on cytochrome P-450 or cyanide-sensitive factor, and associated with a new oxygen-activating enzyme, as well as the NADH-dependent deethylation of *p*-nitrophenetole. This new type of NADH-dependent oxygenase is, therefore, very likely to be also involved in common in other O-dealkylations with rabbit liver microsomes.

NADH plus NADPH-dependent demethylation activity of *p*-nitroanisole was increased more than NADH-dependent activity plus NADPH-dependent (Fig. 3). This activity was inhibited little more by CO than NADPH-dependent activity, although this mechanism was not known at the present.

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Metabolism of Budralazine, a New Antihypertensive Agent. II.[1] Metabolic Pathways of Budralazine in Rats[2]

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The metabolic pathways of budralazine were studied *in vivo* and *in vitro* experiments and characterized as follows; 1) 3-methyl-*s*-triazolo[3,4-*a*]phthalazine (M-1), one of the major metabolites of budralazine, was mainly biotransformed *via* 1-hydrazinophthalazine. 2) *s*-triazolo[3,4-*a*]phthalazine (M-3) was also biotransformed *via* 1-hydrazinophthalazine. 3) Experiments using liver preparations of rat were also discussed briefly.

Keywords——metabolism; budralazine; antihypertensive agent; metabolic pathway; *in vivo*; *in vitro*

Budralazine (1-[2-(1,3-dimethyl-2-butenylidene)hydrazino]phthalazine, DJ-1461) is an orally active antihypertensive agent.[4] In our previous work,[1] ten metabolites of the drug were isolated from rat urine and these structures were characterized by several physical methods. However, the metabolic pathway has not been established because two of the metabolites, 3-methyl-s-triazolo[3,4-*a*]phthalazine (M-1) and s-triazolo[3,4-*a*]phthalazine (M-3) can be presumably produced through several metabolic pathways as shown in Chart I and II, respectively.

In this report, we wish to present the metabolic pathways of budrazine by the confirmation of the formation routes of M-1 and M-3.

Experimental

Materials——Deuterated budralazine (budralazine-d_{10}) was synthesized by using mesityl oxide-d_{10} by the method of Ueno, *et al.*[5] The existence ratio of budralazine-d_{10} in the deuterated budralazine was

1) Part I: R. Moroi, K. Ono, T. Saito, M. Sano, and T. Akimoto, *Chem. Pharm. Bull.* (Tokyo), 24, 2850 (1976).
2) A part of this work was presented at the 95th Annual Meeting of Pharmaceutical Society of Japan, Nishinomiya, April 1975.
3) Location: *Minamifunabori-cho, Edogawa-ku, Tokyo, 132, Japan.*
4) A. Akashi, T. Chiba, and A. Kasahara, *Europ. J. Pharmacol.*, 29, 161 (1974).
5) K. Ueno, R. Moroi, M. Kitagawa, K. Asano, and S. Miyazaki, *Chem. Pharm. Bull.* (Tokyo), 24, 1068 (1976).

90% and d_0 form was less than 0.1%, which were calculated from the intensity of molecular ion peaks (*m/e* 240 and *m/e* 250) corresponding to each form on mass spectra shown in Fig. 1. Physicochemical properties of the labelled compound, melting point, ultraviolet (UV) spectrum and *Rf* values in three different thin-layer chromatography (TLC) solvent systems, were identical to those[5] of unlabelled compound.

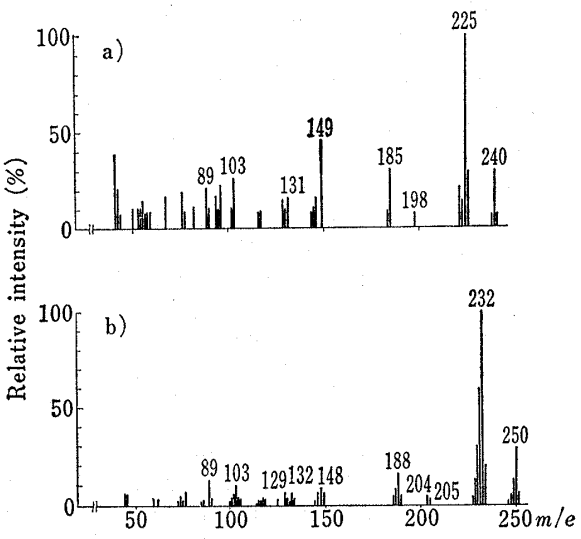

Fig. 1. Mass Spectra of Budralazine

a) none-labelled budralazine
b) budralazine-d_{10}

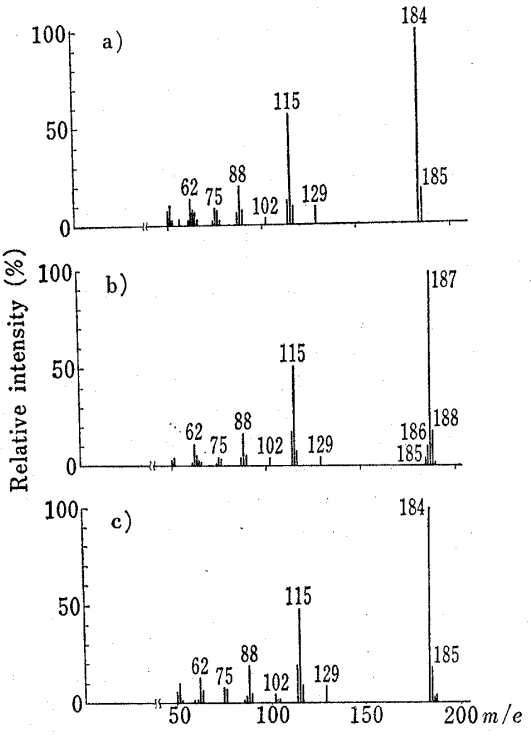

Fig. 2. Mass Spectra of M-1

a) none labelled authentic M-1
b) authentic M-1-d_3
c) M-1 biotransformed from budralazine-d_{10}

1-Hydrazinophthalazine (M-10) was synthesized according to the method of Druey, *et al.*[6] M-10 labelled with ^{14}C (M-10-^{14}C) at the 1- and 4-positions of the phthalazine ring was supplied from Daiichi Pure Chemical Co. Ltd (specific activity: 101.5 μCi/mg). The radiochemical purity was 98% by TLC.

M-1 was synthesized according to the method of Druey, *et al.*[6] M-1 labelled with ^{14}C (M-1-^{14}C) was prepared from the urine of rats after oral administration of budralazine-^{14}C by the method of Moroi, *et al.*[1] The radiochemical purity of M-1-^{14}C analyzed by TLC was over 98% (labelled at 1- and 4-positions of the phthalazine ring, specific activity: 3 μCi/mg). Deuterated M-1 (M-1-d_3) was synthesized as follows: A mixture of 1-hydrazinophthalazine (850 mg) and acetyl chloride-d_3 (2 ml) in benzene was heated under reflux for 3 hr, cooled and poured into ice-water. The mixture was neutralized with $NaHCO_3$ and extracted with benzene. After concentration of the extract, the residue was chromatographed on silica gel and eluted with benzene–$CHCl_3$ (1: 1). An obtained compound was crystallized from AcOEt to give M-1-d_3 (430 mg), mp 171—172°. The existence ratio of M-1-d_3 and -d_0 in the deuterated M-1 was 80.5% and less than 0.1%, respectively, which were calculated by similar way as described above. These mass spectra are shown in Fig. 2(a), 2(b). Elemental analysis was carried out by the thermal conductive detection method. *Anal.* Calcd. for $C_{10}H_5D_3N_4$: C, 64.16; H, 4.31; N, 29.93. Found: C, 64.15; H, 4.51; N, 29.81. The analytical value of deuterium was measured as hydrogen, because of the same thermal conductivity between deuterium oxide and hydrogen oxide.

Ethyl *s*-triazolo[3,4-*a*]phthalazine-3-carboxylate (1) was synthesized from 1-hydrazinophthalazine (3.2 g) and ethoxalyl chloride[7] by heating under reflux for 5 hr in benzene. The precipitate from the reaction mixture after cooling was recrystallized from EtOH to give (1) (2.2 g), mp 223—225°. *Anal.* Calcd. for $C_{12}H_{10}O_2N_4$: C, 59.50; H, 4.16; N, 23.13. Found: C, 59.68; H, 4.27; N, 23.27.

s-Triazolo[3,4-*a*]phthalazine-3-carboxylic acid (M-5) was obtained as follows: To a solution of (1) (1.0 g) in EtOH (30 ml) was added 1N-NaOH (6 ml). The solution was refluxed for 1 hr, cooled and neutralized with 2N-HCl. A precipitate was collected and recrystallized from EtOH–H_2O to give M-5 (85 mg), mp

6) J. Druey and B.H. Ringier, *Helv. Chem. Acta.*, 34, 195 (1951).
7) P.L. Southwick and L.L. Seivard, *J. Am. Chem. Soc.*, 71, 2532 (1949).

240—245°. *Anal.* Calcd. for $C_{10}H_6O_2N_4$: C, 56.07; H, 2.82; N, 26.16. Found: C, 55.78; H, 3.00; N, 26.40. M-5 labelled with ^{14}C (M-5-^{14}C) was prepared from the urine after oral administration of budralazine-^{14}C in rat as described in M-1-^{14}C. Radiochemical purity was 98% (specific activity 0.3 μCi/mg).

Animal Experiments——i) Budralazine-d_{10} suspended in 0.5% CMC was administered orally to two male Wistar rats (200 g) in a dose of 400 mg/kg. The animals were housed singly for 48 hr in metabolism cage which allowed the separate collection of urine and feces. ii) M-1-^{14}C suspended in 0.5% CMC was administered orally to three male Wistar rats in a dose of 10 mg/kg. The animals were kept for 48 hr in a similar manner as described above.

TLC——TLC was carried out on precoated 0.25 mm silica gel HF_{254} plate (Merck). The solvent systems used were a) $CHCl_3$–MeOH (1: 1, v/v), b) $CHCl_3$–acetone (1: 9, v/v), c) acetone–conc. NH_4OH (10: 1, v/v), d) acetone–AcOH (30: 1.5, v/v).

Liver Preparation——Freshly removed Wistar rat liver was homogenized in 4 volumes of ice-cold (0—4°) 1.15% KCl. The homogenate was centrifuged at $9000\times g$ for 20 min and the supernatant was processed further by differential centrifugation at $105000\times g$ for 1 hr. The microsomal pellet was suspended in a volume of 0.1M phosphate buffer pH 7.4, corresponding to 10 ml for each gram of liver.

Incubation——Incubations were conducted at 37° for 1 hr in 25 ml Erlenmeyer flasks containing 10 mM of $MgCl_2$, 0.1 mM of NADP, 0.1 mM of NAD, 0.1 mM of EDTA, 1 mM of glucose-6-phosphate, 1 mM of M-1-^{14}C (or M-10-^{14}C or M-5-^{14}C) and enzyme preparation ($9000\times g$ sup for M-1-^{14}C, or $105000\times g$ sup for M-10-^{14}C and M-5-^{14}C) equivalent to 750 mg of liver in a total volume of 5 ml of 0.2M potassium phosphate buffer pH 7.4.

Binding Spectrum in Microsomes——Microsome suspension (1 mg protein per ml) in 0.1M phosphate buffer was divided into several portions. To each of them was dissolved M-1 in the concentration range of 0.25 mM to 5 mM. The binding spectra (between 350 and 500 nm) of these solution were determined with reference to the microsome suspension with a Hitachi 365 Two-Wavelength Double Beam Spectrophotometer weared head-on type photomultiplier.

Identification of the Metabolites——i) *In Vivo*: a) Thirty milliters of urine obtained from 2 rats administered the budralazine-d_{10} was filtered and the filtrate was adsorbed onto a column of Amberlite XAD-2 resin (20 ml). After the column was washed with water (20 ml), the metabolites were eluted with $CHCl_3$–MeOH (1: 1, 40 ml). The eluate was evaporated to dryness in a stream of nitrogen. The residue was dissolved in H_2O (30 ml) and adjusted to pH 10—11 with 1N–NaOH (2 ml). The aqueous solution was extracted continuously for 20 hr with $CHCl_3$. After concentration of the $CHCl_3$ extract, the residue was applied on silica gel plate and developed with the solvent system (a) for M-1. The band corresponding to M-1 (*Rf*: 0.3) detected under UV light was scrapped off and extracted with $CHCl_3$–MeOH (1: 1). After concentration of the extract, the residue was crystallized from AcOEt to give the metabolite M-1 (2 mg). b) Forty milliters of urine obtained from 3 rats administered M-1-^{14}C was filtered and the filtrate was onto a column of Amberlite XAD-2 resin (30 ml) in a similar manner as described above. The eluate with $CHCl_3$–MeOH (1: 1) was evaporated to dryness in a stream of nitrogen. The residue was dissolved in H_2O (40 ml) and adjusted to pH 2—3 with 1N HCl and extracted with $CHCl_3$. The $CHCl_3$ layer was evaporated to dryness. The residue was applied on silica gel plate and developed with the solvent system (d) for M-3.

ii) *In Vitro*: The incubation mixture was dissolved in 2-fold volume of MeOH. After concentration of the MeOH solution, the residue was analyzed by radioscannogram of TLC. The bands on TLC were scrapped off and analyzed by high resolution mass spectrometry, using a JEOL JMC OISG Spectrometer.

Results and Discussion

Biotransformation Route of M-1

There were two possible pathways in the biotransformation of M-1. One of them was a route *via* the metabolite M-10 including acetylation and dehydration. The acylation of of M-10 gives a ring closed compound and not gives an acylated compound even in a mild condition[6] or *in vitro* experiment.[8] The other was oxidative ring closure of budralazine as shown in Chart 1. The methyl group of M-1 is originated from acetyl group introduced by bioacetylation in route A or from the butenylidene group of budralazine itself in route B. In order to clarify the route, budralazine-d_{10} was administered orally in rats. The crystalline sample isolated from the urine was identified by mass spectrometry as shown in Fig. 2 (c).

8) Douglass, *et al.* reported that the rat liver homogenate with 1-hydrazinophthalazine gave acetylhydrazine. But later this structure was corrected by Zimmer, *et al.* as 3-methyl-*s*-triazolo[3,4-*a*]phthalazine. *a*) C.D. Douglass and R. Hogan, *Proc. Soc. Exptl. Bio. Med.*, **100**, 446 (1959); *b*) H. Zimmer, J. McManus, T. Novison, E.V. Hess, and A.H. Litwin, *Arzneim.-Forsch.* (Drug Res.) **20**, 1586 (1970).

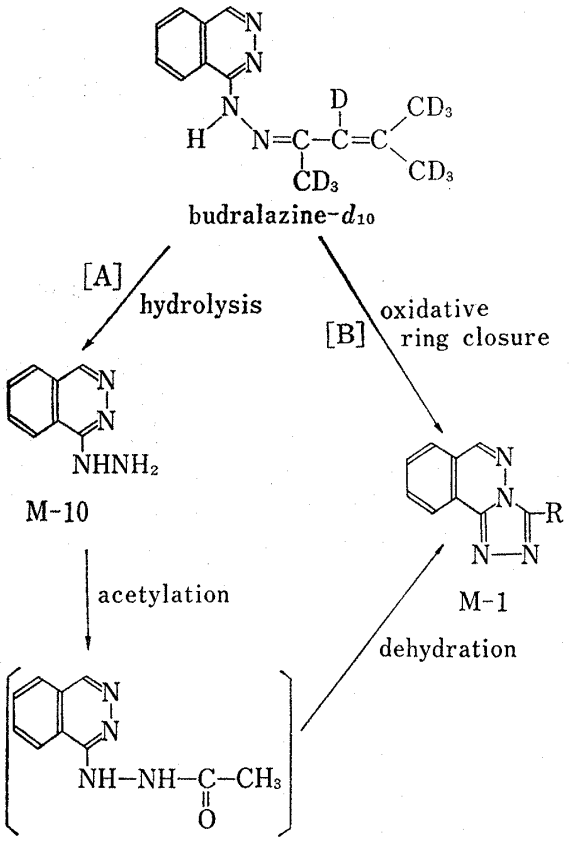

Chart 1. Presumable Transformation Routes of M-1 from Budralazine-d_{10}

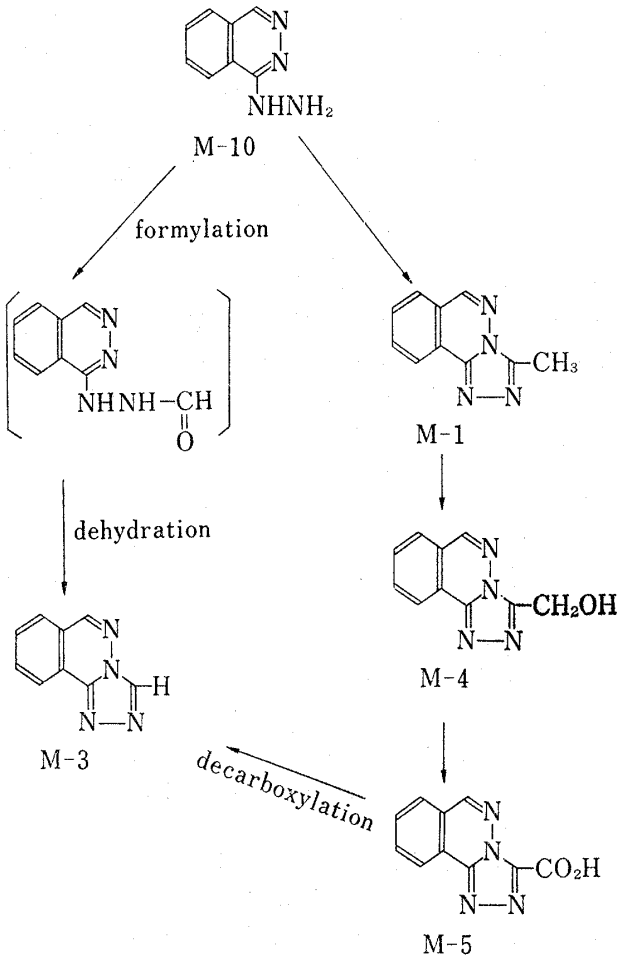

Chart 2. Presumable Transformation Routes of M-3 from M-10

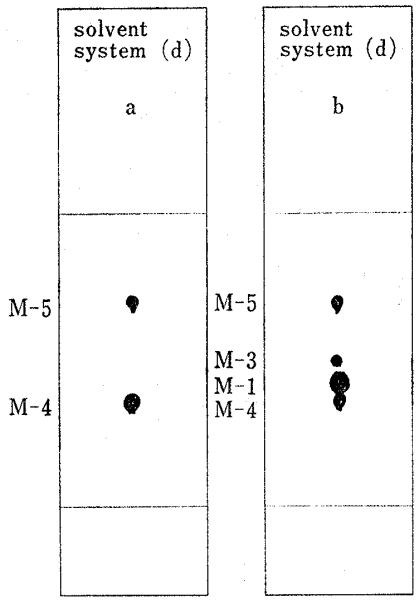

Fig. 3. TLC Pattern of Urine Extracts

a) after oral administration of M-1-^{14}C
b) after oral administration of budralazine-^{14}C

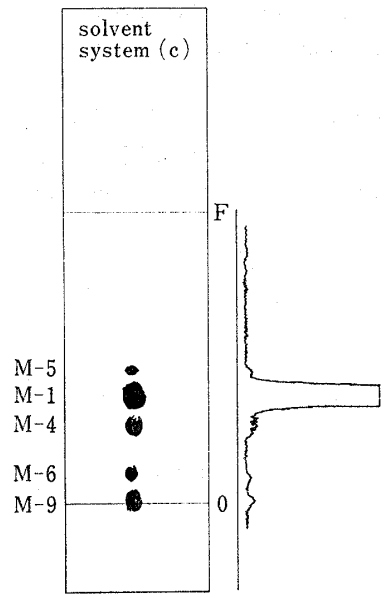

Fig. 4. TLC Pattern of the Mixture incubated with M-1-^{14}C and 9000×*g* Supernatant

Molecular ion cluster of the sample possessed the significant ion at *m/e* 184 and 187. The elemental compositions of them were confirmed by high resolution mass spectrometry as follows; $C_{10}H_8N_4$ calcd: 184.0748, obsd: 184.0716. $C_{10}H_5D_3N_4$ calcd: 187.0904, obsd: 187.0937. The existence ratio of *m/e* 184 and 187 was estimated as approximately 100: 4 from their ion intensity which was recorded with on-line computer system and corrected with isotopic purity of administered budralazine-d_{10}. The results suggested that M-1 was produced not only by route A, but also by route B.

Biotransformation Route of M-3

The biotransformation of M-3 was assumed to proceed through two routes. One was a dehydrocyclization of formylated M-10 which was a postulated intermediate, and the other was a decarboxylation of M-5 as shown in Chart 2. To elucidate the biotransformation route of M-3, the presence of M-3 in the urinary metabolites was examined after administration of M-1-^{14}C to rats. According to the previous report,[1] M-3 should be extractable from the urine with chloroform in a weakly acidic condition (pH 2—3). The TLC pattern of the extract is shown with that of a similar extract after administrating budralazine-^{14}C in Fig. 3 (a) and (b). The detected metabolites in the case of administration of M-1-^{14}C lacked M-3. So M-3 was seem to be biotransformed via formylated M-10 which was immediately cyclized as explained above.

***In Vitro* Expreiments**

The enzymatic reaction of M-1-^{14}C with 9000×***g*** supernatant produced the metabolites, M-4, M-5, M-6 and M-9 as shown in Fig. 4. Those metabolites were obtained *in vivo* experiment.[1] This fact suggested that the hepatic microsomes played a major role in the metabolism of budralazine in rats. A preliminary experiment concerned on the binding spectrum of M-1 in 105000×***g*** pellet, microsomal fraction, was performed. The spectrum ranging on concentration from 0.25 mM to 5 mM of M-1 showed a typical type II pattern as shown in Fig. 5. The oxidation of M-1 would be dependent on P-450. The reaction

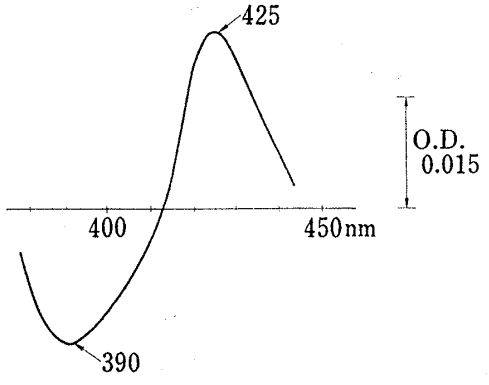

Fig. 5. Binding Spectrum of Microsome with M-1 in Concentration of 1mM

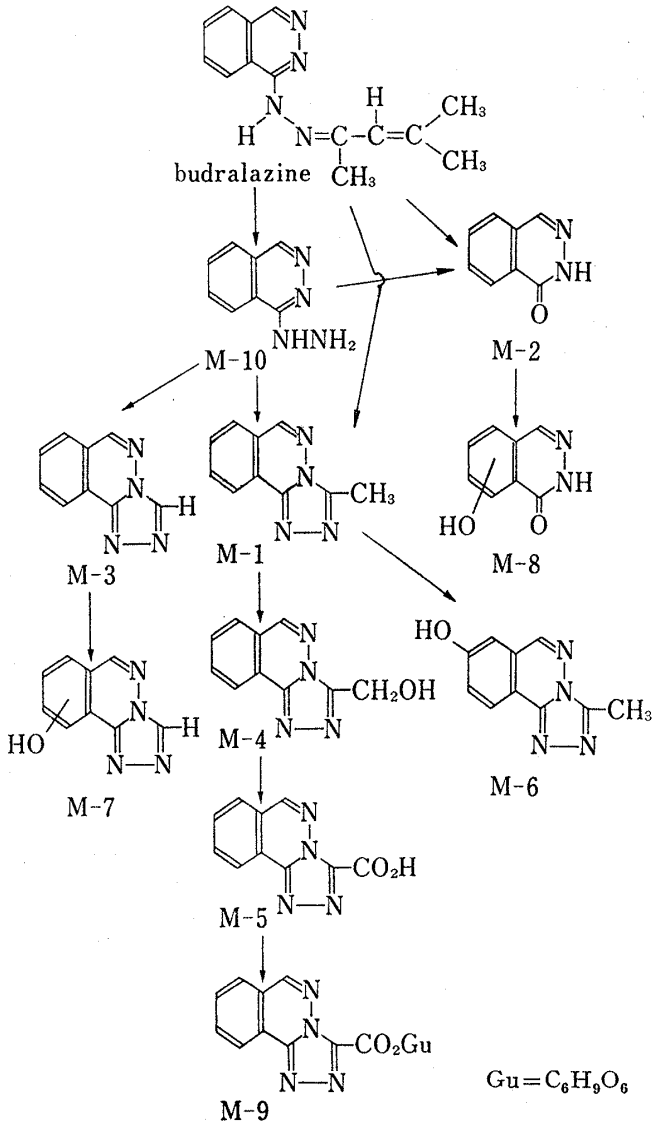

Chart 3. Established Metabolic Pathways of Budralazine

of M-10- ^{14}C with 105000×*g* supernatant produced M-1 and M-3, but the reaction of M-5- ^{14}C with 105000×*g* supernatant did not produce M-3. As explained above, the formation process of M-3 was deduced to be a dehydrocylization *via* formylated M-10. This formate as intermediate wolud be generated from a C_1-unit of tetrahydrofolic acid.

Metabolic Pathways of Budralazine

In conclusion, the pathways produced for the biotransformation of budralazine were presented in Chart 3.

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