolus i.v. Administration of SDB-Na in Rats

■: 5 mg/kg; ▲: 15 mg/kg; ●: 27 mg/kg.

K^+-ATPase activity was restored by dilution (data not shown), indicating that inhibition by SDB was reversible. Next, we investigated the effect of SDB on proton uptake into gastric vesicles. SDB at concentrations higher than 100 μM completely abolished proton uptake (Fig. 3). Thus, SDB inhibited both proton uptake and ATP hydrolysis of the enzyme.

The inhibitory effect of SDB on H^+, K^+-ATPase may play an important role in the pharmacological action of this herb, since proton pump inhibitors such as omeprazole and SCH28080 are useful for the treatment of gastric ulcers.[15]

Pharmacokinetics of SDB in Rats In order to evaluate SDB as a remedy, it is important to elucidate its pharmacokinetic behaviour in the body. So far, very little data concerning the pharmacokinetic characteristics of diterpenoids has been reported. Since SDB is insoluble in water, it was converted into sodium salt (SDB-Na) and its plasma concentration profile in rats was examined.

The plasma concentrations of SDB after i.v. bolus administration of SDB-Na (5, 15, 27 mg/kg) are shown in Fig. 4, as semi-logarithmic plots. Although a plasma concentration profile of SDB showed a typical two exponential decline for each dose, the slope of the terminal phase decreased with an increase of dose. Statistical moment analysis was applied to the plasma concentration of SDB at each dose, and apparent values for the area under the plasma concentration time curve (AUC), the mean residence time (MRT), the variance of residence time (VRT), the total body clearance (CL) and the volume of distribution at steady-state (V_{ss}) were estimated. The results are shown in Table II. Apparent values for AUC, CL and MRT suggested that non-linear kinetics are involved in the disposition of SDB in the rat. Accordingly, the plasma concentrations of SDB for all doses were fitted simultaneously to a two-compartment open model with Michaelis–Menten

TABLE II. Non-compartmental Analysis of SDB Plasma Concentration in Rats[a]

Drug	SDN-Na	SDB-Na	SDB-Na	SDB-Na	SDB
Dose (mg/kg)	5 i.v. (4)	15 i.v. (4)	27 i.v. (3)	200 *p.o.* (3)	200 *p.o.* (4)
$AUC(0)$ ((μg/ml)h)	6.143 ±0.527	19.55 ±1.00	82.54 ±15.62	314.5 ±29.3	420.2 ±99.3
MRT (h)	0.455 ±0.123	0.524 ±0.041	0.934 ±0.193	11.33 ±2.63	16.01 ±2.59
VRT (h^2)	0.405 ±0.190	0.404 ±0.038	1.182 ±0.493	145.0 ±64.2	145.3 ±58.6
CL ((ml/h)/kg)	835.5 ±83.9	773.4 ±39.25	349.1 ±58.7	—	—
V_{ss} (ml/kg)	370.3 ±94.5	403.1 ±30.4	303.1 ±9.0	—	—

a) Results are given as the mean ± S.D. of 3 to 4 rats. The number of experiments is shown in parentheses.

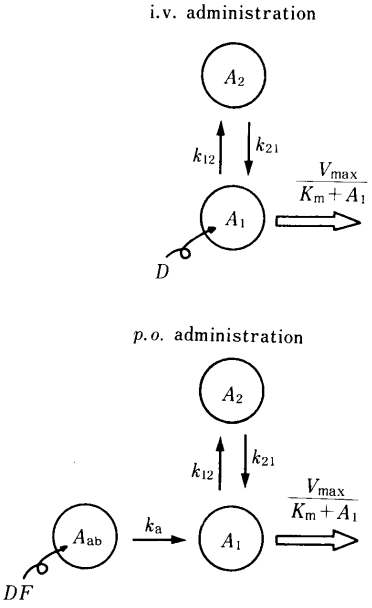

Fig. 5. Pharmacokinetic Models

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TABLE III. Computer Estimated Pharmacokinetic Parameters of SDB in Rats[a)]

	Dose (mg/kg)			
	5 i.v.	15 i.v.	27 i.v.	200 *p.o.*
k_{12} (h^{-1})	16.0691 ±3.4898			
k_{21} (h^{-1})	5.5178 ±0.5971			
K_m (mg)	1.2616 ±0.6384			
V_{max} (mg/h)	16.3719 ±3.3783			
V_c (ml/kg)	116.3 ±20.1	152.2 ±23.7	95.13 ±16.9	34.2 ±17.0
F	—	—	—	0.533
k_a	—	—	—	0.0739 ±0.0104

a) Results are shown as the computer estimate ± S.D.

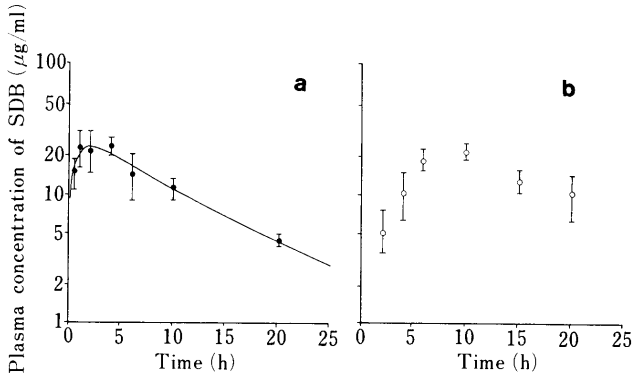

Fig. 6. Plasma Concentrations of SDB after *p.o.* Administration of SDB-Na (a) or SDB (b) in Rats (200 mg/kg)

elimination kinetics, as shown in the Experimental section (Fig. 5 and Eqs. 1 to 3). Since noncompartmental analysis merely suggested that nonlinear kinetics exist in SDB disposition, there are many possible models such as one-, two- or three-compartment models with nonlinear distribution or elimination kinetics, etc. Among these models, we chose a model with the minimum AIC[16] as the best fitting model. The solid lines in Fig. 5 show the fitted values, and the estimated parameters are listed in Table III. The results indicate that the two-compartment model with Michaelis–Menten elimination kinetics adequately describe the disposition of SDB after intravenous administration of SDB-Na in rats. Also, the values for the distribution volume of the central compartment (V_c)varied with doses. Plasma protein binding might be the possible reason for this, and therefore, further investigation is required in this respect.

Since the crude drug, "Typychá kuratũ" is only used orally, SDB-Na or SDB itself was also administered orally (200 mg/kg) and plasma concentrations were determined. As shown in Fig. 6a, the slope of the terminal phase of SDB plasma concentration was much smaller than what was expected from the i.v. study, suggesting relatively slow gastro-intestinal absorption. Then, these data were fitted to a two-compartment model with first-order absorption and Michaelis–Menten elimination kinetics (Eqs. 4 to 7 in Experimental section), and apparent bioavailability F was estimated. The solid line in Fig. 6a represents the calculated values and estimated parameters listed in Table III. The result indicated that orally-administered SDB-Na was efficiently absorbed from the gastro-intestinal tract (more than fifty percent of dose). Fig. 6b shows the plasma concentration profile of SDB after oral administration of SDB itself. The apparent value of AUC indicated that SDB was absorbed at the same rate as SDB-Na.

Next, urinary excretion of SDB after i.v. or oral administration of SDB-Na was also determined. However, no appreciable amount of unchanged SDB was detected in the urine. Although the metabolic pathway of SDB has not been clarified, glucuronide, sulfate or glycine conjugates are the most probable metabolites of SDB in rats. Consequently, urine samples were hydrolyzed by acid and the SDB level was then analyzed, but no SDB was detected. These facts suggest that renal excretion is the minor route for SDB excretion in the rat. Bioavailability of SDB-Na after oral administration (200 mg/kg) was estimated by the model fitting technique, not by the ratio of $AUC_{p.o.}$ to $AUC_{i.v.}$. Because of the nonlinear disposition of SDB, AUC could not be estimated appropriately in the conventional way, especially in the oral study. The estimated value for bioavailability was 53% and this value is considerably high, because it may include the first pass metabolism. However, with the limited data it was not possible to individually estimate the hepatic extraction ratio in the present study.

In conclusion, SDB can be absorbed pretty well from gastro-intestinal tract in the rat. The elimination of SDB from plasma shows rapid but capacity-limited kinetics, and the main elimination route may be attributable to biliary excretion.

Experimental

1. Structure Determination Apparatus Melting points were determined on a Yanagimoto micro-melting point apparatus and uncorrected. UV spectra were recorded on a Hitachi 220S spectrophotometer, and IR spectra were taken on a Hitachi 260—10 infrared spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were measured with JEOL GX-270 (270 MHz) and Varian XL-200 (50.3 MHz) spectrometers, respectively. ^{1}H-^{1}H COSY, ^{1}H-^{13}C COSY and NOE difference spectra were taken on a JEOL GX-400 (^{1}H, 400 MHz; ^{13}C, 100 MHz) spectrometer. Chemical shifts are given in δ-values (ppm) with tetramethylsilane as an internal standard. Mass spectra were measured with a JEOL JMS-D200 mass spectrometer at an inoization voltage of 70 eV. Optical rotations were taken on a JASCO DIP-140 polarimeter. CD spectrum was recorded on a JASCO J-500 spectrophotometer.

Plant Materials *Scoparia dulcis* was collected near Asuncion, Paraguay, in April 1986. Voucher specimens have been deposited in the herbarium of the herbal garden at Toyama Medical and Pharmaceutical University.

Isolation of SDB SDB (**2**) was isolated from a 70% EtOH extract of *Scoparia dulcis* as reported previously.[8] Yield 0.46%. mp 228—232 °C, $[\alpha]_D$ −49.6° (c=1.02, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3200, 1730, 1710, 1600, 1580. UV λ_{max}^{MeOH} nm (log ε): 229 (3.99), 265 (2.81), 272 (2.83), 280 (2.74). CD $[\theta]_{297}^{CHCl_3}$: +2490. EIMS m/z: 438 (M^+), 333 ($M^+ - C_6H_5CO$), 315 ($M^+ - C_6H_5COOH$–H), 301, 287, 271, 257, 105 ($C_6H_5CO^+$, base). ^{1}H- and ^{13}C-NMR: see Table I. HRMS m/z: 438.2420 (M^+, $C_{27}H_{34}O_5$ requires 438.2415). *Anal.* Calcd for $C_{27}H_{34}O_5$: C, 73.93; H, 7.82. Found: C, 74.11; H, 7.92.

Methylation of SDB SDB (100 mg) was treated with etherial diazomethane (CH_2N_2) for 5 h. After evaporation to a dry state, a crystalline material was purified by recrystallization from $CHCl_3$–MeOH to furnish methyl ester **3** as colorless needles (90 mg). mp 190—191 °C. $[\alpha]_D$ −42.0° (c=0.5, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 1710, 1600, 1580. UV λ_{max}^{MeOH} nm (log ε): 229 (4.42), 272 (3.05), 280 (2.99). ^{1}H-NMR ($CDCl_3$) δ: 1.09 (3H, s), 1.35 (3H, s), 1.54 (3H, s), 3.63 (3H, s), 5.20 (1H, d, J=2.0 Hz),

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7.46 (2H, t, J=7.3 Hz), 7.56 (1H, t, J=7.3 Hz). 8.02 (2H, d, J=7.3 Hz). *Anal.* Calcd for $C_{28}H_{36}O_5$: C, 74.30; H, 8.02. Found: C, 74.11; H, 7.92.

Hydrolysis of SDB A solution of SDB (100 mg) in dimethyl sulfoxide (DMSO) (0.2 ml) was added to a saturated solution of KOH in MeOH (1 ml) and the mixture was heated at 120 °C for 15 h in a sealed tube. The reaction mixture was then allowed to cool. The reaction product was diluted with water (1.2 ml) and the whole solution was acidified with 1 N HCl. The solution was extracted with EtOAc and the EtOAc layer was concentrated *in vacuo*. The residue was chromatographed on a silica gel column with $CHCl_3$ to produce benzoic acid (12 mg) and debenzoylated product (**4**) as colorless needles (34 mg). mp 224—288°C. $[\alpha]_D$+5.5° (c=0.4, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3500, 3420, 3250, 1710, 1690. ^{1}H-NMR ($CDCl_3$) δ: 1.08 (3H, s), 1.36 (3H, s), 1.58 (3H, s), 4.02 (1H, d, J=2.0 Hz). HRMS m/z: 334.2156 (M^+, $C_{20}H_{30}O_4$ requires 334.2142). *Anal.* Calcd for $C_{20}H_{30}O_4 \cdot 0.25\ H_2O$: C, 70.86: H, 9.07. Found: C, 71.10; H, 9.04.

2. Biological Activity Gastric Vesicles Hog gastric vesicles enriched in H^+,K^+-ATPase were isolated as described elsewhere.[17]

Enzyme Assay H^+,K^+-ATPase activity was measured in 1 ml of solution containing 10 μg protein, 3 mM $MgSO_4$, 3 mM ATP, 15 mM KCl and 40 mM Tris/HCl (pH 7.4) as described elsewhere.[14e] The reaction was done at 37 °C for 10 min.

Proton Transport Activity into Gastric Vesicles Proton transport into gastric vesicles was monitored by the quenching of acridine orange fluorescence.[18] Twenty micrograms of gastric vesicles were incubated in 1 ml of solution containing 150 mM KCl, 2 mM $MgCl_2$, 4 μM acridine orange, 10 μg of valinomycin and 5 mM Pipes/NaOH (pH 7.4) at 25 °C for 1 min. Proton uptake was started by the addition of 0.3 mM ATP. Fluorescence of acridine orange was excited at 493 nm and emitted at 530 nm.

3. Pharmacokinetic Experiments Materials SDB-Na was obtained from SDB as follows: SDB (1 g, 2.28 mmol) was mixed with $NaHCO_3$ (0.19 g, 2.25 mmol) and the mixture was stirred under dropping water. The reaction mixture was then evaporated to dryness to yield a colorless powder. After dissolving this powder in water, the solution was filtered. Evaporation of the filtrate furnished SDB-Na (0.94 g) as colorless powder. SDB-Na, $C_{27}H_{33}O_5Na$, positive FAB fast atom bombardment MS[19]: m/z 483 $(M+23)^+$, 461 $(M+1)^+$.

Animals Seven week-old male Wistar rats (Shizuoka Laboratory Animal Center, Hamamatsu, Japan) were used. The rats were allowed free access to water and laboratory food and were housed in a room with a 12/12 light-dark cycle for at least 2 weeks before the day of the experiment. One day before the experiment, rats were cannulated in the right jugular vein for blood sampling and drug administration, under light ether anesthesia. The catheter (Silastic Medical Grade Tube, Dow Corning Crop., Midland, MI and Intramedic PE-50, Clay Adams, Parsippany, NJ) was exteriorized at the back of the neck, filled with heparinized saline and plugged with a stainless-steel pin. After a 19 h recovery period, the rats were placed in individual metabolic cages (TP-85M, Tokyo Riko Co., Tokyo, Japan) and SDB-Na or SDB was administered intravenously or orally.

Plasma Disposition In the intravenous study, SDB-Na (5, 15, 27 mg/kg) was dissolved in distilled water, adjusting the pH of the solution to around 8 by 1 N HCl. This was then injected into the right jugular vein of each rat. Blood samples (0.5 ml each) were collected from the right jugular vein using a heparinized syringe at 2, 5, 10, 15 and 20 min (5 mg/kg dose) or at 2, 5, 10, 15, 20, 30, 60 and 90 min (15 mg/kg dose) or 5, 15, 30, 60 and 120 min (27 mg/kg dose) after the dosing. In the case of oral administration, SDB-Na (200 mg/kg) was dissolved in purified water, whereas SDB (200 mg/kg) was suspended in 30% polyethylene glycol 400, and was administered with a gastric catheter. The total volume of oral administration was less than 1 ml per rat. Blood samples were withdrawn from the right jugular vein through the cannula at 0.5, 1, 2, 4, 6, 10, 20 and 30 h after the administration. Plasma was separated by centrifugation at 10000 rpm for 2 min and was stored at −20°C until analysis.

Assay Method for SDB Plasma concentration of SDB was determined by high performance liquid chromatography (HPLC). The plasma sample (0.2 ml) was mixed with 1 ml of MeOH and centrifuged at 3000 rpm for 10 min. The supernatant was acidified with 1 N HCl and was evaporated to dryness. The residue was dissolved in 1 ml of MeOH containing 0.2% bezene as an internal standard, and 5 μl of the solution was injected into an HPLC. A Shimadzu LC-6A HPLC system (Kyoto, Japan) equipped with a UV detector (Shimadzu, model SPD-MIA) was used. The column used was a Chemco Nucleosil-7ph (150 × 4.6 mm, Takatsuki, Japan) and was maintained at 30 °C. The mobile phase consisted of 0.05% AcOH in CH_3CN–H_2O (56:44, v/v) and its flow rate was 0.5 ml/min. The eluent was monitored at 230 nm. For quantitative calculations, a computer software package program was used on a personal computer (PC-9801VM, NEC Corp., Tokyo, Japan). The urine was collected for 48 h after oral administration of SDB-Na (200 mg/kg) by metabolic cage. The urine sample (10 ml) was acidified with 1 N HCl and extracted with n-hexane (10 ml) three times. The n-hexane-insoluble part was lyophilized and dissolved in 10% HCl (7 ml). The acidic solution was then heated at 100°C in a sealed tube for 1.5 h. The reaction mixture was extracted with $CHCl_3$ (10 ml) three times and the $CHCl_3$ layer was evaporated to dryness. The n-hexane and $CHCl_3$ soluble parts were used for analysis of SDB by HPLC.

Pharmacokinetic Analysis The AUC and the area under the first moment time curve ($AUMC$) were calculated by trapezoidal intergration with extrapolation to finite time as described previously.[20] The CL was determined from the ratio of dose to AUC. The MRT was estimated by dividing $AUMC$ by AUC. The volume of distribution at steady state was calculated as the product of CL and MRT. The plasma concentrations of SDB following i.v. administration of SDB-Na were fitted to a two-compartment open mocel with Michaelis–Meten elimination, as shown in Fig. 5. The differential equations are as follows:

$$\frac{dA_1}{dt} = -\left(k_{12} + \frac{V_{max}}{K_m + A_1}\right)A_1 + k_{21}A_2 \tag{1}$$

$$\frac{dA_2}{dt} = k_{12}A_1 - k_{21}A_2 \tag{2}$$

$$C = \frac{A_1}{V_c} \tag{3}$$

at $t=0$, $A_1=D$, $A_2=C=0$

where A_1 and A_2 are the amount of SDB in the central compartment and in the peripheral compartment, respectively. k_{12} and k_{21} are the inter-compartmental first-order rate constants. V_c is the volume of distribution at the central compartment. V_{max} and K_m are the maximum rate and the Michaelis constant, respectively. C is the concentration of SDB at central (plasma) concentration and D is the dose divided by the total body mass (in mg/kg). After oral administration of SDB-Na, plasma concentration is described by the following Eqs. 4 through 7.

$$\frac{dA_{ab}}{dt} = -k_a A_{ab} \tag{4}$$

$$\frac{dA_1}{dt} = -k_a A_{ab} - \left(k_{12} + \frac{V_{max}}{K_m + A_1}\right)A_1 + k_{21}A_2 \tag{5}$$

$$\frac{dA_2}{dt} = k_{12}A_1 - k_{21}A_2 \tag{6}$$

$$C = \frac{A_1}{V_c} \tag{7}$$

at $t=0$, $A_{ab}=DF$, $A_1=A_2=C=0$

where A_{ab} is the amount of SDB at the absorption site, k_a is the first-order absorption rate constant and F is the fraction of dose to be absorbed.

Model fitting by nonlinear regression of Eqs. 1—3 in this intravenous study of SDB for 5, 15 and 27 mg/kg dose was performed on a Micro-vax II minicomputer (DEC Corp. Maynard, MS) using the FKDM program[21] to generate parameter estimates for k_{12}, k_{21}, V_{max}, K_m and V_c. The parameters for F and k_a were separately estimated for oral dose using Eqs. 4—7. The numerical integration of the differential equations was performed using the Runge–Kutta–Gill procedure, and all data used in the nonlinear regression analysis were weighted with the reciprocals of the data values. The condition of convergency was described elsewhere.[22]

Statistical Analysis The differences among various experimental conditions were compared by using ANOVA and the Student's t-test. A 0.05 level of probability was used as the level of significance.

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