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Analysis of 1,4-Dimorpholino-7-phenylpyrido[3,4-*d*]pyridazine (DS-511) and Its Metabolites in Biological Specimens. I. Identification of Metabolites in Rat Urine and Dog Urine and Bile

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The metabolic fate of 1,4-dimorpholino-7-phenylpyrido[3,4-*d*]pyridazine (DS-511) was studied in the rat and the dog. Urine and bile were extracted with ethyl acetate before and after enzymatic hydrolysis and separated into lipophilic and hydrophilic fractions. The structures of seven lipophilic and two hydrophilic metabolites obtained on thin-layer chromatograms were investigated by comparing their ultraviolet, infrared and nuclear magnetic resonance spectra, *Rf* values on thin-layer chromatograms and other physicochemical properties with those of synthetic samples. The main metabolites were 7-(4-hydroxyphenyl)-1,4-dimorpholinopyrido[3,4-*d*]pyridazine and 4-[N-carboxymethyl-N-(2-hydroxyethyl)amino]-1-morpholino-7-phenylpyrido[3,4-*d*]pyridazine. In addition, 7-(4-hydroxy-3-methoxyphenyl)-1,4-dimorpholinopyrido[3,4-*d*]pyridazine and 7-(4-hydroxy-3-methoxyphenyl)-4-morpholinopyrido[3,4-*d*]pyridazin-1(2H)-one were observed as minor metabolites in the dog and the rat, respectively.

Keywords——1,4-dimorpholino-7-phenylpyrido[3,4-*d*]pyridazine; DS-511; metabolism; two-dimensional thin-layer chromatography; ultraviolet spectrum; fluorescence; gas chromatography–mass spectrometry

A large number of pyrimido[4,5-*d*]pyridazines and pyrido[3,4-*d*]pyridazines, synthesized by Yurugi and his collaborators,[2] showed distinct diuretic activity.[3] Among them, 1,4-dimorpholino-7-phenylpyrido[3,4-*d*]pyridazine (DS-511, **1**) was selected for further study because of its characteristic diuretic action[4] and low toxicity.[5] The present paper describes a study on metabolites of **1** in the urine and bile after oral administration to experimental animals.

Experimental

Urine and Bile——Twenty male Sprague-Dawley rats weighing about 260 g, 7 to 8 weeks old, were housed in metabolic cages and given orally 10 ml/kg of a 50 mg/ml suspension of **1** prepared using gum arabic. In 24 hr, a total of 444 ml of urine was collected from the rats, which had received a total of 2.575 g of **1**. Dog urine was obtained from a male beagle dog weighing 10.6 kg fed about 20 g of dog food mixed with 530 mg of **1**. A total of 230 ml of urine was collected in 24 hr. Bile samples were obtained from beagle dogs used for studies on the chronic toxicity of **1**. Dogs of either sex weighing about 10 kg were given **1** with a dosage regimen of 500 mg/kg/day for a year. The bile in the gall-bladder, 11 ml, was collected by sacrifice 24 hr after the final dose.

1) Location: *Jusohonmachi, Yodogawa-ku, Osaka 532, Japan.*
2) S. Yurugi, M. Hieda, T. Fushimi, Y. Kawamatsu, H. Sugihara, and M. Tomimoto, *Chem. Pharm. Bull.* (Tokyo), **20**, 1528 (1972); S. Yurugi, T. Fushimi, H. Sugihara, and M. Hieda, *Yakugaku Zasshi*, **92**, 1333 (1972); S. Yurugi, *Takeda Kenkyusho Nempo*, **34**, 53 (1975).
3) K. Nishikawa, H. Shimakawa, Y. Inada, Y. Shibouta, S. Kikuchi, S. Yurugi, and Y. Oka, *Chem. Pharm. Bull.* (Tokyo), **24**, 2057 (1976).
4) K. Nishikawa, Y. Inada, H. Shimakawa, I. Kuramoto, M. Isono, and S. Kikuchi, *Takeda Kenkyusho Nempo*, **32**, 539 (1973); Y. Inada, Y. Shibouta, H. Shimakawa, K. Nishikawa, and S. Kikuchi, *Arzneim.-Forsch.*, **28** (II), 1105 (1978).
5) H. Kawaji, R. Tsukuda, K. Nishikawa, S. Kikuchi, and T. Hirano, *Takeda Kenkyusho Nempo*, **32**, 299 (1973).

Thin-Layer Chromatography (TLC)——Silica gel plates with a 0.5 or 0.3 mm layer of E. Merck Kieselgel HF_{254} type 60 containing fluorescent indicator were activated at 105° for 2 hr. The solvent systems used were:

Solvent I (S-I) : chloroform–methyl alcohol (10: 1).
Solvent II (S-II) : ethyl acetate–benzene (3: 2).
Solvent III (S-III): butyl alcohol–ammonia water (2.8% NH_3)–ethyl alcohol (5: 2: 1).
Solvent IV (S-IV) : butyl acetate–acetic acid–water–acetone (10: 6: 4: 3).
Solvent V (S-V) : chloroform–methyl alcohol (2: 1).

Gas Chromatography (GC)——A Shimadzu 5A gas chromatograph with a flame ionization detector was used with a U-shaped, glass column (3 mm i.d., 1 m long), packed with 1% SE-30 on Chromosorb W (80 to 100 mesh). Gas flows were held constant at 80 (helium), 50 (hydrogen) and 800 (air) ml/min. The temperature of the column bath was 250° and that of the injection and detector regions was 290°.

Apparatus——The instruments used for the determination of chemical structure were: a Shimadzu UV-200 ultraviolet (UV) spectrometer, a Hitachi EPI-S2 infrared (IR) spectrometer, a Varian EM-390 nuclear magnetic resonance (NMR) spectrometer, a Hitachi RMU-6D mass spectrometer and a Japan Electronic JGC-20K-JMSOISC gas chromatograph–mass spectrometer.

Fractionation of Metabolites——The fractionation procedure is shown schematically in Chart 1. The urine or bile was adjusted to pH 7 and extracted with two volumes of AcOEt. The AcOEt layer (Fr. I) was washed with water and evaporated to dryness under a vacuum. The residue was reconstituted in 1 ml of AcOEt and separated by two-dimensional TLC using Solvents I and II. Each development was repeated twice to obtain larger *Rf* values. The aqueous layer was adjusted to pH 6.8 and incubated with 15 mg of β-glucuronidase (Sigma Chemical, Type I: 63250 units/g) at 37° for 24 hr. The pH of the resulting solution was adjusted to 5.0 and the solution was similarly incubated with 15 mg of arylsulfatase (Sigma Chemical, Type H-1: 31000 units/g). The mixture was then extracted at pH 7 with two volumes of AcOEt, and the AcOEt layer (Fr. II) was worked up as described for Fr. I. The separation procedure for the aqueous layer (Fr. III) differed in the cases of urine and bile. In the case of urine, Fr. III was adsorbed on a column of Amberlite XAD-2 and eluted with 50 ml of EtOH after being washed with water. The concentrated eluate was separated by two-dimensional TLC developed with Solvents III and IV. To separate metabolites in Fr. III obtained from bile, column treatment was unnecessary. Fr. III was evaporated to dryness by repeated addition of EtOH to the residue and re-evaporation. The dried residue was mixed with 5 ml of EtOH and the EtOH-soluble portion was concentrated and subjected to two-dimensional TLC using Solvents

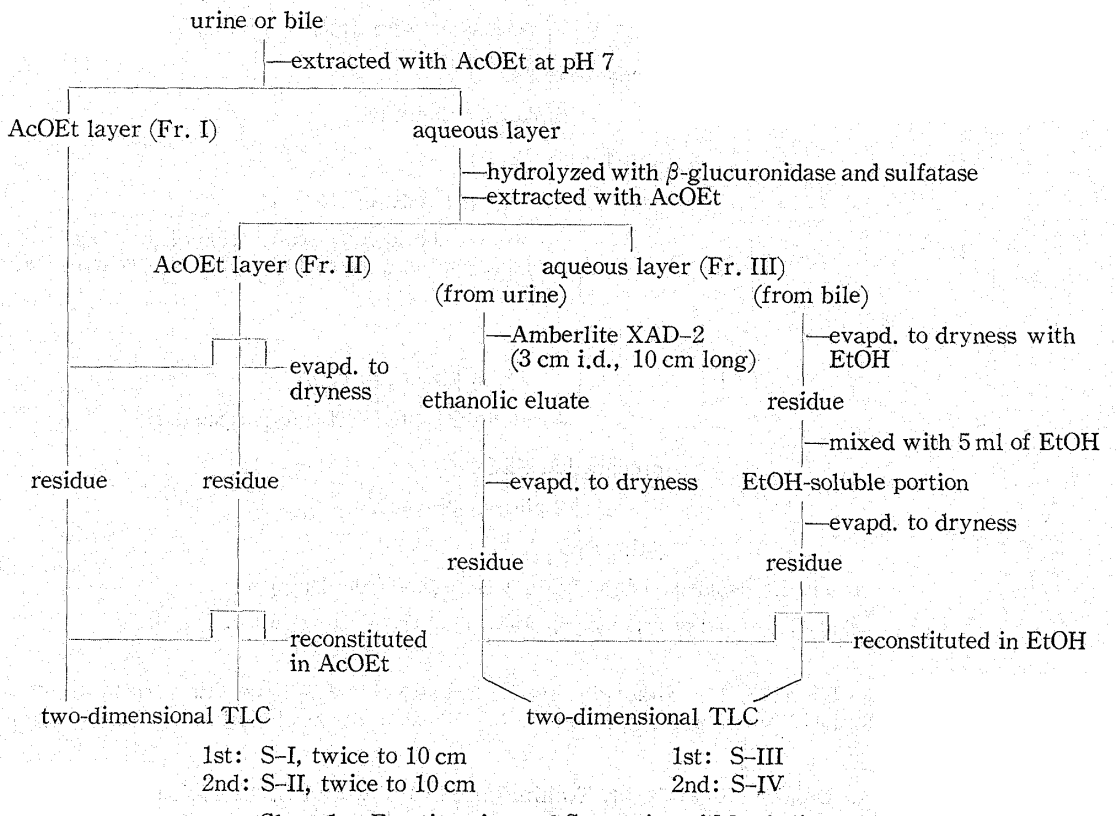

Chart 1. Fractionation and Separation of Metabolites

III and IV. The chromatograms were observed under UV light and the spots attributable to metabolites were selected by comparison with control specimens.

Isolation of the Main Metabolites——Dog bile was processed as outlined in Chart 2. Bile, 10 ml, was mixed with an equal volume of water and after adjusting the pH, was subjected to enzymolysis as described in the previous section. The mixture was extracted with AcOEt and the extract was evaporated to dryness, dissolved in $CHCl_3$ and subjected to preparative TLC developed with Solvent I. Each metabolite spot was eluted with MeOH and the centrifuged supernatant of the extract was evaporated to dryness. The residue was dissolved in $CHCl_3$ and subjected to a second preparative TLC using Solvent II, followed by a similar work-up. The aqueous layer remaining after extraction with AcOEt was evaporated to dryness by repeated addition of EtOH and re-evaporation. The dried residue was dissolved in EtOH and passed through a column of Sephadex LH-20 monitored by UV. Ten ml fractions of the eluate were collected in tubes; the first six tubes were discarded, and then the contents of groups of ten tubes were examined by TLC (Solvent III) for the presence of metabolites. The first and second pooled fractions, Frs. A and B, in which metabolites were detected, were each evaporated to dryness and the residue was purified by preparative TLC using Solvent III after being dissolved in MeOH. The MeOH eluate was reconstituted in 1 ml of MeOH and rechromatographed using Solvent IV followed by a similar work-up. The MeOH eluate, after evaporation to dryness, was mixed with 5 ml of 1% $NaHCO_3$ and the supernatant was adsorbed on a column of Amberlite XAD-2 after washing with AcOEt. The column was washed with water and eluted with EtOH. Finally the eluate was purified for the third time by preparative TLC using Solvent V.

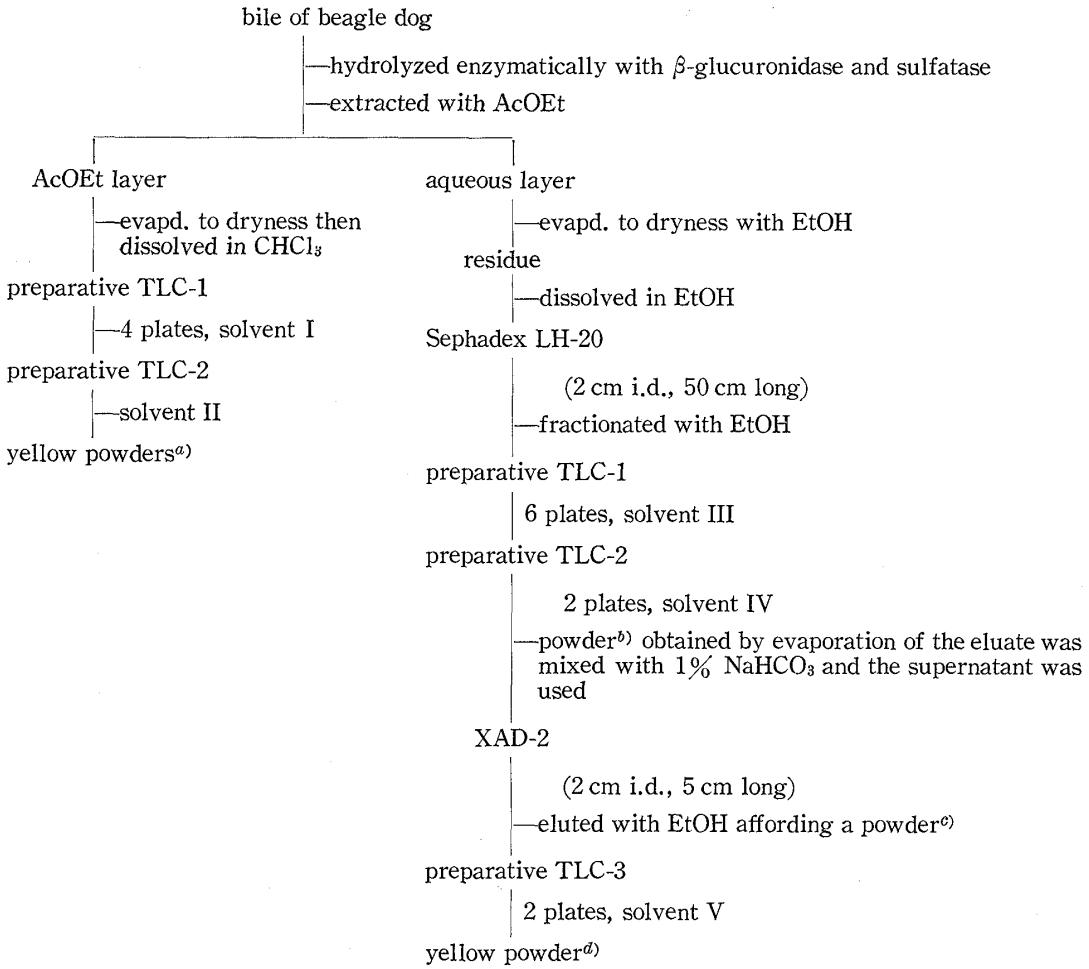

Chart 2. Isolation of the Main Metabolites from Dog Bile

a) spot b_o, 2.5 mg; spot c, 7 mg. *b*) 35 mg. *c*) 17 mg. *d*) 14 mg (spot e).

Authentic Samples——DS-511 (1), 4-morpholino-7-phenylpyrido[3,4-*d*]pyridazin-1(2H)-one[6] (2), 1-morpholino-7-phenylpyrido[3,4-*d*]pyridazin-4(3H)-one[6] (3), 7-(3-hydroxyphenyl)-1,4-dimorpholinopyrido-

6) Y. Oka, K. Omura, A. Miyake, K. Itoh, M. Tomimoto, N. Tada, and S. Yurugi, *Chem. Pharm. Bull.* (Tokyo), 23, 2239 (1975).

[3,4-*d*]pyridazine[7] (**4**) and 7-(4-hydroxyphenyl)-1,4-dimorpholinopyrido[3,4-*d*]pyridazine[7] (**5**) were kindly provided by Dr. Oka of this division. 7-(4-Hydroxy-3-methoxyphenyl)-1,4-dimorpholinopyrido[3,4-*d*]-pyridazine (**6**) was synthesized from acetovanillon benzyl ether[8] by a procedure similar to that described by Oka *et al.*[7] for the synthesis of **4** and **5**. The compound thus obtained was a yellow crystalline powder. mp 133—137°. *Anal.* Calcd. for $C_{22}H_{25}N_5O_4 \cdot H_2O$: C, 59.85; H, 6.16; N, 15.86. Found: C, 59.73; H, 5.97; N, 15.57. UV λ_{max}^{MeOH} nm: 325. IR ν_{max}^{KBr} cm^{-1}: 3500—3200 (OH), 2970—2800 (CH), 1605, 1515 (C=C), 1280, 1120 (C–N, C–O: N O). MS *m/e*: 423 (M^+), 366 ($M^+-C_3H_5O$), 365 ($M^+-C_3H_6O$), 252 (M^+-2N O$+H$), 86 (N O). 7-(4-Hydroxy-3-methoxyphenyl)-4-morpholinopyrido[3,4-*d*]pyridazin-1(2H)-one (**7**) and 7-(4-hydroxy-3-methoxyphenyl)-1-morpholinopyrido[3,4-*d*]pyridazin-4(3H)-one (**8**) were prepared by the hydrolysis of **6** followed by TLC separation using Solvent II described above. The separated compounds were tentatively assigned by analogy with the *Rf* values of **2** and **3**. The *Rf* values of **7** and **8** were 0.29 and 0.26, respectively. 7-(4-Hydroxyphenyl)-4-morpholinopyrido[3,4-*d*]pyridazin-1(2H)-one (**9**) and 7-(4-hydroxyphenyl)-1-morpholinopyrido[3,4-*d*]pyridazin-4(3H)-one (**10**) were similarly prepared from **5**; the *Rf* values of **9** and **10**, assigned as above, were 0.29 and 0.26, respectively. 4-[N-Carboxymethyl-N-(2-hydroxyethyl)amino]-1-morpholino-7-phenylpyrido[3,4-*d*]pyridazine sodium salt (**11**) and 1-[N-carboxymethyl-N-(2-hydroxyethyl)amino]-4-morpholino-7-phenylpyrido[3,4-*d*]pyridazine sodium salt (**12**) were synthesized from 4-chloro-1-morpholino-7-phenylpyrido[3,4-*d*]pyridazine[7] (**13**) and 1-chloro-4-morpholino-7-phenylpyrido[3,4-*d*]pyridazine[7] (**14**), respectively, by replacing the Cl atom with an $-NHCH_2CH_2OH$ group by heating with ethanolamine to 130°, then introducing a $-CH_2COOCH_3$ group on this NH by reaction with methyl bromoacetate in DMF followed by hydrolysis with $NaHCO_3$. The compounds thus obtained were both hygroscopic yellow powders. **11**, mp about 265° (dec.). *Anal.* Calcd. for $C_{21}H_{22}N_5NaO_4 \cdot H_2O$: C, 56.12; H, 5.38; N, 15.58. Found: C, 56.80; H, 5.38; N, 15.52. UV λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$): 275 (580). IR ν_{max}^{KBr} cm^{-1}: 3550—3200 (OH), 2950—2800 (CH), 1610 (COO^-), 1120 (CO: N O). **12**, mp about 210° (dec.). *Anal.* Calcd. for $C_{21}H_{22}N_5NaO_4 \cdot H_2O$: C, 56.12; H, 5.38; N, 15.58. Found: C, 56.58; H, 5.04; N, 15.20. UV

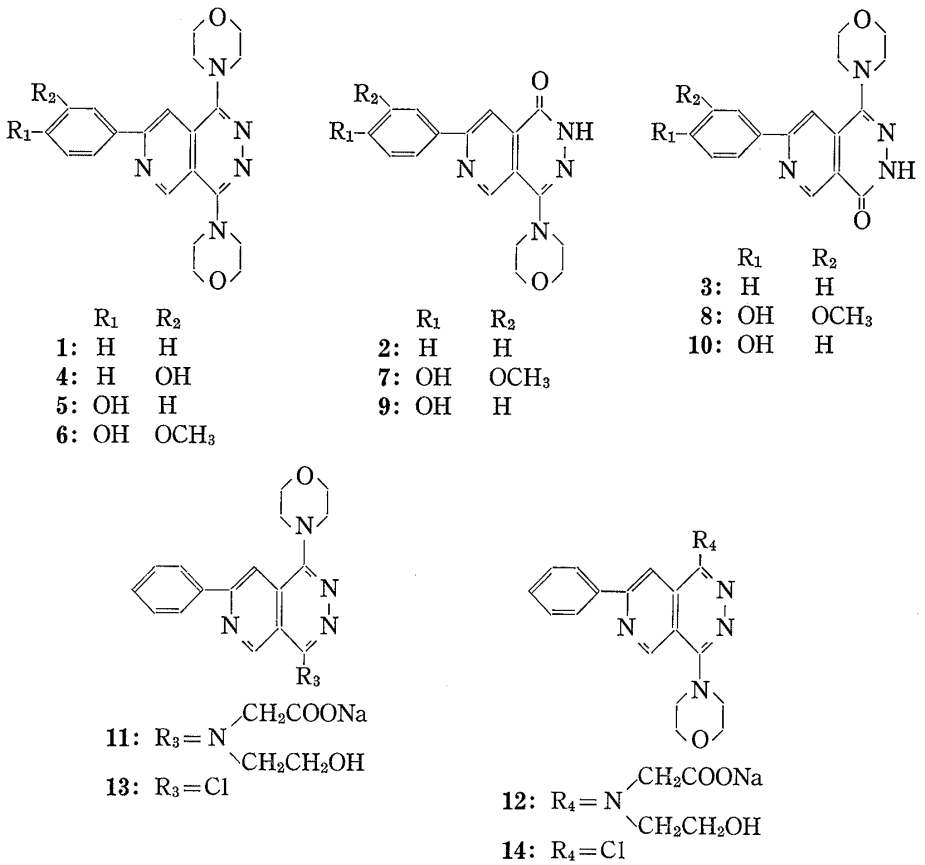

Chart 3. Structures of DS-511 and Related Compounds

7) Y. Oka, K. Itoh, A. Miyake, N. Tada, K. Omura, M. Tomimoto, and S. Yurugi, *Chem. Pharm. Bull.* (Tokyo), 23, 2306 (1975).
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λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$): 280 (421). IR ν_{max}^{KBr} cm^{-1}: 3550—3200 (OH), 2950—2800 (CH), 1610 (COO^-), 1120 (CO: ). (Chart 3).

UV absorption data and *Rf* values of the authentic compounds are given in Table I.

Table I. Physico-chemical Characteristics of DS-511 and Related Compounds

Compound	Ultraviolet absorption (nm)			*Rf* value on TLC (Kieselgel HF_{254})	
	λ_{max}^{MeOH}	Acidic λ_{max}^{MeOH}	Basic λ_{max}^{MeOH}	$CHCl_3$–MeOH (10:1)	AcOEt–C_6H_6 (3:2)
1	285	307	285	0.76	0.20
2	283	283	283	0.65	0.45
3	270	270	270	0.59	0.39
4	283	308	293	0.53	0.13
5	313	340	365	0.52	0.13
6	325	355	385	0.62	0.14
7	330	330	390	0.54	0.29
8	315	315	390	0.52	0.26
9	315	318	375	0.33	0.29
10	305	307	375	0.30	0.26
11	275	295	275	0.01	0
12	280	295	280	0.01	0

Results

Detection of Metabolites by Two-dimensional TLC

Two-dimensional chromatograms of Frs. I, II and III obtained from rat urine are shown in Fig. 1. Figs. 2 and 3 show those of dog urine and bile, respectively. Chromatograms of Fr. II resembled those of Fr. I except for the absence of spots due to **1** (spot 1) and spot 9. The pattern of each fraction was almost the same in dog urine and bile but was different for dog and rat. About ten spots were found in the chromatograms of the lipophilic fractions (Frs. I and II) obtained from rat urine, but only five spots in those from dog urine or bile. Among these five spots, spot 11 was specific to the dog. No marked difference was observed among the chromatograms of the hydrophilic fractions from rat urine, dog urine and dog bile.

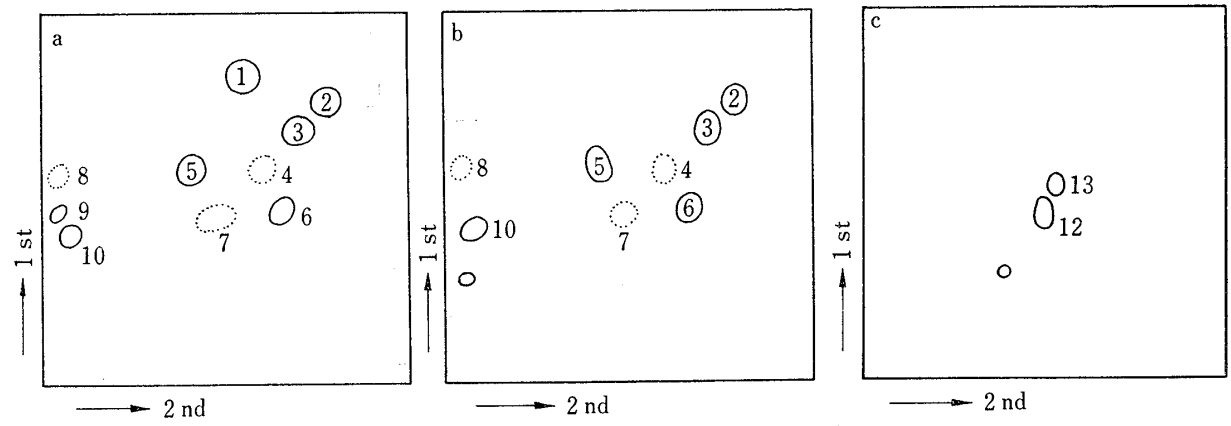

Fig. 1. Two-dimensional Thin-Layer Chromatograms of Rat Urine[a)]

a : Fr. I, solvent: 1st, S-I; 2nd, S-II.
b : Fr. II, solvent: as above.
c : Fr. III, solvent: 1st, S-III; 2nd, S-IV.
a) The rats were given a dosage of 500 mg/kg orally.

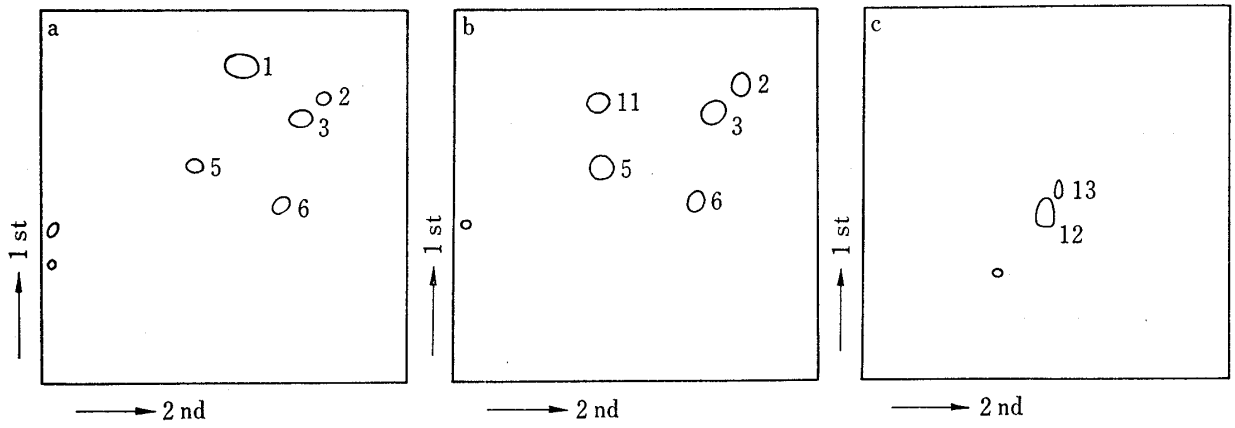

Fig. 2. Two-dimensional Thin-Layer Chromatograms of Dog Urine[a)]

a, b, c: Frs. I, II and III, respectively. The solvents are given in Fig. 1.
a) The dog was given a dosage of 50 mg/kg orally.

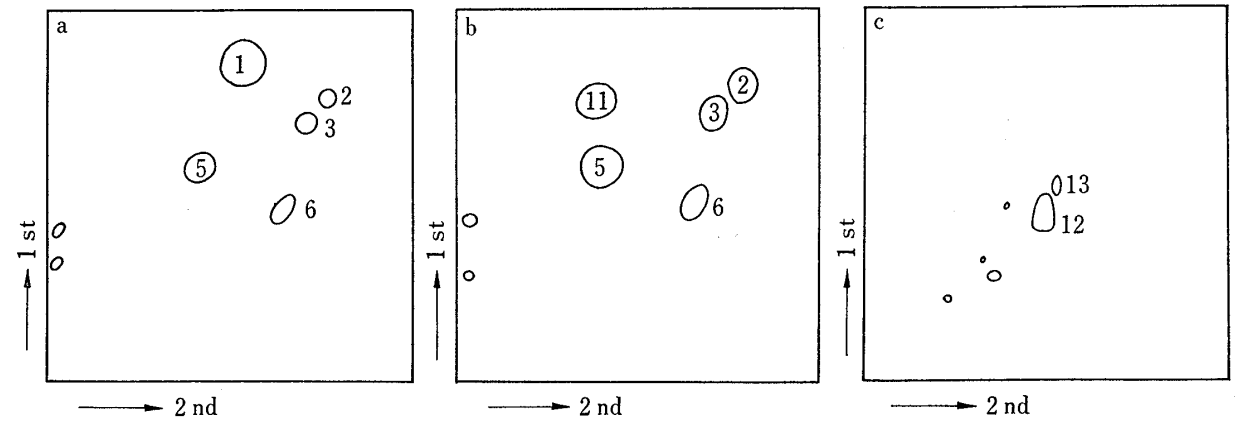

Fig. 3. Two-dimensional Thin-Layer Chromatograms of Dog Bile[a)]

a, b, c: Frs. I, II and III, respectively. The solvents are given in Fig. 1.
a) The dogs were given a dosage of 500 mg/kg/day orally for a year.

Isolation of Metabolites

For isolation of the main metabolites, dog bile was chosen as a source since it was available in the largest amount. Spots a, b and c in Fig. 4 obtained on a preparative TLC plate (solvent I) of the ethyl acetate extract of enzymatically hydrolyzed bile were assigned as spot 1, an overlapped spot of spots 2, 3 and 11, and spot 5 in Fig. 3, respectively, from their *Rf* values. There was no distinct spot in Fig. 3 which corresponded to spot d in Fig. 4. A second preparative TLC using solvent II separated spot b into three spots which showed *Rf* values that coincided with those of spots 2, 3 and 11, respectively. Among them, spot b_0 with the smallest *Rf* value was the major component, and after extraction with methanol followed by transfer to chloroform, 2.5 mg of fairly pure compound was obtained as a yellow powder. This corresponded to spot 11 in Fig. 3. Preparative TLC (solvent II) of spot c,

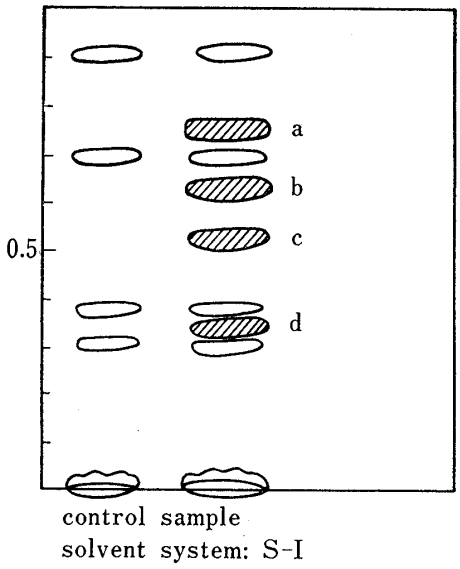

Fig. 4. Preparative Thin-Layer Chromatogram of the AcOEt Layer

corresponding to spot 5, gave only one spot which afforded 7 mg of yellow powder on similar work-up. Spot d gave a spot of weak UV absorption at the origin on TLC when developed with solvent II. Though isolation of this substance was impossible, we assumed that it corresponded to the weak spot at the middle left region of Fig. 3b. From the water-soluble portion of the enzyme-treated bile, 14 mg of yellow powder, spot e (Chart 2), was obtained. The *Rf* value in TLC showed that this compound corresponded to spot 12 and was the main component of both Frs. A and B.

Identification of Metabolites

Metabolites in quantities too small to be isolated were eluted from the TLC plates with 10 ml of methanol and directly subjected to spectroscopic analysis. To determine chemical structures, UV spectra, fluorescent properties, *Rf* values, mass spectra and other physicochemical properties of authentic compounds **1**—**5** as well as **6**—**12** were used.

Spot 1: This spot was identified as **1** from its *Rf* values, UV absorption maximum at 285 nm and its characteristic bathochromic shift of 20 nm in an acid solution.

Spot 2: This spot was identified as **2** from its *Rf* values, mass spectrum and UV maximum at 283 nm, which did not exhibit any significant shift in either alkaline or acid medium.

Spot 3: This spot was identified as **3** in a similar manner (UV maximum at 270 nm).

Spot 4: A large bathochromic shift, 55 nm, of the UV maximum (330 nm) in alkaline solution suggested the existence of a *para*-hydroxy substituent in the phenyl group at the 7-position of **1**. Loss of one of the morpholino groups was also deduced from the disappearance of the shift of the UV maximum in an acidic medium, as shown in the change from **1** to **2** or **3**. However, **9** and **10** prepared from **5** showed UV maxima at 315 and 305 nm, respectively, which were at about 10—20 nm shorter wavelength than that of spot 4. This difference suggested that the phenyl group was substituted by a second functional group. Later, compounds **7** and **8** were synthesized from **6** and the UV maximum, pH changes, and *Rf* value as well as the retention time (t_R) of the trimethylsilyl (TMS) derivative of **7** coincided with those of spot 4. This compound has not been detected in dog urine or bile.

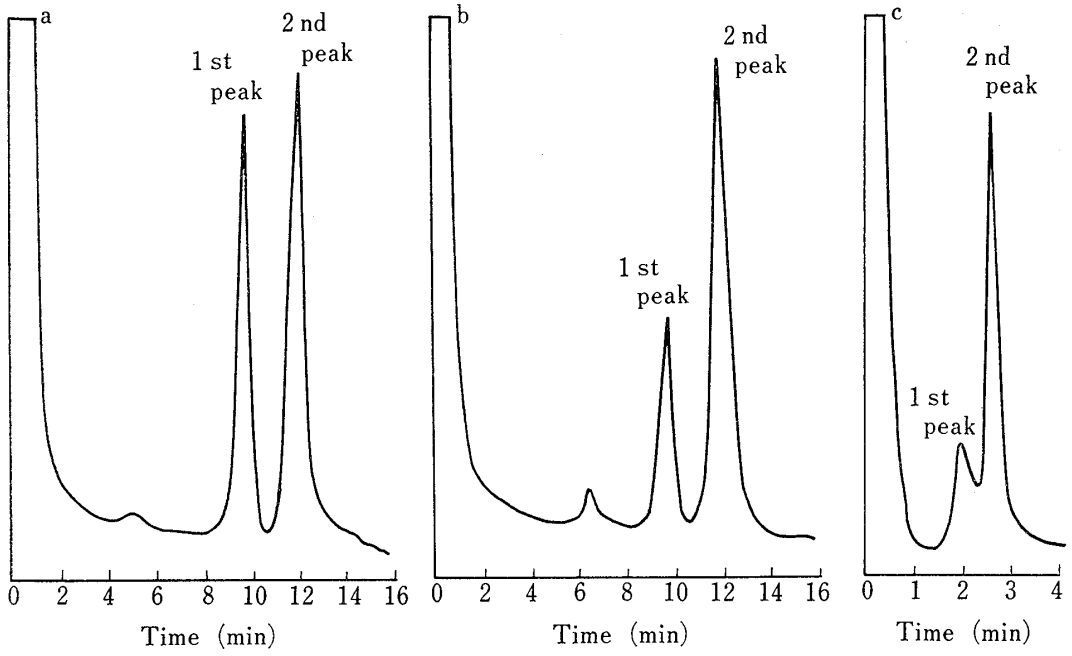

Fig. 5. Gas Chromatograms of Trimethylsilyl Derivatives of Spots 5 and 6 on Two-dimensional Thin-Layer Chromatograms

a: spot 5 (rat urine). b: spot 5 (dog bile). c: spot 6.

Spot 5: GC of the TMS derivative showed two peaks (t_R=9.7 and 12.0 min), both of which gave M^+ at *m/e* 465 in mass spectrometry (MS), suggesting that this spot was a mixture of **4** and **5**. On comparison with gas chromatograms of trimethylsilylated **4** and **5**, the peak at the shorter retention time (9.7 min) was assigned to **4** whereas that at t_R=12.0 min coincided with **5**. The *Rf* values were also identical. From the peak area of the gas chromatogram, the content of **5** was estimated to be nearly equal to that of **4** in rat urine (Fig. 5a). Spot 5 from dog bile was composed of the same compounds but the ratio of **5**: **4** was 7: 3 in this case (Fig. 5b).

Spot 6: The UV maximum (315 nm) and its bathochromic shift of 55 nm in alkaline solution, together with the slight shift in acid, suggested the structure **9** or **10**. The gas chromatogram of its TMS derivative gave two peaks, at t_R=2.0 min and t_R=2.7 min, which were identified as those of trimethylsilylated **10** and **9**, respectively. As shown in Fig. 5c, the content of **9** was much larger than that of **10**. Their relative contents were similar in dog and rat.

Spots 7, 8, 9 and 10: The structures of these compounds were not determined. They have UV maxima at a shorter wavelength than **1** and showed bathochromic shifts of 10—20 nm in acid but not in alkaline solution. On treatment with 1 N sodium hydroxide at 100° for 1 hr, spots 9 and 10 were hydrolyzed to give spots with *Rf* values which coincided with those of **2** and **3**. Since **1** resisted the same treatment, spots 9 and 10 were assumed to be produced by metabolic conversion of one of the morpholino groups in **1**.

Spot 11: This compound was identified as spot b_0 obtained above. A UV maximum at longer wavelength than that of **5** and bathochromic shifts of 30 nm in acid and 60 nm in alkali suggested the structure **6**. *Rf* values in TLC coincided with those of **6**. Furthermore, GC–MS of the TMS derivative of spot 11 showed M^+ at *m/e*=495, suggesting a methoxy derivative of **5**.

Spot 12: This spot was identified as spot e, as described above. The IR spectrum showed an absorption band due to a carboxylate anion (1610 cm^{-1}). The NMR spectrum in D_2O showed a multiplet at δ=2.9—3.2 ppm of four protons instead of eight protons as in **1**, **4** or **5**. One of the morpholine rings may be cleaved to give a carboxyl group. On methylation with diazomethane, a broad one-proton signal which disappeared on treatment with D_2O was located at δ=6.54—6.86 ppm in the NMR spectrum (in $CDCl_3$), suggesting the existence of a hydroxyl group. Thus, a partial structure of $N\begin{matrix}\diagup CH_2CH_2OH \\ \diagdown CH_2COONa\end{matrix}$ for spot 12 was assumed. Multiplets of four protons at δ=3.24—3.49 ppm and eight protons at δ=3.82—4.10 ppm and a singlet of two protons at δ=4.17 ppm supported this structure. To determine which morpholine ring was opened, spot 12 was hydrolyzed with 1 N sodium hydroxide at 100° and, after extraction with ethyl acetate, the resulting product was identified as **3**. Thus, the structure of spot 12 was determined to be **11**, and this was confirmed by the synthesis of **11** from **13**.

Spot 13: The similarity of the UV and *Rf* values with those of spot 12 suggested the structure **12**. The physico-chemical properties coincided with those of **12** subsequently synthesized.

Discussion

No significant difference seems to exist between the metabolic pathways of **1** in the rat and dog. The metabolites found in dog urine and bile were also similar.

The main metabolic pathways of **1** in these experimental animals were *para* hydroxylation of the phenyl group at position 7 and ring cleavage of one of the morpholino groups, especially the one at position 4. Hydroxylation of the phenyl group also seems to take place

at the *meta* position and some metabolites which were assumed to have both *p*-hydroxy and *m*-methoxy groups were detected. Catechol-type compounds, *i.e.*, *p*- and *m*-dihydroxy derivatives may be formed as metabolites, but they may be too unstable to be isolated by the method used in this work. The rupture of the morpholine ring may proceed through oxidation of the methylene group α to the oxygen atom.[9] We identified **11** and **12** from the hydrophilic fraction of urine and bile. These compounds might, however, be artifacts produced from 2-oxo-morpholine derivatives of **1** formed by oxidation during the incubation with enzymes or other separation processes. It is not clear whether the metabolites which have only one morpholino group, *i.e.*, **2**, **3**, **7**, **9** and **10** arise by direct hydrolysis of **1**, **6** and **5** or whether the hydrolysis proceeded through compounds having **11**- or **12**-type structures.

Quantitative analysis of these metabolites in biological specimens will be described in a forthcoming paper.

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