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Metabolic Fate of 1,1-Dimethyl-5-methoxy-3-(dithien-2-ylmethylene)piperidinium Bromide (SA-504). I. Biliary Metabolites in Rats

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Isolation and characterization of biliary metabolites of SA-504, a new anticholinergic drug, were studied in rats. More than 70% of the radioactivity excreted in 3 hr bile of rats given ^{14}C-SA-504 was found to be due to metabolites. Fifteen metabolites were separated from bile of rats dosed with ^{14}C-SA-504, and eleven metabolites were characterized. Most of characterized metabolites were conjugated with GSH, cysteinylglycine, cysteine or N-acetylcysteine. The presence of the glycol derivative of SA-504 and tertiary amine N-oxide of SA-504 as minor metabolites was demonstrated. The oxidation of SA-504 by rat liver microsomes suggested that the epoxide was formed as an intermediate. From these facts, it seems likely to conclude that metabolic pathways of SA-504 consists of epoxidation, GSH conjugation and hydration of the epoxide, cleavage of C–C bond, N-demethylation and N-oxidation and that the main metabolic pathway of SA-504 is epoxidation followed by GSH conjugation.

1,1-Dimethyl-5-methoxy-3-(dithien-2-ylmethylene)piperidinium bromide (SA-504)[2] has been shown by Tamaki, *et al.*,[3] to have a potent anticholinergic activity and a low potency of side effects of mydriasis and dry mouth. Studies on the absorption, distribution and excretion of SA-504 have shown that, after *i.v.* injection of ^{14}C-SA-504 to rats, the radioactivity is more largely excreted in the feces than in the urine and about 30% of the administered radioactivity is excreted in the bile within 3 hr.[4] Therefore, in the present study on metabolism of SA-504, the bile samples of rats given ^{14}C-SA-504 were employed. The present report deals with the separation and characterization of biliary metabolites of SA-504 in rats.

Experimental

Synthesis of ^{14}C-SA-504 and Metabolite——The synthesis of ^{14}C-SA-504 was carried out by the quaternization of tertiary nitrogen of piperidine with ^{14}C-methyl bromide, as shown in Fig. 1. 1-Methyl-5-methoxy-3-(dithien-2-ylmethylene)piperidine (132 mg) was dissolved in 2 ml of acetone, and ^{14}C-methyl bromide (1.0 mCi, 0.43 mmole) in 2 ml of acetone was added. The mixture was stirred for 3 hr. Crystals which appeared in the mixture were collected and recrystallized from methanol. The specific activity of the product was 4.5 μCi/mg and the radiochemical purity was more than 98% as determined by thin-layer chromatography (TLC).

1,1-Dimethyl-5-methoxy-3-hydroxy-3-[dithien-2-yl(hydroxy)]methylpiperidinium bromide (SA-504 glycol) which was a metabolite of SA-504 was prepared as follows. Tertiary amine (2.35 g) of SA-504 in ether (60 ml) was treated with osmium tetroxide (2 g) and pyridine (2 g) at room temperature. A rapid reaction set in, and after 3 hr the solid osmium complex was filtered off and dissolved in dichloromethane (100 ml). This solution was shaken with an excess of a solution of mannitol (10%) and KOH (1%) in water for 4 hr. The dichloromethane layer was separated and dried (Na_2SO_4), and the solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography using 4% methanol–chloroform for elution to afford a crystalline material. The product obtained was converted by treating with methyl bromide in acetone to a quaternary ammonium compound, which was recrystallized from acetone–isopropyl ether to give a colorless crystalline substance, mp 213—215°. *Anal.* Calcd. for $C_{17}H_{24}O_3NS_2Br \cdot H_2O$:

1) Location: *Toda-shi, Saitama.*
2) M. Kawazu, T. Kanno, S. Saito and H. Tamaki, *J. Med. Chem.*, **15**, 914 (1972).
3) H. Tamaki, M. Tanaka, S. Murata, S. Harigaya and A. Kiyomoto, *Jap. J. Pharmacol.*, **22**, 689 (1972).
4) M. Yoshikawa and Y. Sato, *Radioisotopes*, in press.

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C, 45.13; H, 5.79; N, 3.10. Found: C, 45.25; H, 5.57; N, 3.12. UV λ_{max}^{MeOH} mμ(ε): 235 (16600). IR ν_{max}^{Nujol} cm^{-1}: 3250 (OH). Mass Spectrum *m/e*: 339 ($M^+ - CH_3Br$).

An isomer of SA-504 glycol was also isolated from reaction products above described. However, the configuration of both isomers could not be established.

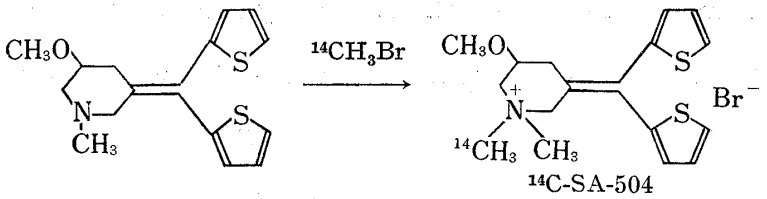

Fig. 1. Synthesis of ^{14}C-SA-504

Collection of Bile——Male Sprague-Dawley rats weighing about 200 g were used in all the experiments. The bile duct was cannulated with a polyethylene tubing under anaesthesia of urethane. ^{14}C-SA-504 which was diluted with nonradioactive SA-504 was dissolved in distilled water and administered intraperitoneally to 100 rats in a dose of 25 mg/kg. (The intraperitoneal LD_{50} for SA-504 is 55.1 mg/kg in rat.[2]) The bile was collected for 3 hr after *i.p.* injection of ^{14}C-SA-504. (More than 30% of the administered radioactivity was excreted in 3 hr bile).

Separation of Biliary Metabolites——The procedure for the separation of biliary metabolites is outlined in Chart 1. The bile sample was adjusted to pH 8 with NH_4OH and extracted three times with an equal volume of chloroform. The chloroform extract (fraction A) was concentrated to a small volume and spotted on Silica Gel GF_{254} plates (0.25 mm thick), activated at 110° for 60 min. The plates were developed with solvent systems described in the legend to Table I.

The aqueous solution above mentioned was passed through the column (3×30 cm) of Amberlite XAD-2, the effluent being retained. After washing the column with distilled water, methanol was used to elute metabolites. Methanol eluate was concentrated to dryness *in vacuo*. The residue was dissolved in water, and then passed through the column (2.5×30 cm) of Amberlite CG-50 (H^+ type). The effluent (fraction B) was concentrated to a small volume under vacuum and spotted on Toyo Roshi No. 51. The papers were developed with the solvent systems described in the legend to Table I. After washing the column of Amberlite CG-50 with distilled water, 1N NH_4OH was used to elute metabolites. The eluate (fraction C) was concentrated to a small volume and subjected to paper chromatography (PPC).

The effluent from Amberlite XAD-2 above mentioned was passed through the column (2.5×30 cm) of Dowex 50 (H^+ type). After washing the column with distilled water, 1N NH_4OH was used to elute metabolites. The eluate (fraction D) was concentrated to a small volume and subjected to PPC.

Radioactive metabolites on the chromatograms were detected with an Aloka thin-layer chromatogram scanner TRM-1B or an Aloka paper chromatogram scanner PCB-4. Color development of metabolites was carried out with platinic iodide[5] and ninhydrin. After scanning the chromatograms, the various radioactive zones were separately scraped (or cut out) from the plates (or the papers), and the metabolites were eluted from the silica gel (or the papers) with methanol or water. The eluates were concentrated to dryness under vacuum, and the residues were extracted with a mixture of chloroform–methanol (1:1) or water. After removal of the solvent, the extracts were used for ultraviolet (UV) and mass spectrometry.

Hydrolysis and Hydrogenolysis of Amino Acid Conjugates——Samples of the materials obtained as described above were heated to 100° with HBr (sp. gr. 1.7) for 4 hr, and the excess of the acid was removed by evaporation. The residues obtained were examined for the amino acids both by direct comparison with authentic materials on paper chromatograms and, after conversion into 2,4-dinitrophenyl derivatives,[6] by comparison with authentic derivatives.[7] The samples in 30% ethanol were heated under reflux with Raney nickel catalyst (W-2 grade) for 6 hr. The mixtures were filtered, and the filtrates were evaporated. The residues were hydrolyzed with HBr and then examined for amino acids as described above.

Identification of Biliary Metabolites——Identification of separated metabolites was accomplished by co-chromatography with the authentic samples and by spectrometric analysis including UV and mass spectrometry. UV spectra were taken of SA-504 and the separated metabolites dissolved in water. The mass spectra of SA-504 and its metabolites were taken on a RMS-4 mass spectrometer (Hitachi Co.) with a direct solids inlet operating at 125°. For mass spectrometric analyses of conjugated metabolites, nonvolatile amino acid conjugates were esterified by treating wtih *n*-propanol and sulfuric acid and then trifluoroacetylated by treating with trifluoroacetic anhydride.

Quantitative Determination of Biliary Metabolites——Three hr bile samples of four male and female rats *i.p.* dosed with ^{14}C-SA-504 (5 mg/kg) were used for quantitative determination of the metabolites.

5) G. Toennies and J.J. Kolb, *Anal. Chem.*, **23**, 823 (1951).
6) F. Sanger, *Biochem. J.*, **39**, 507 (1945).
7) D.M.P. Phillips, *Biochem. J.*, **68**, 35 (1958).

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Separation of the metabolites was carried out in a similar manner as described for isolation of biliary metabolites. After scanning thin-layer or paper chromatograms, radioactive spot areas were scraped or cut out into counting vials and extracted with 1 ml of methanol or water. Fifteen ml of solution of 7 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene in 1 liter of 50% ethanol–toluene was added and the vials were counted in an Aloka liquid scintillation spectrometer LSC-502 equipped with an automatic quenching monitor system. This procedure resulted in quantitative detection of SA-504 and all metabolites originally spotted on the plates and papers.

Epoxidation of SA-504 by Rat Liver Microsomes——Rat liver microsomes were obtained from animals which had been given intraperitoneal injections of phenobarbital (40 mg/kg) for 4 days in order to induce

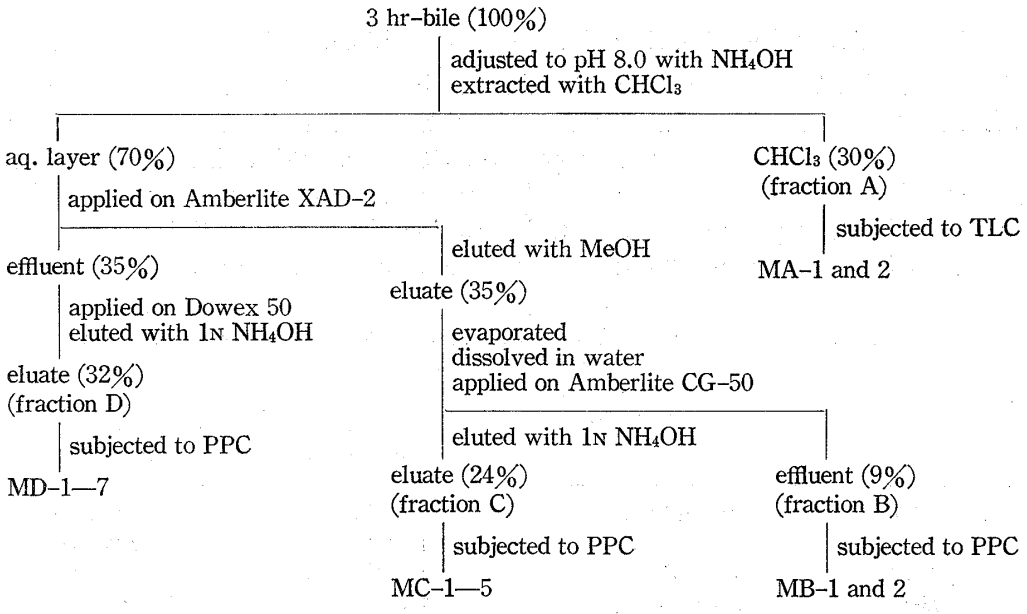

Chart 1. Major Steps Used for Separation of Metabolites of ^{14}C-SA-504

Recoveries are expressed in terms of ^{14}C.

TABLE I. *Rf* Values of SA-504 and Its Metabolites

	Thin-layer chromatograms		Paper chromatograms		
	Solvent I	Solvent II	Solvent III	Solvent IV	Solvent V
SA-504	0.42	0.20	0.66	0.85	0.77
Fraction A of bile	0.28(MA-1)	0.16			
	0.42(MA-2)	0.20			
Fraction B of bile			0.05(MB-1)		
			0.19(MB-2)		
Fraction C of bile			0.04(MC-1)		
			0.17(MC-2)		
			0.28(MC-3)		
			0.49(MC-4)		
			0.68(MC-5)		
Fraction D of bile				0.02(MD-1)	0.0
				0.16(MD-2)	0.05
				0.28(MD-3)	0.05
				0.40(MD-4)	0.24
				0.65(MD-5)	0.24
				0.75(MD-6)	0.55
				0.86(MD-7)	0.55

TLC was performed on Silica Gel GF_{254} with solvent systems: I, $CHCl_3$–MeOH–AcOH (75:20:5); II, $CHCl_3$–MeOH (4:1), and PPC was done on Toyo Roshi No. 51 with solvent systems: III, *n*-BuOH–H_2O–EtOH–AcOH (32:12:8:1); IV, *n*-PrOH–H_2O–AcOH (10:5:1); V, *tert*-BuOH–H_2O (1.73:1). Motabolites of ^{14}C-SA-504 were detected by thin-layer or paper chromatogram scanning. Bile sample was collected for 3 hr after *i.p.* injection of ^{14}C-SA-504. Separation of bile sample into fraction A, B, C, and D was described under method.

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high levels of drug-metabolizing enzymess. Microsomes were prepared according to the procedure of Mitoma, *et al.*[8] The incubation mixture contained microsomes (from 250 mg of rat liver) in 3.0 ml of phosphate buffer (0.1M, pH 8.0), 4.8 μmoles of NADPH, 1 μmole of ^{14}C-SA-504 and 25 μmoles of magnesium chloride; final volume 5.0 ml. The suspension was agitated under air for 5 min at 37° and the reaction was stopped by extraction with 1 volume of chilled chloroform containing 0.5% triethylamine. The chloroform extracts were subjected to TLC directly or after heating with 1N HCl for 30 min. Incubations with heat-denatured microsomes served as controls.

Result

Isolation and Characterization of Biliary Metabolites of ^{14}C-SA-504

As shown in Chart 1, the bile sample of rats given ^{14}C-SA-504 was separated into four fractions (A, B, C and D) by means of solvent extraction and column chromatography.

When fraction A, B, C and D were subjected to TLC or PPC with the appropriate solvent systems, two radioactive peaks (MA-1 and MA-2) were observed in fraction A, two radioactive peaks (MB-1 and MB-2) were in fraction B, five radioactive peaks (MC-1—5) were in fraction C, and seven radioactive peaks (MD-1—7) were in fraction D, as shown in Table I.

Table II. UV Spectra and Color Reactions of SA-504 and Its Metabolites

	UV spectra λ_{max} mμ	Color reaction	
		Platinic iodide	Ninhydrin
SA-504	249 and 285	purple	negative
MA-1	234	purple	negative
MA-2	249 and 285	purple	negative
MB-1	233	purple	negative
MB-2	246 and 285	purple	negative
MC-1	232	yellow	positive
MC-2	232	yellow	positive
MC-3	232	yellow	positive
MC-4	233, 266 and 293	purple-yellow	positive
MC-5	234, 266 and 295	purple-yellow	negative
MD-1	—	yellow	positive
MD-2	—	yellow	positive
MD-3	—	yellow	positive
MD-4	—	yellow	positive
MD-5	—	yellow	positive
MD-6	—	yellow	negative
MD-7	—	yellow	negative

UV spectra were run with the aqueous solutions.

MA-1——Table II shows absorption maxima of UV spectra and color reactions to spray reagents for SA-504 and its metabolites. The UV spectrum of MA-1 was different from that of SA-504. The mass spectrum of MA-1 indicated the presence of a small peak at *m/e* 339, which probably belongs to a molecular ion of MA-1. The increase in weight of 34 mass units of the molecular ion of MA-1 as compared with SA-504 suggested that this compound was a dihydroxylated derivative of SA-504. The base peak of MA-1 appeared at *m/e* 305, which corresponded to the molecular ion of tertiary amine of SA-504. Characteristic fragment ions appeared at *m/e* 195, *m/e* 144 and *m/e* 111, the probable structures of which are depicted in Fig. 2. The presence of the fragment ions at *m/e* 195 ($C_9H_7OS_2$) and *m/e* 144 ($C_7H_{14}O_2N$), which are formed probably by the cleavage of glycol C-C bond of MA-1, suggested that two hydroxy groups were introduced into the double bond bridge of SA-504. From these data,

8) C. Mitoma, H.S. Posner, H.C. Reitz and S. Udenfriend, *Arch. Biochem. Biophys.*, **61**, 431 (1956).

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it seems likely to conclude that MA-1 is formed by the addition of two hydroxyl groups into the double bond bridge of SA-504. Indeed, the UV and mass spectra of MA-1 were identical with those of a synthetic sample of SA-504 glycol.

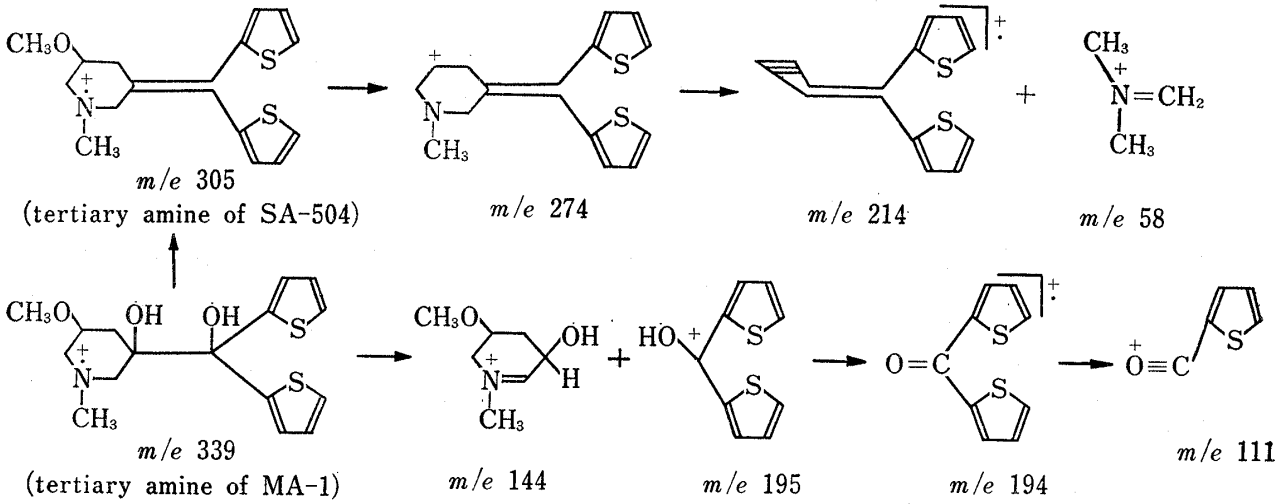

Fig. 2. Structures of Selected Fragments of SA-504 and MA-1

MA-2——MA-2 had the same *Rf* values (in solvent systems I and II) as the authentic sample of SA-504. The UV and mass spectra of MA-2 were identical with those of SA-504. From these data, it could be concluded that MA-2 was intact SA-504.

MB-1——MB-1 responded positively to naphthoresorcinol indicative of glucuronide. When MB-1 was incubated with β-glucuronidase and then extracted with chloroform, about 60% of the radioactivity in the incubation mixture was transferred into chloroform. TLC of chloroform extract showed that hydrolyzed product of MB-1 had the same *Rf* value as the authentic sample of SA-504 glycol. The UV and mass spectra of hydrolyzed product of MB-1 were identical with those of SA-504 glycol. From these facts, it could be concluded that MB-1 was a glucuronide of SA-504 glycol.

MB-2——The UV spectra of MB-2 resembled that of SA-504. The mass spectrum of MB-2 gave a molecular ion at *m/e* 321. The increase in weight of 16 mass units of the molecular ion of MB-2 as compared with the tertiary amine of SA-504 suggested that this metabolite might be an oxidized derivative of SA-504. Comparison of MB-2 and SA-504 mass spectra showed a striking similarity in the fragmentation and in the variation of intensity with mass number, with the exception of the molecular ion at 321. This fact suggested that the oxygen was attached to double bond bridge or nitrogen atom. When MB-2 was treated with zinc powder in the presence of acetic acid and then subjected to TLC, it was found that MB-2 was converted to tertiary amine of SA-504. This fact suggested that the oxygen was not attached to double bond bridge but to nitrogen atom. From these data, it seems likely that MB-1 is a N-oxide derivative of tertiary amine of SA-504.

MC-1—5——When sprayed with platinic iodide, MC-1—5 gave yellow colored spots while SA-504 gave a purple colored spot. MC-1—4 responded positively to ninhydrin but SA-504 did not. These facts suggested that MC-1—5 were sulfur-containing amino acid conjugates.

When MC-1—5 was heated with 1N HCl and then subjected to TLC, it was found that all of them gave SA-504 which was confirmed by the UV and mass spectra. PPC of the hydrolyzed products of MC-2—4 indicated the presence of ninhydrin-positive compounds, which were corresponded in *Rf* values to oxidized glutathione (GSSG), oxidized cysteinylglycine, and cystine, respectively. MC-5 gave N,N'-diacetylcystine which was visualized by spraying with platinic iodide. When MC-1—5 were hydrolyzed with HBr and examined for amino

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Table III. Degradation Products of Amino Acid Conjugates Present in Fraction C of the Bile of Rats Dosed with SA-504

Metabolites	Products obtained	
	By hydrolysis with 1N HCl	By hydrolysis with HBr
MC-1	SA-504; unknown	cystine; glycine; unknown
MC-2	SA-504; GSSG	cystine; glycine; glutamic acid
MC-3	SA-504; oxidized cysteinylglycine	cystine; glycine
MC-4	SA-504; cystine	cystine
MC-5	SA-504; N,N'-diacetylcystine	cystine

acids, cyctine, glycine and an unknown compound were detected in the hydrolysis products of MC-1; cystine, glycine and glutamic acid were in MC-2; cystine and glycine were in MC-3; cystine was in both MC-4 and MC-5, as shown in Table III. The mass spectra of MC-1—5 did not show any parent molecular ion probably because of their low volatilities. However, the top mass peak of MC-5 was seen at *m/e* 467 corresponding to loss of hydroxyl group from the parent molecular ion. This suggested that MC-5 might be an N-acetylcysteine conjugate of SA-504. Prominent fragment ions appeared at *m/e* 194 ($C_9H_6OS_2$) and *m/e* 273 ($C_{12}H_{21}O_3N_2S$), the probable structures of which are depicted in Fig. 3. The presence of these fragment ions at *m/e* 194 and *m/e* 273, which are formed probably by the cleavage of C-C bond bridge of the ion at *m/e* 467, suggested that a hydroxyl group attached to the methylene of SA-504 and that N-acetylcysteine attached to 3 position of piperidine ring. From these data, it seems likely that MC-5 is an N-acetylcysteine conjugate formed by the addition of N-acetylcysteine and hydroxyl group into the double bond bridge of SA-504. Consequently, it seems that MC-2 is a glutathione (GSH) conjugate of SA-504, MC-3 is a cysteinylglycine conjugate, and MC-4 is a cysteine conjugate.

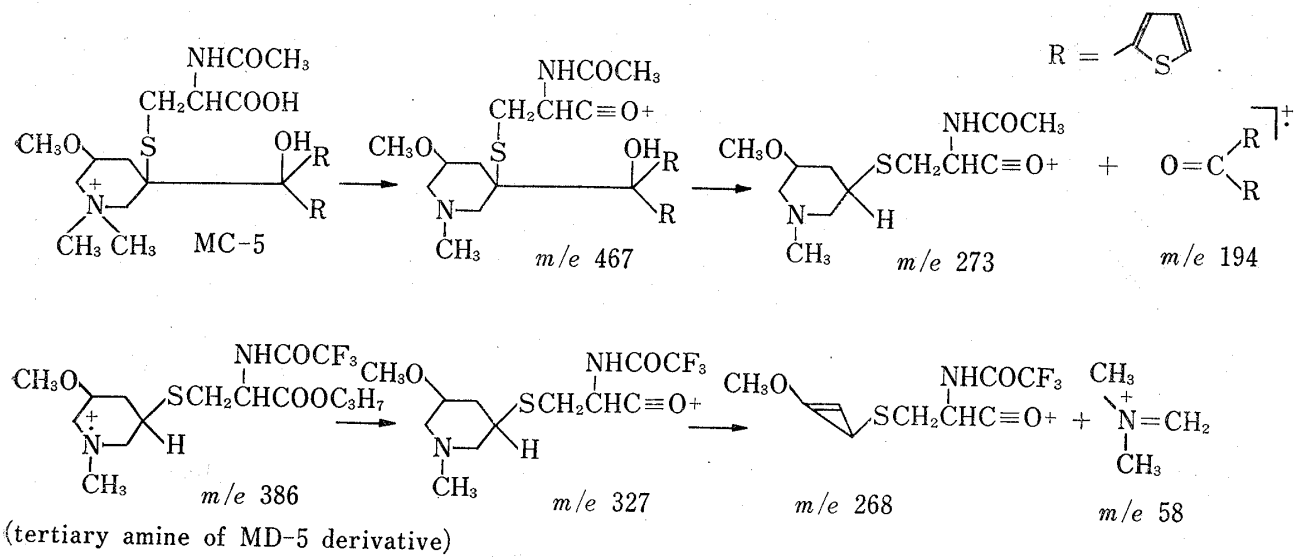

Fig. 3. Structures of Selected Fragments of MC-5 and *n*-Propyl Ester of Trifluoroacetylated MD-5 (MD-5 derivative)

MD-1—7——UV spectra of MD-1—7 did not show any absorption between 230—360 mμ. This fact indicated that MD-1—7 had no thiophene in their molecules. MD-1—7 gave yellow colored spots by spraying with platinic iodide. MD-1—5 were positive to ninhydrin, but MD-6 and 7 were not. These data suggested that MD-1—7 were sulfur containing amino acid conjugates. MD-1, 4 and 7 which were excreted in small amounts could not be analyzed further. The mass spectra of MD-2, 3, 5 and 6 did not show any prominent peak at *m/e* larger

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than 100 probably because of their low volatilities. When MD-2, 3, 5 and 6 were hydrolyzed with HBr and examined for amino acids, cystine, glycine and glutamic acid were detected in the hydrolysis products of MD-2; cystine and glycine were in MD-3; cystine was in both MD-5 and MD-6, as shown in Table IV. On the other hand, wnen MD-2, 3, 5 and 6 were treated with Raney nickel and then hydrolyzed with HBr, alanine was detected instead of cystine.

Table IV. Degradation Products of the Amino Acid Conjugates Present in Fraction D of the Bile of Rats Dosed with SA-504

Metabolites	Products obtained	
	By hydrolysis with HBr	By hydrogenolysis with Raney Ni and hydrolysis of the products with HBr
MD-2	MD-5; cystine; glycine; glutamic acid	alanine; glycine; glutamic acid
MD-3	MD-5; cystine; glycine	alanine; glycine
MD-5	MD-5; cystine	alanine
MD-6	MD-5; cystine	alanine

PPC of hydrolyzed products of MD-2, 3, 5 and 6 showed the presence of a radioactive spot corresponding in *Rf* value to MD-5. MD-5 was esterified with *n*-propanol and sulfuric acid, trifluoroacetylated with trifluoroacetic anhydride and then examined by mass spectrometry. The mass spectrum of the propyl ester of trifluoroacetylated MD-5 showed small peaks at *m/e* 386 and 327 corresponding to the molecular ion and a species produced by loss of *n*-propyloxy group from the molecular ion respectively. Prominent fragment ions appeared at *m/e* 268 ($C_{12}H_{18}O_3N_2SF_3$) and *m/e* 58 (C_3H_8N), the probable structures of which are depicted in Fig. 3. The presence of the fragment ion at *m/e* 58, which is also found characteristically for SA-504, suggested that MD-5 had a piperidine ring in its molecule. From these data, it seems likely that MD-5 is a cysteine conjugate of 1,1-dimethyl-5-methoxypiperidinium (DMP). Consequently, it seems that MD-2 is a GSH conjugate of DMP, MD-3 is a cysteinyl-glycine conjugate of DMP and MD-6 is an N-acetylcysteine conjugate of DMP.

Quantitative Determination of Biliary Metabolites——Table V shows quantitative determination of biliary metabolites of ^{14}C-SA-504 in male and female rats. Approximately 35% of the administered radioactivity was excreted in 3 hr bile of male rats *i.p.* given ^{14}C-SA-504. About 25% of the biliary radioactivity was present as intact ^{14}C-SA-504. Most of characterized metabolites were amino acid conjugates which accounted for more than 60% of the biliary radioactivity. Of the radioactivity excreted in 3 hr bile, 30% was amino acid conjugates (MC-2—5) of SA-504 and about 35% was amino acid conjugates (MD-2, 3, 5, 6) of DMP. SA-504 glycol and its glucuronide accounted for 9% of the biliary radioactivity.

It was found that the biliary excretion of intact SA-504 was significantly greater in female rats than in male rats.

Epoxidation of SA-504 by Rat Liver Microsomes——It was not possible to separate SA-504 and SA-504 epoxide in the chloroform extracts of incubation mixture by TLC with various solvent systems. When the chloroform extracts were treated with 1N HCl to convert the epoxide into the glycol and then subjected to TLC in solvent system I, the band corresponding to SA-504 glycol was found on the chromatograms although a very small amount of SA-504 glycol was present in chloroform extracts before acid treatment. No SA-504 glycol was separated from the heat-denatured control incubation. Therefore, the chloroform extracts were assayed for SA-504 and SA-504 glycol by TLC before and after acid treatment. The amounts of the epoxide were calculated from the difference between the two measurements. The oxidation of SA-504 occurred only in the presence of NADPH, as shown in Table VI. Approximately 10% of incubated SA-504 was converted to the epoxide by liver microsomes

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of phenobarbital-treated rats. The activity of SA-504 epoxidase in liver microsomes was significantly higher in phenobarbital-treated rats than in untreated rats.

Table V. Quantitative Determination of Biliary Metabolites of ^{14}C-SA-504

Metabolites	Corresponding compound	Male (36.0±3.1%) %	Female (37.9±3.9%) %
MA-1	SA-504 glycol	3.6±0.6	3.9±0.5
MA-2	SA-504	24.0±2.4	32.4±2.6[a]
MB-1	glucuronide of SA-504 glycol	5.4	4.7
MB-2	tertiary amine N-oxide of SA-504	3.2	2.6
MC-1	unknown	2.7	1.4
MC-2	GSH conjugate of SA-504	6.0	6.5
MC-3	cysteinylglycine conjugate of SA-504	3.9	3.4
MC-4	cysteine conjugate of SA-504	7.4	7.7
MC-5	N-acetylcysteine conjugate of SA-504	8.0	8.7
MD-1	unknown	2.0	1.2
MD-2	GSH conjugate of DMP[b]	5.3	4.4
MD-3	cysteinylglycine conjugate of DMP	8.7	7.5
MD-4	unknown	3.7	2.1
MD-5	cysteine conjugate of DMP	6.2	5.7
MD-6	N-acetylcysteine conjugate of DMP	7.1	6.5
MD-7	unknown	2.8	1.3

Bile samples were collected for 3 hr after *i.p.* injection of ^{14}C-SA-504 (5 mg/kg) to eight rats of both sexes. Quantitative determination of ^{14}C-SA-504 and its metabolites was accomplished by combining solvent extraction, column chromatography, TLC and PPC. Data were expressed as the percentages of the total radioactivity excreted in bile. Values in parentheses indicate per cent recovery of the administered radioactivity.

a) Significantly different ($P < 0.05$) from the comparable value for the male.

b) 1,1-dimethyl-5-methoxypiperidinium.

Table VI. Epoxidation of SA-504 by Rat Liver Microsomes

	μmoles of SA-504 epoxide	
	Untreated rat	Phenobarbital-treated rat
Complete system	0.046±0.011	0.114±0.025[a]
NADPH omitted	<0.008	<0.010

The reaction mixture contained 1 μmole of ^{14}C-SA-504, 4.8 μmoles of NADPH, 25 μmoles of $MgCl_2$, microsomes (equivalent to 250 mg of rat liver) in 3.0 ml of 0.1M phosphate buffer at pH 8.0, and water to make a final volume of 5.0 ml and was incubated for 5 min at 37°. The epoxide was converted to the glycol by treating with 1N HCl and then separated from SA-504 by TLC.

Data represent the mean (±standard deviation) of four experiments.

a) Significantly different ($P < 0.01$) from comparable values for untreated rat.

Discussion

It is commonly assumed that most quaternary ammonium compounds are not metabolized, since the lipophilic endoplasmic reticulum may restrict the penetration and limit the metabolism of polar compounds.[9] However, SA-504 which is a quaternary ammonium compound appears to be readily metabolized by the rat as compared with other quaternary ammonium compounds, since the present work showed that more than 70% of the radioactivity excreted in 3 hr bile of rat *i.p.* dosed with ^{14}C-SA-504 was present as ^{14}C-metabolites.

The presence of SA-504 glycol, which was excreted partly free and partly conjugate with glucuronic acid, was demonstrated in the bile of rat given ^{14}C-SA-504. This metabolite is

9) L.E. Gaudette and B.B. Brodie, *Biochem. Pharmacol.*, 2, 89 (1959).

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assumed to be formed either by the addition of hydrogen peroxide into the double bond bridge of SA-504 or by the epoxidation of SA-504 followed by the hydration of the epoxide.

Epoxides were proposed by Boyland[10] to be intermediates in the addition of the elements of hydrogen peroxide across aromatic double bonds. Epoxides have also been postulated as intermediates in the production of glycols from nonaromatic cyclenes.[11–13] Indene[14,15] and anthracene[16] have been shown to be converted to corresponding dihydroxy derivatives. In none of the above cases has an epoxide intermediate been demonstrated. However, the formation of 1,2-naphthalene oxide from naphthalene in the microsomal system was demonstrated both by radiotracer trapping technique and by isolation, although 1,2-naphthalene oxide is very unstable.[17] Recently, the oxidation in rabbit liver microsomes of styrene, cyclohexene and indene to epoxides had been demonstrated by gas chromatography, by thin-layer chromatography of the epoxide picrate derivatives and by accumulation of radioactivity in a styrene oxide pool from labeled styrene.[18] Wong and Terriere[19] reported that aldrin, isodrin and heptachlor were converted to their respective epoxides by rat liver microsomes. In the current work, the oxidation of SA-504 by rat liver microsomes suggested that the epoxide was formed as an intermediate, although the presence of the epoxide could not be demonstrated in the bile of rat given SA-504. The fact that SA-504 epoxide was not detected in the bile of rat given SA-504 may be explained by taking account of that SA-504 epoxide formed from SA-504 is immediately converted to such compounds as GSH conjugate. From a consideration of these facts, it seems likely that SA-504 glycol is formed by the hyration of SA-504 epoxide. The hydration of alkene oxides in liver microsomes has already been demonstrated.[20,21]

Hydrophilic metabolites, which were conjugated with GSH, cysteinylglycine, cysteine or N-acetylcysteine, could be divided into two groups, one of which was adsorbed on Amberlite XAD-2 and the other was not. The former (MC-2—5) had thiophene in their molecules while the latter (MD-2, 3, 5 and 6) had no thiophene. MC-2, GSH conjugate of SA-504, seems to be formed by GSH conjugation of SA-504 epoxide, since the presence of glutathione S-epoxidetransferase, which is involved in the catalysis of the conjugation of epoxides and glutathione, has been demonstrated in liver of rats and ferrets.[22] The GSH conjugation of epoxides has already been shown to occur in several compounds.[16,17,23,24]

It is generally accepted that the GSH conjugates formed *in vivo* are converted to S-substituted cysteine, which is further acetylated to form mercapturic acids.[25,26] Therefore, it seems reasonable to consider that MC-3 is formed from MC-2, MC-4 is from MC-3, and MC-5 is from MC-4.

The formation of MD-2, 3, 5 and 6 from SA-504 indicates that the cleavage of C-C bond of SA-504 occurs *in vivo*. At present, the pathway by which MD-2 is formed is uncertain.

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26) S.P. James, D.J. Jeffery, R.H. Waring and D.A. White, *Biochem. Pharmacol.*, **20**, 897 (1971).

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However, it may be considered that there are two pathways for the formation of MD-2; one is that SA-504 or SA-504 glycol is converted by the cleavage of C-C bond to an unknown piperidinium compound, which is conjugated with GSH, and the other is the cleavage of C-C bond of GSH conjugate (MC-2) of SA-504. In the current experiments, the presence of GSH conjugate of SA-504 was demonstrated. This fact suggests that MD-2 is formed by the cleavage of C-C bond of MC-2. Consequently, it seems that MD-3, 5 and 6 are formed by the cleavage of C-C bond of their respective MC-3—5. However, it can not be completely ruled out that MD-3, 5 and 6 are formed from MD-2.

Oesch and Daly[27] reported that 1,1,1-trimethyl-2-propene oxide reacted nonenzymatically with GSH to form two products, one (major product) of which was S-(2-hydroxy-3,3,3-trichloro-1-propyl)glutathione and the other (minor product) was S-(1-hydroxy-3,3,3-trichloro-2-propyl)-glutathione. Therefore, it is possible to consider that the reaction of SA-504 epoxide and GSH gives two products. However, in the present work, only one product, in which GSH attached to 3 position of piperidine ring, was detected.

Tertiary amine N-oxide (MB-2) of SA-504 was separated as a minor metabolite from the bile of rats given ^{14}C-SA-504. This fact indicated that SA-504 underwent N-demethylation in the body of rat. This was supported by the report[4] of Yoshikawa and Sato that about 2% of the *i.v.* administered ^{14}C-SA-504 was excreted as $^{14}CO_2$ in the expired air of rats within 24 hr. TLC of chloroform extract of bile of rats given ^{14}C-SA-504 showed the presence of a very small amount of tertiary amine of SA-504. The biological N-oxidation of tertiary amine is a common metabolic reaction during drug metabolism.[28,29] Therefore, it seems likely to

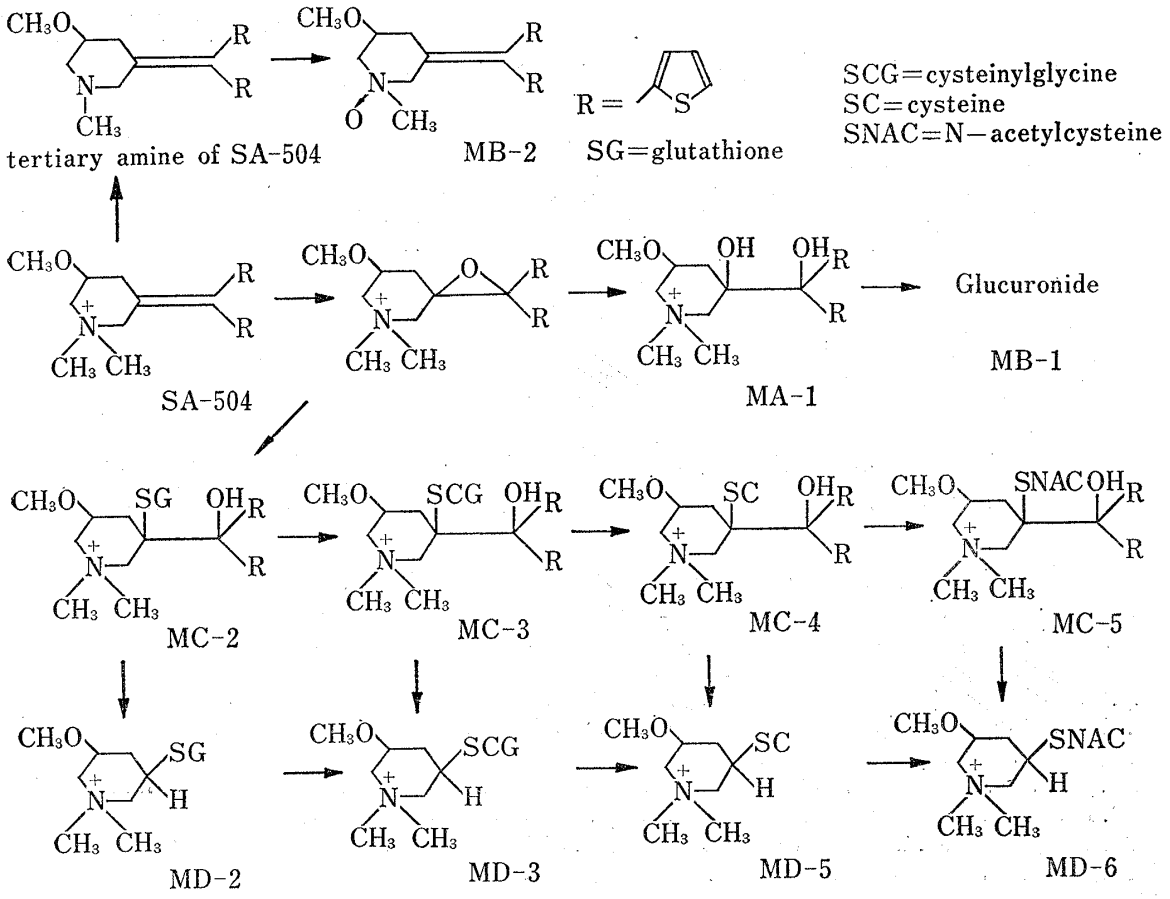

Fig. 4. Possible Metabolic Pathways of SA-504

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28) T. Meshi, J. Sugihara and Y. Sato, *Chem. Pharm. Bull.* (Tokyo), **19**, 1546 (1971).
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consider that tertiary amine N-oxide of SA-504 is formed by N-demethylation of SA-504 followed by N-oxidation.

From a consideration of the facts described above, it seems most reasonable to conclude that the metabolic fate of SA-504 includes 6 different reactions of epoxidation, GSH conjugation and hydration of the epoxide, cleavage of C-C bond, N-demethylation and N-oxidation and that the main metabolic pathway is epoxidation followed by GSH conjugation. Therefore, reactions shown in Fig. 4 may be proposed for the metabolism of SA-504.

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