CHEMICAL & PHARMACEUTICAL BULLETIN

Vol. 23, No. 6 June 1975

Regular Articles

[Chem. Pharm. Bull. 23(6)1173—1183(1975)]

UDC 547.587.51.09 : 615.217.24.015.4.076.9

Metabolic Studies on CS-359, a New β-Adrenergic Blocking Agent. I. Isolation and Identification of the Urinary Metabolites of CS-359[1]

Ryozo Hayashi, Minoru Okada, Tadamasa Nakazawa, Yorihisa Tanaka, Yasuo Shimoji, and Yasunobu Sato

Central Research Laboratories, Sankyo Co., Ltd.[2]

(Received August 13, 1974)

On the basis of the spectral examinations of the isolated materials and direct comparisons with chemically synthesized samples, the main metabolites of 5-methyl-8-(2-hydroxy-3-*t*-butylaminopropoxy)coumarin (CS-359) in excreta of rats and dogs were established as the 5-hydroxymethyl and 5-carboxyl compounds, resulting from oxidation of the 5-methyl group of the parent compound. 8-Oxyacetic acid was also identified as a minor metabolite. Thin-layer chromatograms of the samples from urine and feces exhibited the similar pattern, irrespective of animal species or routes of administrations. Results from fractionation by AcOEt extraction and paper electrophoresis suggested that both neutral and acidic metabolites may be minor components. Experiments on enzymatic hydrolysis indicated that neither glucuronide (s) nor sulfate(s) occurred in significant amounts in excreta.

Possible metabolic pathways of CS-359 are presented.

Synthesis and pharmacology of 5-methyl-8-(2-hydroxy-3-*t*-butylaminopropoxy)coumarin (CS-359), a new β-adrenergic blocking agent developed in our laboratories, have been reported by Sato, *et al.*[3] and Oshima, *et al.*[4] The present paper deals with studies on isolation and identification of the urinary metabolites of CS-359 in dogs, together with their chemical synthesis.

Experimental

Spectral and Gas Chromatographic Determinations——Proton nuclear magnetic resonance (NMR) spectra were determined with Varian A-100, mass spectra with TEOL TMS OISG Mass Spectroscopy and infrared (IR) spectra with Hitachi EPI-S2 or with JASCO J-0004. Gas chromatography–mass spectrometry (GC–MS) was carried out with LKB-9000 and gas chromatography (GC) with Hewlett Packard FDM 400. Trimethylsilyl (TMS) derivatives for gas chromatographic determinations were obtained either by heating sample solutions in N,O-bis(trimethylsilyl)acetamide (BSA) at 60° for 1 hr or by treatment of the samples in a mixture of BSA and trimethylchlorosilane (TMCS) at room temperature overnight.

---

1) This work was presented at the 93rd Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, April, 1973.

2) Location: *1-2-58, Hiromachi, Shinagawa-ku, Tokyo, 140, Japan.*

3) Y. Sato, Y. Kobayashi, T. Nagasaki, T. Oshima, S. Kumakura, K. Nakayama, H. Koike, and H. Takagi, *Chem. Pharm. Bull.* (Tokyo), **20**, 905 (1972).

4) T. Oshima, S. Kumakura, H. Koike, and K. Nakayama, *Japan J. Pharmacol.*, 23, 497 (1973).

Thin-Layer Chromatography (TLC)——Samples of urine and feces from rats or dogs administered with CS-359-5-$^{14}CH_3$ were applied on Kieselgel F_{254} plates (Merck) and TLC developed with the following solvent systems either by double development in one direction or by two-dimensional technique: No. 1 $CHCl_3$–C_6H_6–EtOH–NH_4OH (6: 14: 8: 1), No. 2 BuOH–AcOH–$(C_2H_5)_2O$–H_2O (5: 3: 10: 1), No. 3 $CHCl_3$–$(CH_3)_2$-CO–Et_2NH–EtOH–H_2O (40: 32: 8: 8: 1), No. 4 iso-PrOH–$CHCl_3$–NH_4OH (80: 5: 15), No. 5 BuOH–EtOH–NH_4OH (10: 5: 2).

Animals——Wistar-Imamichi male rats weighing *ca.* 150 g were used after 18 hr starvation. Crystals of CS-359-5-$^{14}CH_3$ (HCl salt) were dissolved in 0.5% physiological saline and administered to animals orally or intravenously at the doses of 10 mg/kg or 30 mg/kg. Beagles weighing *ca.* 10 kg, after fasting for 18 hr, were dosed with 10 mg/kg of the radioactive drug in gelatin capsules. Each animal was kept in a metabolic cage. Urine and feces were collected separately. For isolation studies, dog urine, which was collected during 0—24 hr after administration of 30 mg/kg of non-labeled CS-359, was employed.

Procedure for Isolation of CS-359 and Its Metabolites from Dog Urine——The isolation procedures are presented in Chart 1. Combined urine samples (600 ml) from four dogs (30 mg/kg *p.o.*) were applied to a Amberlite XAD-2 resin column (one liter suspension), washed with water (2 l) and finally eluted with MeOH (1.2 l). The MeOH effluent, which contained 93% of the urinary activity applied to the resin, was concentrated to dryness *in vacuo* below 30°. The residue obtained was dissolved in borate buffer (pH 9, 20 ml) and extracted with AcOEt (40 ml) 3 times. The combined extracts were concentrated *in vacuo* to dryness, dissolved in a small volume of 70% EtOH. The solution was applied on Kieselgel F_{254} plates in streaks and doubly developed in one direction with solvent system No. 1 followed by No. 2. The band areas having *Rf* values of 0.45 and 0.60 were located with ultraviolet (UV) light, scraped from the plates, powdered and extracted with MeOH. The MeOH extract from the band corresponding to *Rf* 0.6 was concentrated to dryness, giving an oily material (M-1, CS-359). The other MeOH extract from the band having *Rf* 0.45, after concentration to a small volume, was further chromatographed on Kieselgel F_{254} plates with the same

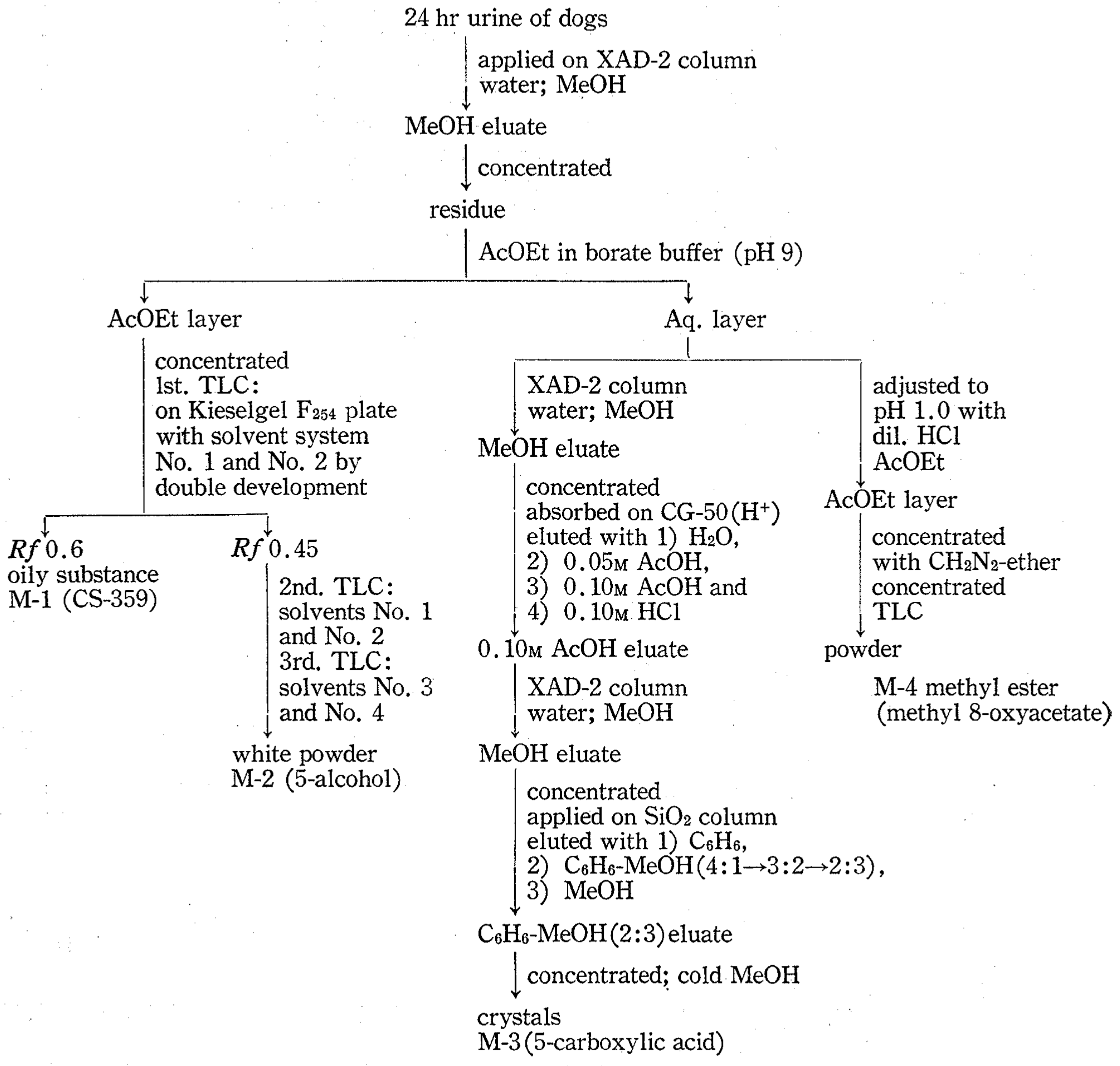

Chart 1. Procedures for Isolation of CS-359 and Its Metabolites from Dog Urine

solvent systems as above, and finally with first solvent system No. 3 and then No. 4 by double development technique. Evaporation of the solvent from the MeOH extract afforded a white powder of M-2 (5-alcohol).

The aqueous layer from AcOEt extraction was fed on a XAD-2 column (40 ml suspension) and after washing with water (100 ml), eluted with MeOH (60 ml). The MeOH eluate was concentrated, absorbed on an Amberlite CG 50 (H^+ type) column (2 × 30 cm) and eluted with the following solvent systems: 1) H_2O (300 ml), 2) 0.05 M AcOH (100 ml), 3) 0.10M AcOH (300 ml) and 4) 0.10 M HCl (100 ml). The 0.10 M AcOH effluent was passed through an Amberlite XAD-2 column (300 ml suspension), washed with water (300 ml) and then with MeOH (200 ml).

The MeOH eluate, after being concentrated to a small volume, was applied on a SiO_2 (*ca.* 100 g) column and developed with the following solvent systems (for each: 50 ml × 10): 1) C_6H_6, 2) C_6H_6–MeOH (4: 1), 3) C_6H_6–MeOH (3: 2), 4) C_6H_6–MeOH (2: 3), 5) MeOH. The C_6H_6–MeOH (2: 3) eluates, after evaporation of the solvents, afforded an oily residue (*ca.* 200 mg), which was crystallized from MeOH to give microcrystals (M-3, 5-carboxylic acid).

A portion of the remaining aqueous layer from AcOEt extraction was acidified to pH 1.0 with dil. HCl and extracted with AcOEt 3 times. The combined extracts were concentrated to dryness and dissolved in

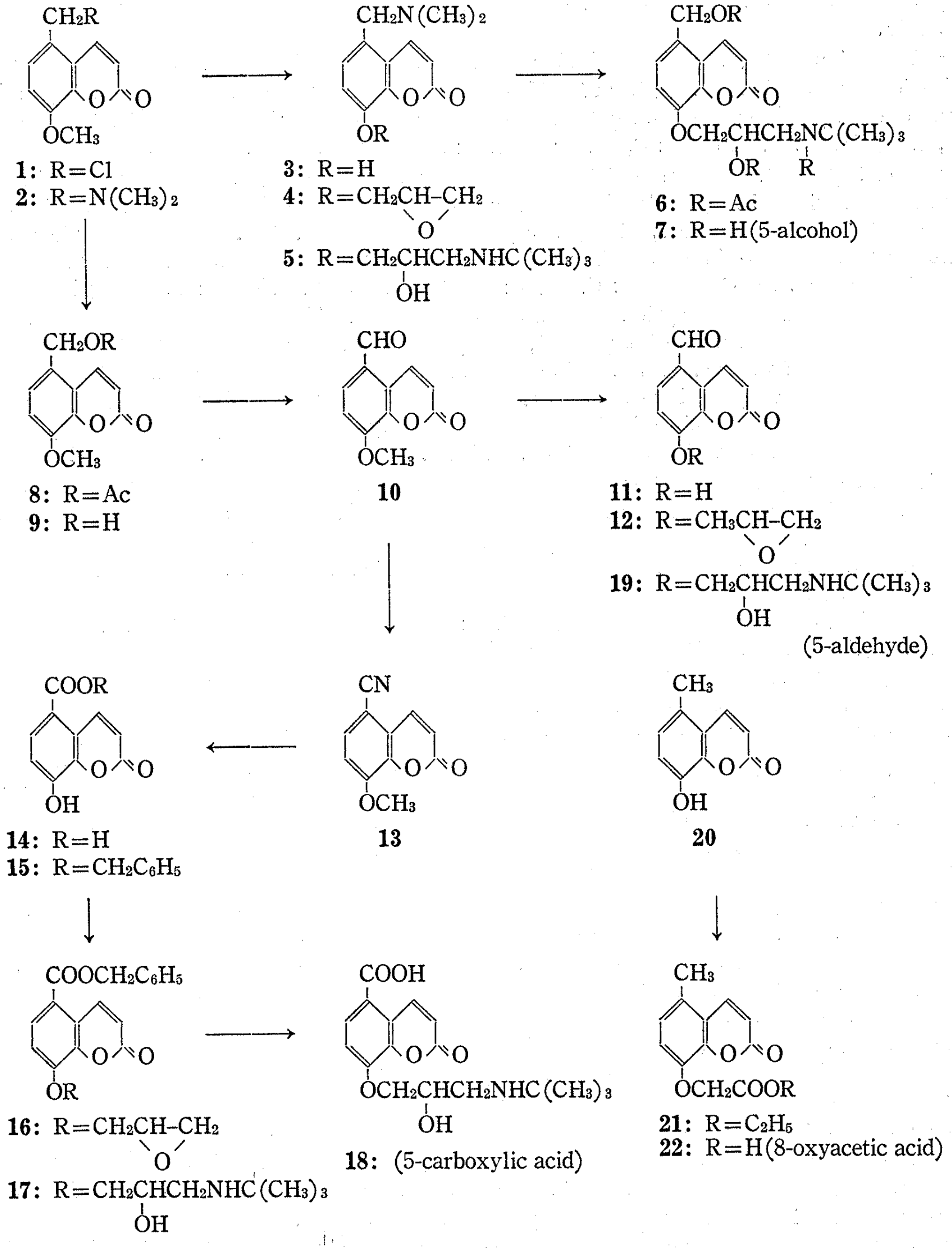

Chart 2. Synthetic Routes for Metabolites of CS-359

MeOH. The MeOH solution was treated with an excess of CH_2N_2–ether solution under ice-cooling for 2 hr. The reaction mixture was concentrated, giving an oily residue. This was streaked on Kieselgel F_{254} plates and developed with a mixture of solvents: C_6H_6–AcOEt–*n*-hexane–AcOH (24: 12: 4: 1). The area corresponding to *Rf ca.* 0.54 was scraped off and extracted with MeOH. Evaporation of the solvent from the MeOH extract afforded a white powder, the methyl ester of M-4 (methyl 8-oxyacetate).

Chemical Synthesis of Urinary Metabolites of CS-359——Synthetic routes for the urinary metabolites are shown in Chart 2. 5-Formyl-8-(2-hydroxy-3-*t*-butylaminopropoxy)coumarin (19, 5-aldehyde) was synthesized as a possible metabolic intermediate.

5-Hydroxymethyl-8-(2-hydroxy-3-*t*-butylaminopropoxy)coumarin (7, 5-Alcohol)——A stirred mixture of 5-chloromethyl-8-methoxycoumarin (**1**, 6.3 g), dimethylamine hydrochloride (4.9 g), triethylamine (10 ml) and EtOH (50 ml) was heated to reflux for 3.5 hr. The reaction mixture was concentrated *in vacuo*, diluted with water and made alkaline with K_2CO_3. The crystalline precipitate was collected, dissolved in 10% HCl, and the resulting solution was filtered.

The filtrate was made alkaline with conc. aqueous Na_2CO_3. After filtration the crystalline product was recrystallized from iso-PrOH, giving pale yellow crystals (4 g) of 5-dimethylaminomethyl-8-methoxycoumarin (**2**), mp 123—124°. *Anal.* Calcd. for $C_{13}H_{15}O_3N$: C, 66.93; H, 6.48; N, 6.01. Found: C, 67.06; H, 6.40; N, 6.17.

A stirred solution of **2** (6.4 g) in 48% HBr (64 ml) was refluxed for 2 hr and concentrated *in vacuo*. Recrystallization of the residue (7.2 g) from MeOH afforded colorless needles (6 g) of 5-dimethylaminomethyl-8-hydroxycoumarin hydrobromide (**3**), mp 246.5—247° (decomp.). *Anal.* Calcd. for $C_{12}H_{14}O_3NBr$; C, 48.02: H, 4.07; N, 4.67; Br, 26.62. Found: C, 48.13; H, 4.71; N, 4.76; Br, 26.52.

A mixture of **3** (1.85 g), epibromohydrin (0.94 g), anhyd. K_2CO_3 (4.2 g) and methyl ethyl ketone (40 ml) was refluxed for 8 hr with stirring. The reaction mixture was filtered while hot and the filtrate was concentrated *in vacuo* at room temperature, giving a crystalline residue (1.12 g). Recrystallization from EtOH yielded micro-crystals of 5-dimethylaminomethyl-8-(2,3-epoxypropoxy)coumarin (**4**), mp 114.5—115.5° (decomp.). *Anal.* Calcd. for $C_{15}H_{17}O_4N$: C, 65.44; H, 6.22; N, 5.09. Found: C, 63.60; H, 6.32; N, 4.84. The IR spectrum showed no absorption band due to OH group. The epoxide (**4**) was found to be somewhat unstable. A stirred mixture of **4** (3.85 g), *t*-butylamine (5.1 g) and EtOH (150 ml) was refluxed for 2.5 hr and concentrated *in vacuo*. The residue obtained was chromatographed on Al_2O_3 with $CHCl_3$, furnishing crystals (3 g) after evaporating the solvent. Recrystallization from *n*-hexane–isopropyl ether afforded colorless crystalline powder of 5-dimethylaminomethyl-8-(2-hydroxy-3-*t*-butylaminopropoxy)coumarin (**5**), mp 77—79°. *Anal.* Calcd. for $C_{19}H_{28}O_4N_2$: C, 65.49; H, 8.10; N, 8.04. Found: C, 65.21; H, 8.19; N, 7.74.

A solution of **5** (5.4 g) in Ac_2O (128 ml) was refluxed for 3 hr. The reaction mixture was concentrated *in vacuo*, giving an oily residue (**6**, 6.3 g): IR spectrum ν max cm^{-1}: 1640 (amide), 1740 (acetate and lactone). A stirred mixture of **6** (4.7 g) in EtOH (75 ml) and 2 N K_2CO_3 (25 ml) was refluxed for 1.5 hr, then diluted with water and extracted with $CHCl_3$. The $CHCl_3$ layer was dried, concentrated to yield a crystalline residue (0.3 g). Recrystallization from benzene afforded pale yellow microcrystals (**7**, 0.19 g, 5.6% yield), mp 136.5—138°. *Anal.* Calcd. for $C_{17}H_{23}O_5N$: C, 63.53; H, 7.21; N, 4.36. Found: C, 63.74; H, 7.10; N, 4.33. NMR (in $CDCl_3$) δ: 1.14 (singlet, 9H, $C(CH_3)_3$), 2.80 (broad, 2H, $-C\underline{H}_2-NH-$), 3.05 (singlet, 3H, $-CH_2O\underline{H}$, $-CH(O\underline{H})-$ and $-NH-$), 4.03 (broad singlet, 3H, $-O-C\underline{H}_2-C\underline{H}(OH)-$), 4.80 (singlet, 2H, $-C\underline{H}_2OH$), 6.35 (doublet, $J=9.8$ Hz, 1H, 3–H), 7.05 (doublet, 6– and 7–H), 8.1 (doublet, $J=9.8$ Hz, 1H, 4–H).

8-(2-Hydroxy-3-*t*-butylaminopropoxy)coumarin-5-carboxylic Acid (18, 5-Carboxylic Acid)——A mixture of 5-chloromethyl-8-methoxycoumarin (**1**, 14.7 g) and anhydrous AcOK in AcOH (14.8 g) was refluxed for 4 hr with stirring. The reaction mixture was poured onto ice-water, forming a crystalline precipitate (13 g) melting at 118.5—120°. Recrystallization from 30% aqueous EtOH gave colorless needles of 5-acetoxymethyl-8-methoxycoumarin (**8**), mp 122—123°. *Anal.* Calcd. for $C_{13}H_{12}O_5$: C, 62.90; H, 4.87. Found: C, 62.99; H, 5.06.

This compound (**8**) could be also prepared by heating the dimethylamino derivative (**2**) in Ac_2O at 90—100° for 12 hr (45% yield). A mixture of **8** (12.7 g), 3 N K_2CO_3 (51 ml) and EtOH (154 ml) was refluxed for 4 hr, concentrated *in vacuo* and diluted with water. Crystals separated were collected, yielding 8.5 g (80.6%) of 5-hydroxymethyl-8-methoxycoumarin (**9**) melting at 153—154°. Recrystallization from MeOH gave silky needles, mp 153—154°. *Anal.* Calcd. for $C_{11}H_{10}O_4$: C, 64.07; H, 4.89. Found: C, 64.34; H, 4.81. To a mixture of **9** (1.4 g) and xylene (400 ml) was added SeO_2 (0.42 g) and the mixture was refluxed with stirring for 12 hr. After cooling, the reaction mixture was filtered and the filtrate concentrated *in vacuo*. The resulting crystalline residue was recrystallized from methyl ethyl ketone to afford pale brown crystals (0.76 g) of 5-formyl-8-methoxycoumarin (**10**), mp 219—220° (decomp.). *Anal.* Calcd. for $C_{11}H_8O_4$: C, 64.70; H, 3.95. Found: C, 64.49; H, 3.86. A mixture of **10** (0.2 g), AcONa (0.097 g), AcOH (4 ml) and $NH_2OH \cdot HCl$ (0.08 g) was refluxed for 26 hr with stirring and then cooled to room temperature. Crystals were collected and recrystallization from dioxane gave pale brown needles (0.12 g) of 5-cyano-8-methoxycoumarin (**13**), mp 242—243° (decomp.). *Anal.* Calcd. for $C_{11}H_7O_3N$: C, 65.67; H, 3.51; N, 6.96. Found: C, 65.48; H, 3.57; N, 6.56. IR in Nujol cm^{-1}: 2230 ($C\equiv N$). The nitrile (**13**) could be also prepared by heating a mixture of **10** (0.5 g), N,O-bis(trifluoroacetyl)hydroxylamine[5] (0.55 g), pyridine (0.4 g) and dry benzene (35 ml) to reflux for 3.5 hr.

A mixture of **13** (0.9 g) and 48% HBr (30 ml) was refluxed for 3 hr, and then diluted with water. Crystals were collected, dissolved in 10% $NaHCO_3$ and filtered. The filtrate was made acidic with 10% HCl. After addition of solid NaCl, the crystals were collected and recrystallized from 80% aqueous EtOH, affording a crystalline powder (0.44 g, 46.8% yield) of 8-hydroxycoumarin-5-carboxylic acid (**14**), mp 266.5—267.5° (decomp.). *Anal.* Calcd. for $C_{10}H_6O_5$: C, 58.26; H, 2.93. Found: C, 58.51; H, 3.08.

A mixture of **14** (2.1 g) in dimethylformamide (DMF) (80 ml) and powdered $NaHCO_3$ (0.9 g) was heated at 90°. To this was added dropwise benzyl chloride (1.54 g) over 1 hr and the resulting mixture was refluxed with stirring for 3 hr. After adding aqueous NaCl solution, the reaction mixture was extracted with $CHCl_3$. The $CHCl_3$ layer was dried and concentrated to dryness and then purified by chromatography on SiO_2 with $CHCl_3$. The residue left after evaporation of the solvent from the first portion of the eluate was recrystallized from EtOH to give needles of benzyl 8-benzyloxycoumarin-5-carboxylate, mp 124.5—125.5°. The second portion of the eluate was concentrated to yield crude crystals (1.7 g, 56.3% yield) melting at 189—191°, which were recrystallized from EtOH to give needles of benzyl 8-hydroxycoumarin-5-carboxylate (**15**), mp 194—195°. *Anal.* Calcd. for $C_{17}H_{12}O_5$: C, 68.91; H, 4.08. Found: C, 68.92; H, 4.07. To a mixture of **15** (2.5 g) and DMF (70 ml) was added powdered anhydrous K_2CO_3 (1.28 g), followed by epibromohydrin (2.3 g) and the mixture was heated at 90° for 30 min. The reaction mixture was poured onto ice-water and after addition of solid NaCl extracted with $CHCl_3$. The $CHCl_3$ layer was dried over Na_2SO_4 and purified by chromatography on SiO_2 with $CHCl_3$. The eluate was concentrated to give a crystalline residue (2.45 g, 82.4 % yield) melting at 109—111°.

Recrystallization from EtOH gave colorless needles (2.25 g, 75.7% yield) of benzyl 8-(2,3-epoxypropoxy)-coumarin-5-carboxylate (**16**), mp 117.5—118°. *Anal.* Calcd. for $C_{20}H_{16}O_6$: C, 68.18; H, 4.58. Found: C, 68.42; H, 4.60.

A mixture of **16** (2.21 g) and *t*-butylamine (44 ml) in EtOH (110 ml) was stirred at room temperature for 24 hr. The reaction mixture was concentrated *in vacuo*, affording an oily residue (2.8 g) of benzyl 8-(2-hydroxy-3-*t*-butylaminopropoxy)coumarin-5-carboxylate (**17**), which was soluble in dil. HCl and showed a single spot on TLC. The oily material (**17**), without further purification, was submitted to catalytic hydrogenation over 2.8 g of 5% Pd-C in 50% aqueous EtOH (400 ml). In the course of the reaction was added additional 50% EtOH (100 ml) and 5% Pd-C (1 g).

After absorption of hydrogen had been completed, the catalyst was filtered off and the filtrate concentrated *in vacuo* to give crude crystals (1.2 g). Recrystallization from 50% aqueous EtOH afforded needles (**18**, 0.9 g), mp 267—267.5° (decomp.). *Anal.* Calcd. for $C_{17}H_{21}O_6N \cdot 1/2H_2O$: C, 59.29; H, 6.44; N, 4.07. Found: C, 59.67; H, 6.24; N, 4.19.

5-Formyl-8-(2-hydroxy-3-*t*-butylaminopropoxy)coumarin (**19**, **5-Aldehyde**)——A mixture of **10** (6.46 g) and pyridine·HCl (36 g) was heated at 160° for 2 hr and the reaction mixture poured onto ice-water. Crystals separated were collected, washed with water, yielding 4.26 g (71.2% yield) of 5-formyl-8-hydroxycoumarin (**11**). This crude material was purified by chromatography on SiO_2 column with 5% EtOH in $CHCl_3$ and recrystallized from 30% aqueous DMF to give pure crystals of (**11**), mp 263—264°. *Anal.* Calcd. for $C_{10}H_6O_4$: C, 63.16; H, 3.18. Found: C, 63.20; H, 3.22. A stirred mixture of **11** (1 g), epibromohydrin (3.5 g), anhydrous K_2CO_3 (3.6 g) and methyl ethyl ketone (200 ml) was refluxed for 50 hr. The reaction mixture was filtered while hot and the filtrate concentrated *in vacuo*, affording crystals (0.46 g) of 5-formyl-8-(2,3-epoxypropoxy)coumarin (**12**) melting at 179—185°. Further purification of this material was difficult, but the spectral data supported the assigned structure. A mixture of **12** (0.10 g), *t*-butylamine (5 ml) and iso-PrOH (4 ml) was stirred for 24 hr, during which time the crystals went into solution. The reaction mixture was concentrated *in vacuo*. The oily residue was dissolved in hot HCl. The resulting solution was filtered and the filtrate concentrated *in vacuo* to a small volume, made alkaline with 10% Na_2CO_3 and extracted with $CHCl_3$. After drying, the $CHCl_3$ layer was concentrated to afford an oily residue (**19**). This was converted to 5-formyl-8-(2-hydroxy-3-*t*-butylaminopropoxy)coumarin maleate, which was recrystallized from EtOH to give pale yellow crystals, mp 204—205° (decomp.). *Anal.* Calcd. for $C_{17}H_{21}O_5N \cdot C_4H_4O_4$: C, 57.92; H, 5.79; N, 3.22. Found: C, 57.91; H, 5.80; N, 3.60.

(5-Methylcoumarin-8-yloxy)acetic Acid (**22**, **8-Oxyacetic Acid**)——A stirred mixture of **20** (3 g), ethyl bromoacetate (3 g), anhydrous Na_2CO_3 (2.7 g) and methyl ethyl ketone (35 ml) was refluxed for 13 hr. The hot solution was filtered and concentrated *in vacuo*. The residue was recrystallized from MeOH (120 ml), affording needles (3.3 g, 73.8% yield) of ethyl (5-methylcoumarin-8-yloxy)acetate (**21**), mp 151—152.5°. *Anal.* Calcd. for $C_{14}H_{14}O_5$: C, 64.11; H, 5.38. Found: C, 64.24; H, 5.26. A mixture of **21** (1 g), dioxane (15 ml) and conc. HCl (10 ml) was refluxed for 3 hr. The residue left after concentration of the reaction mixture was recrystallized from MeOH to give colorless needles (0.7 g) of **22**, mp 247—249. *Anal.* Calcd. for $C_{12}H_{10}O_5$: C, 61.54; H, 4.30. Found: C, 61.44; H, 4.30.

---

[5] J.H. Pomeroy and C.A. Craig, *J. Am. Chem. Soc.*, **81**, 6340 (1959).

Result

Studies on the Metabolites in Excreta

1) Thin-Layer Chromatography——Fig. 1 depicts schematically the thin-layer chromatograms of urinary and fecal metabolites from rats or dogs dosed with CS-359-5-$^{14}CH_3$. As shown in Fig. 1-1, all of the radiochromatograms obtained by double developments were similar in their chromatographic patterns except for some polar spots near the origin, irrespective of the routes of the drug administration or animal species. Rat urine exhibited a rather

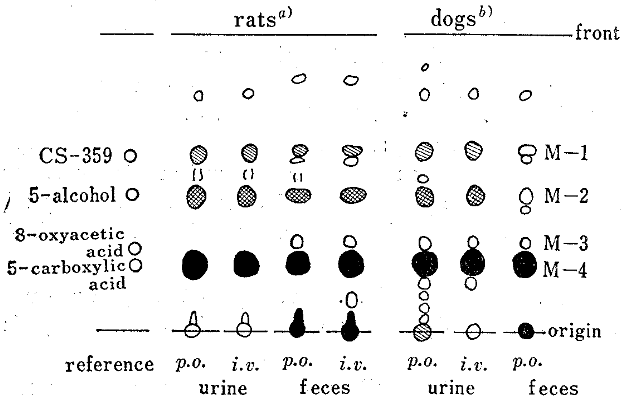

Fig. 1-1. Schematic Presentation of the Radiochromatograms after Thin-Layer Chromatography of the Urinary and Fecal Metabolites of Rats and Dogs

a) 24 hr samples from rats dosed 10 mg/kg of CS-359-5-$^{14}CH_3$
b) 24 hr samples from beagles dosed 10 mg/kg *p.o.* or 1.25 mg/kg *i.v.*

Urine samples were used as the MeOH eluates from the XAD-2 column chromatography and feces samples, as the 70% EtOH extracts.

Doubly developed with solvent system No. 1 and then No. 2.

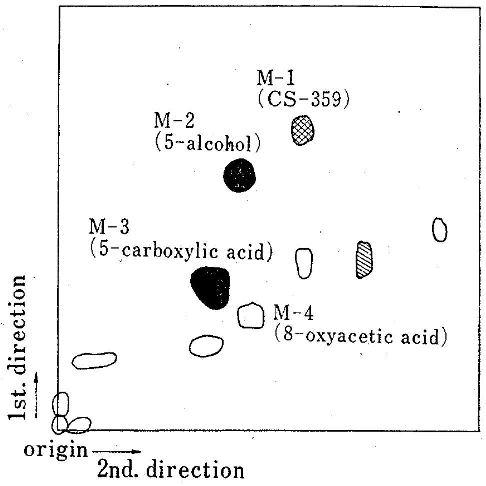

Fig. 1-2. Radiochromatogram after Thin-Layer Chromatography of 24 hr Urine of Rats given CS-359-5-$^{14}CH_3$ orally

Two-dimensionally developed with first solvent system No. 5 and then No. 2.

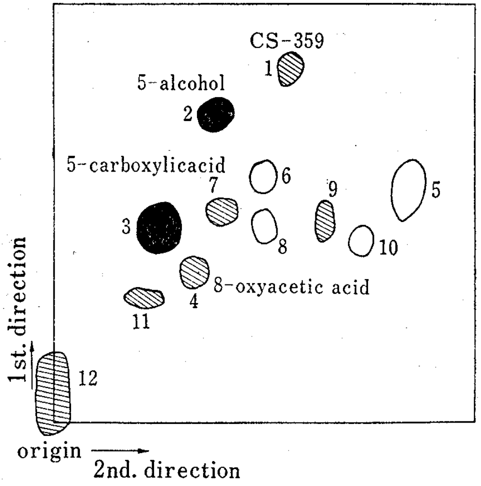

Fig. 1-3. Radiochromatogram after Thin-Layer Chromatography of 24 hr Urine of Dog (No. 49) given a 10 mg/kg Dose of CS-359-5-$^{14}CH_3$

Urine sample was applied on a Kieselgel plate after treatment with XAD-2 column and developed two-dimensionally with the same solvent systems as described in Fig. 1-2.

TABLE I. Determination of the Urinary Metabolites of Dog by Two-Dimensional TLC

Spot No.[a)]			% of the urinary activity
1	M-1	CS-359	1.43
2	M-2	5-alcohol	13.47
3	M-3	5-carboxylic acid	44.87
4	M-4	8-oxyacetic acid	2.44
5		**20**[b)]	0.60
6		unknown	0.37
7		unknown	1.98
8		unknown	1.09
9		unknown	1.53
10		unknown	0.33
11		unknown	4.67
12		unknown	6.79

a) the same as those in Fig. 1-3
b) Spot No. 5 has the same *Rf* value as 5-methyl-8-hydroxycoumarin (**20**), but a sample of CS-359-5-$^{14}CH_3$ used for this experiment contained a small amount of **20** (less than 1.2%).

less complex pattern compared to that of dog urine. Two-dimensional radiochromatograms (Fig. 1-2 and 1-3) of urine samples revealed the presence of 12—14 metabolites in all. Polar spots found near the origin in Fig. 1-1 were separated into 3—4 components in Fig. 1-2. The relative amounts of these spots from dog urine are given in Table I. Spot No. 5 was found to be identical with 5-methyl-8-hydroxycoumarin (**20**), but its metabolic formation in animal body was not conclusive, since the ^{14}C-labeled sample employed was contaminated with a small amount of the phenolic compound (less than 1.2%).

2) Paper Electrophoresis——Rat urine was submitted to paper electrophoresis in both borate buffer (pH 9.0) and formic-acetic buffer (pH 2.0). The radiochromatograms, shown in Fig. 2, revealed that the basic and/or amphoteric components comprise most of the radioactivity from rat urine, and accordingly the acidic components are only minor.

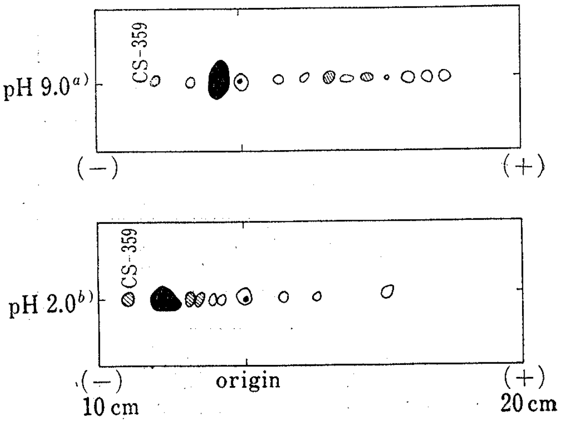

Fig. 2. Radiochromatograms after Paper Electrophoresis of 24 hr Urine Samples of Rats gives 10 mg/kg Dose of CS-359-5-$^{14}CH_3$

Samples were applied on TOYO FILTER PAPER No. 51A.

a) Original urine sample was employed and run for 2.5 hr at 2.0 kV in 0.01M borate buffer (pY 9.0).

b) A MeOH eluate from XAD-2 chromatography of the original urine was employed and run for 1.5 hr at 3.0 kV in formic-acetic buffer (pH 2.0).

3) Fractionation of Urinary and Fecal Activity——Urinary activity from dogs was fractionated by the extraction procedure shown in Chart 3. Neutral and basic fractions were 0.82% and 18.46%, respectively. The radioactivity transferred to the neutral fraction after CH_2N_2 treatment was only 2.86%, thus indicating that the amount of acidic substances was small in the original urine. This was also in accord with the finding from the paper electrophoresis described above. When treated with CH_2N_2–ether under ice-cooling for 2 hr, 5-carboxylic acid was found to remain partially unreacted.

Urine samples of rats or dogs were shaken with AcOEt first at pH 9.5 and then at pH 1. The radioactivity transferred to the respective AcOEt extracts were as follows (%): Rats ($n=3$); 16.90±1.53 (pH 9.5), 16.36±0.47 (pH 1) and 63.58 (remaining water layer). Dogs ($n=2$); 12.02 (pH 9.5), 20.695 (pH 1) and 69.275 (remaining water layer). The radioactivities extracted at pH 1 were nearly the same in their chromatographic patterns on TLC as those from the remaining aqueous layer. Radioactivity in dog feces was analogously fractionated and the results confirmed figures similar to those in urine.

The water-soluble fractions remaining after AcOEt extraction of urine were incubated with β-glucuronidase (bovine liver) or glusulase (*Helix Pomatia*, β-glucuronidase plus aryl sulfatase) in acetate buffer (pH 5.0) for 24 hr. Examination of the reaction mixture by AcOEt extraction as described above showed only minor changes in the ^{14}C-activity of each fraction in comparison with the control sample. Untreated samples of rat and dog urine, or the MeOH eluates from their XAD-2 chromatography were also subjected to enzymatic hydrolysis without obtaining any significant changes in AcOEt fractionation. Treatments with glusulase of 70% EtOH extracts of fecal samples from dogs gave no significant increase in the amounts of CS-359 or other metabolites after TLC separation of the reaction mixture.

M-1 (CS-359)

After purification on TLC with solvent systems containing AcOH, M-1 was isolated as an AcOH salt of CS-359. The UV spectrum (Fig. 3) showed absorption maxima at 252 and 297 nm in EtOH. In an alkaline solution, this changed to a simple strong end-absorption, which on subsequent acidification, reproduced the same spectrum as that in neutral solution.

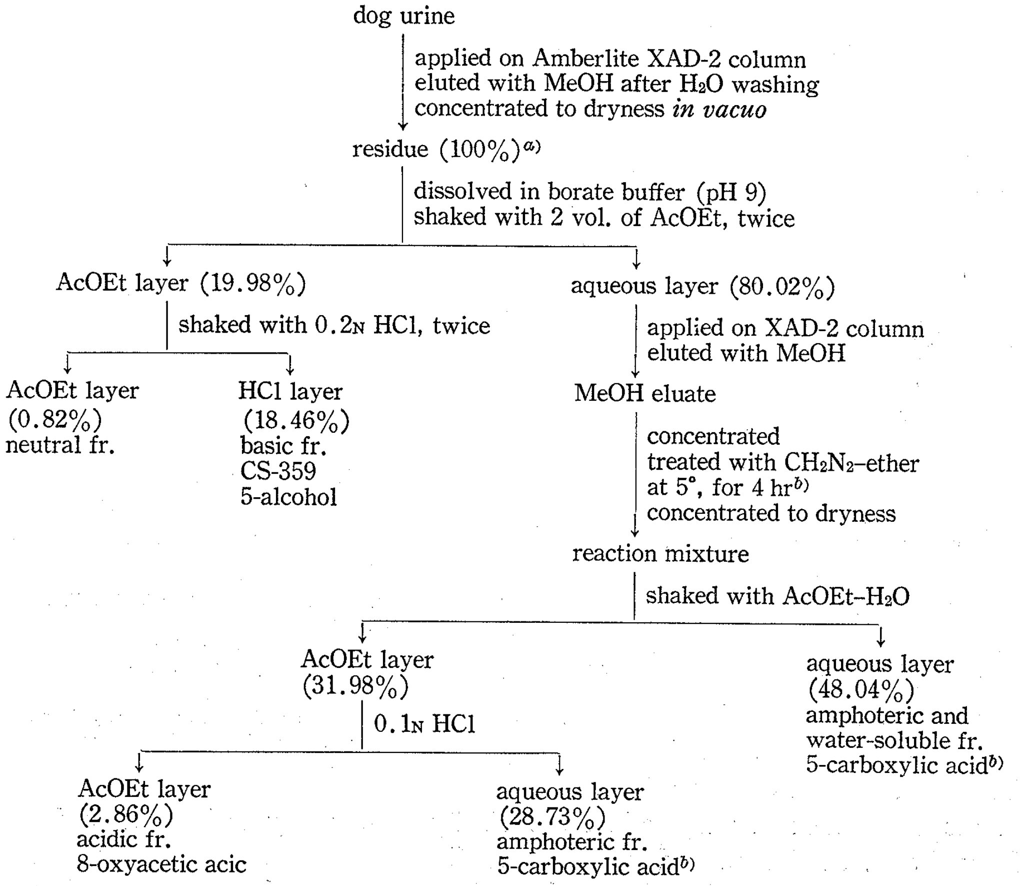

Chart 3. Fractionation of the Urinary Activity of Dogs Given orally 5 mg/kg Dose of CS-359-5-$^{14}CH_3$

a) After chromatography on Amberlite XAD-2 column, 93% of the activity applied was recovered.

b) Under the reaction conditions employed, 5-carboxylic acid seems to remain partially unreacted and was left in the aqueous layer as the free acid.

These spectral changes are characteristic of the coumarin derivatives. The mass spectrum of M-1 had the following fragment ion peaks: *m/e* at 290 (M^+–15), 261 (M^+–44), 176 (M^+–129), 86 ($CH_2{=}N^+H{-}C(CH_3)_3$), and 57 ($C^+(CH_3)_3$). A monoTMS derivative of M-1 gave a M^+–15 ion peak at *m/e* 362 on GC–MS analysis. These spectral data, along with both the *Rf* values of M-1 on TLC and retention times in GC of the monoTMS derivative of M-1, were all confirmed to be identical with those of the authentic CS-359.

M-2 (5-Alcohol)

M-2 had the UV spectrum with absorption maxima at 251 and 295 nm in EtOH, which showed the characteristic pH dependent changes, just similar to CS-359. The mass spectrum of M-2 gave ion peaks at *m/e* 306 (M^+–15), 277 (M^+–44), 192 (M^+–129), 86 ($CH_2{=}N^+HC(CH_3)_3$), 57 ($C^+(CH_3)_3$).

A diTMS derivative of M-2 showed M^+-15 peak at *m/e* 465 on GC–MS.

These spectral data suggested that M-2 has a molecular formula of CS-359 plus one oxygen atom with the intact side chain moiety at the 8 position.

The NMR spectrum of M-2 in CD_3OD had the following signals (δ values): 1.41 (s, $C(CH_3)_3$), 3.11 (m, $-C\underline{H}_2-NH-$), 4.21 (m, $-O-C\underline{H}_2-C\underline{H}(OH)-$), 4.78 (s, 5-$C\underline{H}_2OH$), 6.47 (d, $J=10.0$ Hz, 3-H), 7.25 (s, 6- and 7-H), 8.25 (d, $J=10.0$ Hz, 4-H). Thus, signals assignable both to protons at the 3, 4, 6, and 7 positions on the coumarin ring and to the intact side chain moiety at the 8 position in the CS-359 molecule were all detected. However, the singlet of the 5-CH_3 group which is characteristic of the CS-359 molecule, disappeared and instead, a singlet corresponding

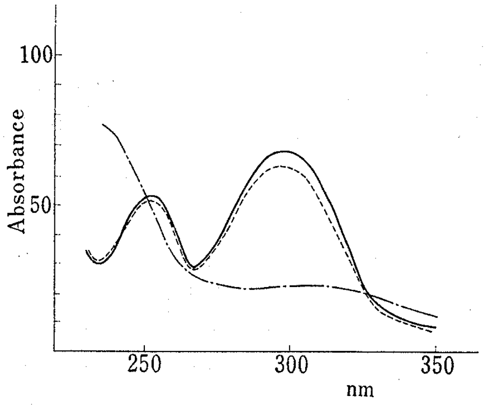

Fig. 3. UV Spectra of M-1 (CS-359)

———: in EtOH
—·—·—: in aqueous NaOH–EtOH
--------: in HCl–EtOH

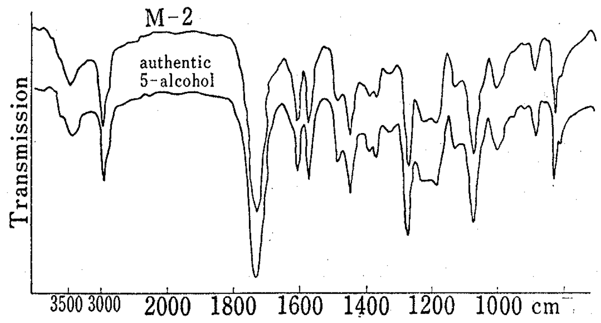

Fig. 4. IR Spectra of M-2 and the Authentic 5-Alcohol in $CHCl_3$

to 2 protons was now observed at δ 4.78. All of these spectral data indicated that M-2 most probably has a 5-hydroxymethyl instead of the 5-methyl group in the CS-359 molecule. Finally, M-2 was identified with an authentic sample of chemically synthesized 5-hydroxymethyl compound (**7**) by comparisons of the UV and IR (in chloroform) spectra (Fig. 4). *Rf* values on TLC and retention times of the diTMS derivative on GC were also confirmed to be the same between M-2 and the authentic specimen.

M-3 (5-Carboxylic Acid)

M-3 was isolated as microcrystals having mp 262—267° (decomp.) and $[\alpha]_D \pm 0$ in water. The UV spectrum of M-3 with absorption maxima at 249 and 293 nm in EtOH showed the characteristic spectral changes of the coumarin structure as described for CS-359. The mass spectrum had a M^+-15 peak at *m/e* 320 along with those at 291 (M^+-44), 206 (M^+-126), 86 ($CH_2=N^+HC(CH_3)_3$) and 57 ($C^+(CH_3)_3$). A diTMS derivative of M-3 gave a M^+-15 ion at *m/e* 479.

These data indicated that M-3 had a molecular formula of CS-359 plus two oxygen atoms with simultaneous loss of two hydrogen atoms, thus having a molecular weight of 335. The NMR spectrum of M-3 in CD_3OD exhibited the following signals both associated with the side chain moiety at the 8 position and protons at the 3- and 4-positions in the CS-359 molecule: 1.40 (s, $C(CH_3)_3$), 3.22 (m, $-\underline{CH_2}-NH$), 4.04 (m, $-O-CH_2-$), 4.28 (m, $-\underline{CH}(OH)-$), 6.20 (d, J= 10 Hz, 3-H), 8.33 (d, J=10 Hz, 4-H). Protons at the 6- and 7-positions appeared as a AB-type quartet at 6.86 (d, J=9 Hz, 7-H) and 7.51 (d, J=9 Hz, 6-H). A singlet signal due to the 5-methyl in CS-359 disappeared in M-3.

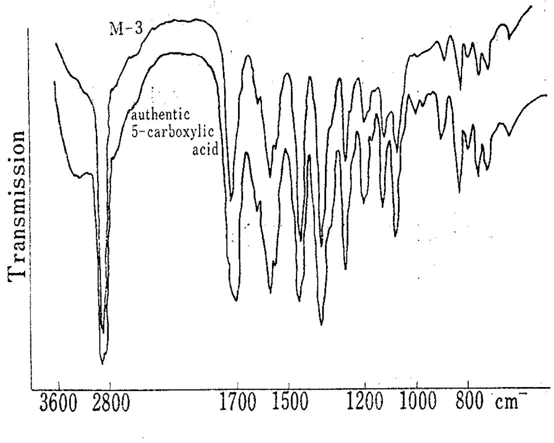

Fig. 5. IR Spetcra of M-3 and the Authentic 5-Carboxylic Acid in Nujol Mull

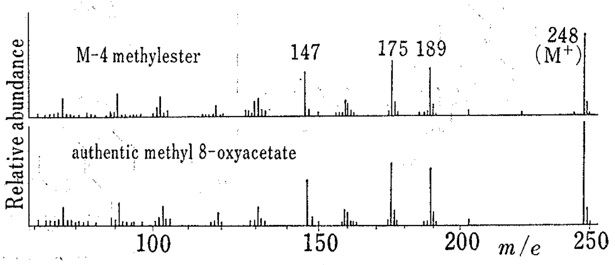

Fig. 6. Mass Spectra of Methyl Esters of M-4 and the Authentic 8-Oxyacetic Acid

These spectral findings strongly suggested that M-3 is the 5-carboxyl derivative of CS-359. The specimen of the synthesized 5-carboxylic acid (**18**) was confirmed to be identical with M-3 by comparison of the IR spectra in Nujol mull (Fig. 5, except for bands due to H_2O present as crystalline water in the synthetic specimen), the UV spectra, *Rf* values on TLC and gas-chromatographic behaviors of the diTMS derivatives.

M-4 (8-Oxyacetic Acid)

The methyl ester of M-4 showed the UV spectrum having absorption maxima at 253 and 293 nm, characteristic of the coumarin structure and gave a prominent M^+ peak at *m/e* 248 in the mass spectrum. Therefore, M-4 was supposed to have the 8-carboxymethoxy structure which can be derived by oxidative fission of the 2-hydroxy-3-*t*-butylaminopropoxy side chain in the CS-359 molecule. Identity with an authentic sample of the synthesized methyl ester of 8-oxyacetic acid (**22**) was confirmed by comparison of the mass spectra (Fig. 6) and *Rf* values on TLC.

Discussion

The main metabolites of CS-359 in dog urine were established as being the 5-hydroxymethyl and 5-carboxyl compounds resulting from oxidation of the 5-methyl group in CS-359. From examination of the radiochromatograms shown in Fig. 1-1, this was also apparently true for the metabolites in dog feces and excreta of rats. The 5-formyl compound, a possible intermediate in the oxidation of 5-alcohol to 5-carboxylic acid could not be detected on the two-dimensional thin-layer chromatograms of the samples from urine, feces and liver.

Chromatographic patterns (Fig. 2) after paper electrophoresis of rat urine revealed that both the neutral and acidic metabolites are minor components. This was also in accord with the findings from the fractionation of the dog urine by AcOEt extraction (Chart 3): an acidic fraction contained 2.80%; a neutral fraction, 0.82% of the urinary ^{14}C-activity. Therefore, it can be deduced that oxidation of the side chain at the 8-position in CS-359, which might be expected to afford some neutral or acidic metabolites, may be a minor course in the metabolism of CS-359. Experiments on the enzymatic hydrolysis of the urinary and fecal samples of rats or dogs indicated that neither glucuronide(s) nor sulfate(s) occurred in significant amounts in the excreta.

Possible metabolic pathways of CS-359 are shown in Chart 4. The 8-oxyacetic acid has been identified as one of the possible metabolites resulting from the oxidation of the 2-hydroxy-3-*t*-butylaminopropoxy side chain. As will be described in a succeeding paper,[6]

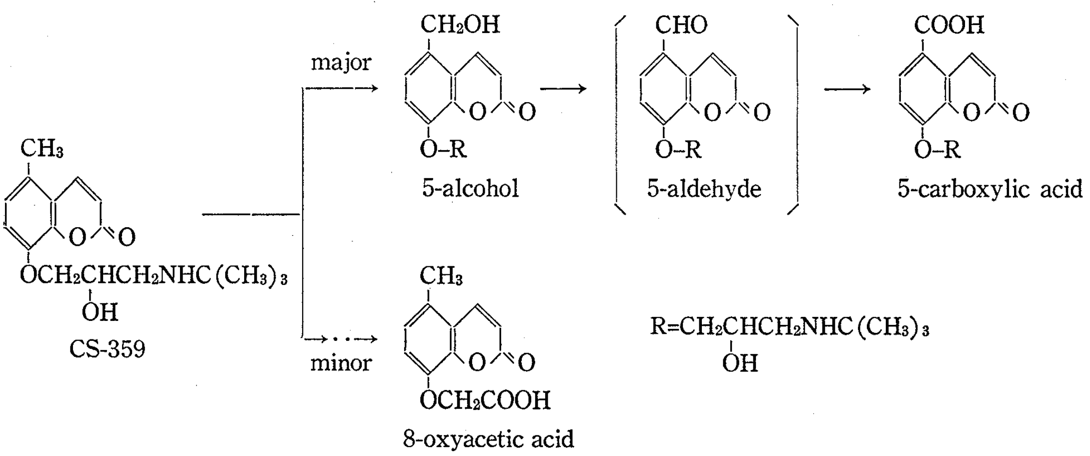

Chart 4. Possible Metabolic Pathways of CS-359

---

[6] R. Hayashi, M. Okada, T. Nakazawa, Y. Tanaka, N. Miyakoshi, and Y. Sato, *Pharmacometrics*, "accepted."

the studies on the blood and tissue concentrations indicated that oxidation of the 5-methyl group in CS-359 proceeded rapidly and predominantly in rat liver and the resulting metabolites, 5-alcohol and 5-carboxylic acid were eliminated rapidly from the blood and tissues. These findings might account for the relatively minor contribution of any oxidative processes other than that of the 5-methyl group in the metabolism of CS-359.

The metabolism of coumarin has been reported to afford some phenolic and acidic compounds, which arise from ring hydroxylation and oxidative fission of the lactone ring, respectively.7) However, any such metabolites formed by the same reactions as with coumarin itself have not been confirmed in the present studies on CS-359. Structures of the minor metabolites are now under investigations.

Acknowledgement The authors express their gratitude to Dr. G. Sunagawa, director of these laboratories and to Dr. K. Tanabe for their kind encouragement. Thanks are also due to Mr. S. Kanno for his technical assistance.

---

7) M. Kaighen and R.T. Williams, *J. Med. Chem.*, **3**, 25 (1961); W.H. Shilling, R.H. Crampton, and R.C. Longland, *Nature*, **221**, 664 (1969).