IDENTIFICATION OF NOVEL *N*-GLUCURONIDES IN RAT BILE AFTER ADMINISTRATION OF 450191-S, A 1*H*-1,2,4-TRIAZOLYL BENZOPHENONE DERIVATIVE

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5-[(2-Aminoacetamido)methyl]-1-[*p*-chloro-2-(*o*-chlorobenzoyl)phenyl]-*N,N*-dimethyl-1*H*-s-triazole-3-carboxamide hydrochloride dihydrate (450191-S) is a ring-opened derivative of 1,4-benzodiazepine, which is activated by desglycylation and subsequent cyclization. After 450191-S administration, rat bile contained three novel conjugates which released active metabolites possessing the 1,4-benzodiazepine structure through β -glucuronidase hydrolysis. Since the released metabolites have no functional groups to conjugate with glucuronic acid, we speculated that the aglycone might be the ring-opened form of 1,4-benzodiazepine which spontaneously cyclizes after the release of glucuronic acid. This possibility was tested by chemically reducing the ketone group of the ring-opened 1,4-benzodiazepine glucuronate conjugates, which would prevent the spontaneous ring closure reaction after the release of the glucuronic acid moiety. The NaBH₄ reduction of the ketone of the benzophenone moiety of the conjugates and subsequent treatment with β -glucuronidase allowed identification of the reduced aglycones with authentic samples using gas chromatography-mass spectrometry.

Keywords — ring-opened 1,4-benzodiazepine derivative; sleep inducer; isolation; identification; *N*-glucuronide; rat bile; GC-MS

INTRODUCTION

5-[(2-Aminoacetamido)methyl]-1-[*p*-chloro-2-(*o*-chlorobenzoyl)phenyl]-*N,N*-dimethyl-1*H*-s-triazole-3-carboxamide hydrochloride dihydrate (450191-S) is a ring-opened derivative of 1,4-benzodiazepine and demonstrates a sleep-inducing effect after metabolic activation.¹⁻³⁾ In examining 450191-S metabolites in rat bile, we found three novel conjugates that released M-1, M-2 and M-A (Fig. 1) *via* enzymatic hydrolysis with β -glucuronidase. Since M-1 and M-2 have no functional groups capable of conjugating with glucuronic acid, the aglycones of these glucuronides must have structures differing from M-1, M-2 and M-A. We recently found that rat plasma contains desglycylation 450191-S (191DG) which is labile and spontaneously cyclizes to the 1,4-benzodiazepine derivative.⁴⁾ When rats received M-1 itself, the rat bile did not contain such glucuronides. From these facts, we considered that the novel conjugates are probably *N*-glucuronides of the ring-opened form of 1,4-benzodiazepine (Chart 1). Namely, if glucuronic acid is hydrolyzed with β -glucuronidase, the ring-closing reaction should spontaneously occur to form the 1,4-benzodiazepine structure,³⁾ in other words, M-1, M-2 and M-A would be formed after β -glucuronidase hydroly-

sis. To confirm this speculation, we planned to chemically modify the conjugates which would prevent spontaneous cyclization without affecting the glucuronic acid moiety. In this study, we succeeded modifying the conjugates by reducing the ketone moiety with NaBH₄, thus preventing the spontaneous ring-closing reaction. The identification of the reduced aglycones permitted the assignment of the chemical structures of the novel glucuronides.

MATERIALS AND METHODS

Materials — Male Jcl-SD rats weighing 250–260 g were used after 24 h of fasting. β -Glucuronidase (calf liver, 13000 UF/ml) was purchased from Tokyo Zoki Chemical Co. (Tokyo, Japan). *N*-Methy-*N*-trimethylsilyl trifluoroacetamide (MSTFA, Pierce Chemical Co., Ill., USA) was used for trimethylsilylation. ¹⁴C-450191-S (specific activity, 4.8 μ Ci/mg; purity, 96.9%) was synthesized by Dr. M. Yoshiooka and his co-workers in our laboratories (labeled positon is denoted in Fig. 1). Saccharo-1,4-lactone was purchased from Sigma Chemical Co. (St. Louis, Mo., USA).

Metabolite Isolation — Rats were anesthetized with i.p. administration of Somnopentyl® (Pitman-Moore, N.J., USA) and polyethylene

FIG. 1. Chemical Structures of 450191-S, M-1, M-2 and M-A

tubing (PE-10, Clay Adams, N.J., USA) was inserted into the common bile duct. 450191-S (50 mg/kg) was intraduodenally administered to the rat and the bile was collected for 1 h. The bile was applied to a Bond Elut® SAX column (Analytichem International, Calif., USA) and the column eluted with 1 N HCl. The eluate was adjusted to pH 6 with sodium acetate and evaporated under reduced pressure. The residue was dissolved in a small amount of water and then NaBH₄ was added to reduce the ketone group of the benzophenones. The reaction mixture was again adjusted to pH 5 with acetic acid and the ketone-reduced glucuronides were enzymatically hydrolyzed with β -glucuronidase (2300 UF/ml of bile) at 37 °C for 18 h. The hydrolysate was extracted with chloroform at pH 2 to remove ring-closed metabolites and then the residual aqueous layer was adjusted to pH 8 and extracted with ethyl acetate. The solvent was removed by evaporation under reduced pressure. The residue was dissolved in methanol and spotted on a silica gel plate (Art. 5715, Merck, Darmstadt, W. Germany). The plate was developed with the solvent mixture, chloroform-methanol-28% ammonium hydroxide (20:6:1 by v/v). All the spots were scraped after detection with ultraviolet (UV) lamp (254 nm) irradiation, and then eluted with the solvent mixture, dichloromethane-ethanol (1:1 by v/v). The eluate was evaporated under reduced pressure and subjected to gas chromatography-mass spectrometry (GC-MS) after trimethylsilylation using MSTFA. To identify the *N*-hydroxymethyl substituted metabolite, the ethyl acetate extracts at an alkaline pH were acetylated using pyridine and acetic anhydride prior to spotting on thin

layer chromatography (TLC) plates.

GC-MS — The gas chromatograph-mass spectrometer was a Varian-MAT 44S coupled to a Varian Model 3700 gas chromatograph equipped with a moving needle injector.

CHART 1

CHART 2

Column: fused silica capillary column SE-54 (25 m length $\times$ 0.3 mm i.d., Hewlett Packard, Calif., USA). Operating temperatures: injector 270 °C, column oven 300 °C, transfer line 295 °C, ion source 170 °C. Carrier gas: He (inlet pressure 20 psi).

SIMS (Secondary Ion Mass Spectrometry)

—A secondary ion mass spectrum was observed with a Hitachi M-68 mass spectrometer using Xe as the primary ion gas.

Inhibition of β -Glucuronidase Hydrolysis

—Bile was collected for 1 h following intraduodenal administration of ^{14}C -450191-S (10 mg/kg) to bile-fistula rats. The bile (0.1 ml) was incubated with β -glucuronidase (1300 UF) and saccharo-1,4-lactone ranging from 10^{-3} to 10^{-6} M in acetate buffer (pH 5.0) for 6 h at 37 °C. After the incubation, the reaction mixture was extracted with ethyl acetate. Then, the organic solvent was evaporated under reduced pressure and subjected to TLC. The determination method for M-1, M-2 and M-A using TLC was described previously.⁵⁾

Synthesis of Authentic Compounds —The synthetic methods are outlined in Chart 2. 1,4-Benzodiazepines, Ia-c, were heated in HBr to

obtain IIa-c and then reduced with $NaBH_4$ to form IIIa-c. Since IIIc itself was too labile to purify, it was acetylated further.

5-Aminomethyl-N,N-dimethyl-1-[4-chloro-2-(α -hydroxy-o-chlorobenzyl)phenyl]-s-triazole-3-carboxamide, IIIa (ORM-1) —Ia (151 mg) was added to 3 ml of 5% HBr and stirred at 60 °C. The crystals of Ia were dissolved and the precipitate which appeared during 0.5-h stirring was collected by filtration and dried. Methanol (5 ml) was rapidly added to the mixture of the precipitate and $NaBH_4$ (152 mg) and the resulting solution was allowed to stand for 15 min. The methanol was removed by evaporation and the residue was extracted with three 10-ml portions of CH_2Cl_2 after addition of ice-chilled water (5 ml). The solvent was washed with saturated sodium chloride, dried over Na_2SO_4 and evaporated under reduced pressure. The residue was purified with TLC using a silica gel plate and the solvent mixture, CH_2Cl_2 -MeOH-EtOAc-*n*-hexane (6:3:3:3 by v/v), which gave IIIa and the yield was 66 mg (47%). Colorless prisms (CH_3CN), mp 184–185 °C. IR ν_{max}^{Nujol} cm^{-1} : 3400–3150 (NH_2 , OH), 1645 ($-CON<$). 1H -NMR ($CDCl_3$) δ : 7.75–7.06 (7H, m, aromatic-

H), 5.68 (1H, s, $>\text{CHOH}$), 4.15, 3.45 (2H, ABq $J = 15.2$ Hz, $=\text{C}-\text{CH}_2-\text{NH}_2$), 3.29 (3H, s, $-\text{CON}(\text{CH}_3)(\text{CH}_3)$), 3.12 (3H, s, $-\text{CON}(\text{CH}_3)(\text{CH}_3)$). MS m/z : 419 (M^+). Anal. Calcd for $\text{C}_{19}\text{H}_{19}\text{Cl}_2\text{N}_5\text{O}_2$: C, 54.30; H, 4.56; Cl, 16.87; N, 16.66. Found: C, 54.13; H, 4.67; Cl, 16.93; N, 16.86.

5-Aminomethyl-N-methyl-1-[4-chloro-2-(α -hydroxy-*o*-chlorobenzyl)phenyl]-s-triazole-3-carboxamide, IIIb (ORM-2) — Ib (33 mg) was refluxed in a solvent mixture of CH_3CN (1 ml) and 5% HBr (1 ml) for 0.5 h. The resultant mixture was evaporated to dryness under reduced pressure and NaBH_4 (35 mg) and methanol were added to the dried residue. The resulting solution was allowed to stand for 0.5 h and then purified by TLC, giving IIIb and the yield was 7 mg (21%). Colorless needles (CHCl_3), mp 207–210 °C. IR $\nu_{\text{max}}^{\text{Nujol}}$ cm^{-1} : 3500–3050 (NH_2 , NH , OH), 1670 ($-\text{CON}<$). $^1\text{H-NMR}$ (CDCl_3) δ : 7.72–6.82 (7H, m, aromatic-H), 5.68 (1H, s, $>\text{CHOH}$), 4.00, 3.42 (2H, ABq, $J = 14.4$ Hz, $=\text{C}-\text{CH}_2-\text{NH}_2$), 3.03 (3H, d, $J = 2.8$ Hz, $-\text{CONHCH}_3$). MS m/z 405 (M^+). Anal. Calcd for $\text{C}_{18}\text{H}_{17}\text{Cl}_2\text{N}_5\text{O}_2$: C, 53.21; H, 4.22; Cl, 17.45; N, 17.24. Found: C, 53.22; H, 4.32; Cl, 17.60; N, 17.21.

5-Aminomethyl-N-hydroxymethyl-1-[4-chloro-2-(α -hydroxy-*o*-chlorobenzyl)phenyl]-s-triazole-3-carboxamide, IIIc (ORM-A) — Ic (58 mg) was added to a solution of CH_3CN (3 ml) and 5%

HBr (1.5 ml) and then stirred at 60 °C for 0.5 h. At room temperature, NaBH_4 (100 mg) was added to the resultant mixture, which was immediately adjusted to pH 4 with acetic acid and then the organic solvent was removed by evaporation. The residual aqueous solution was filtered to remove the precipitate. The filtrate was washed with three 3-ml portions of CH_2Cl_2 , adjusted to pH 8–9 with saturated NaHCO_3 and extracted with three 5 ml of CH_2Cl_2 . The organic layer was washed with saturated sodium chloride, dried over Na_2SO_4 and evaporated giving IIIc. The yield was 22 mg (33%). This was so labile that it was not purified further.

5-Acetamidomethyl-N-acetoxymethyl-1-[4-chloro-2-(α -acetoxy-*o*-chlorobenzyl)phenyl]-s-triazole-3-carboxamide, IVc (Triacetyl ORM-A) — IIIc (20 mg) was stirred in the mixture of acetic anhydride (0.3 ml) and sodium acetate (10 mg) at 60 °C for 1 h. The resultant mixture was evaporated and extracted with CH_2Cl_2 after addition of ice-chilled water (1 ml). The organic layer was washed with saturated sodium chloride, dried over Na_2SO_4 and evaporated to dryness. The extract was purified with TLC, giving IVc. The yield was 13 mg (51%). White powder (*n*-hexane), mp 80–95 °C. IR $\nu_{\text{max}}^{\text{Nujol}}$ cm^{-1} : 3450–3120 (NH), 1740 ($\text{C}=\text{O}$), 1670 ($-\text{CON}<$). $^1\text{H-NMR}$ (CDCl_3) δ : 8.10–6.80 (7H, m, aromatic-H), 6.83 (1H, s, $>\text{CHOCH}_3$), 5.40 (2H, d, $J = 7.2$ Hz, $-\text{CONHCH}_2\text{OCOCH}_3$),

FIG. 2. GC-MS Spectra of Synthetic ORM-1 (Panel A) and Isolated Metabolites from Rat Bile (Panel B) Mass spectra were determined after trimethylsilylation.

FIG. 3. GC-MS Spectra of Synthetic ORM-2 (Panel A) and Isolated Metabolites from Rat Bile (Panel B) Mass spectra were determined after trimethylsilylation.

4.30 (2H, t, $=\text{C}-\text{CH}_2-\text{NHCOCH}_3$), 2.13, 2.06, 1.92 (3H $\times$ 3, s, $>\text{CHO}-\text{COCH}_3$, $-\text{CONHCH}_2-\text{O}-\text{COCH}_3$, $=\text{C}-\text{CH}_2-\text{NH}-\text{COCH}_3$). MS m/z 504 ($(\text{M}-\text{COCH}_3)^+$). SIMS m/z 548 (MH^+). Anal. Calcd for $\text{C}_{24}\text{H}_{23}\text{Cl}_2\text{N}_5\text{O}_6$: C, 52.57; H, 4.23; Cl, 12.93; N, 12.77. Found: C, 52.20; H, 4.58; Cl, 12.67; N, 12.57.

RESULTS AND DISCUSSION

To interrupt the ring-closing reaction, we

examined the reduction of the ketone moiety of 450191-S as a model, using NaBH_4 , and found that it was rapidly reduced in aqueous solution at room temperature and at neutral pH, all of which are favorable conditions to modify the *N*-glucuronides in rat bile. We also found that 191DG was similarly reduced and, as expected, its ring-closing reaction could be inhibited. Then, we applied the reduction to the bile obtained from the rat given 450191-S. Chart 1 out-

FIG. 4. GC-MS Spectra of Synthetic ORM-A (Panel A) and Isolated Metabolites from Rat Bile (Panel B) Mass spectra were determined after acetylation.

TABLE I. Effect of Saccharo-1,4-lactone on β -Glucuronidase Hydrolysis of Ring-Opened *N*-Glucuronides in Rat Bile

Metabolite	Addition of saccharo-1,4-lactone (M)			
	10^{-3}	10^{-4}	10^{-5}	10^{-6}
M-1	0.4	11.6	69.5	101.3
M-2	— ^{a)}	7.1	56.1	100.5
M-A	— ^{a)}	— ^{a)}	48.0	100.6

Each value represents percent of concentrations obtained from the incubation without saccharo-1,4-lactone. a) not detected.

lines, the reaction and the details are given under Materials and Methods.

Figure 2 shows the mass-spectra obtained from the spot (*R_f* 0.72) on the TLC plate and synthetic ORM-1 (IIIa). Figure 3 shows the spectra obtained from the spot (*R_f* 0.64) and synthetic ORM-2 (IIIb). Thus, the bile was demonstrated to contain ORM-1 and ORM-2, showing the presence of *N*-glucuronides of the ring-opened form of 1,4-benzodiazepine.

In the case of M-A, there are two possible types of glucuronides. One is the *N*-glucuronide of a ring-opened form similar to that described above and the other is a ring-closed form, namely M-A itself, with the *N*-hydroxymethyl acting as a functional group for the conjugation. As ORM-A was not detected at the same time when ORM-1 and ORM-2 were found and authentic ORM-A could not be purified because of its instability, we attempted to acetylate ORM-A to purify it as its triacetyl derivative. The extracts from the bile subjected to reduction were similarly acetylated prior to being subjected to TLC to avoid decomposition of ORM-A during TLC. Figure 4 shows the mass spectra obtained from the spot (*R_f* 0.47) on the TLC plate and the authentic triacetyl ORM-A. Thus, ORM-A was identified, suggesting that M-A released through the enzymatic hydrolysis originated from the *N*-glucuronide of the ring-opened form of M-A, which is similar to the cases of M-1 and M-2.

In order to ascertain whether the conjugates were truly glucuronides, we investigated the inhibition of β -glucuronidase using saccharo-1,4-lactone, a specific inhibitor of β -glucuronidase.⁶⁾ As shown in Table I, the hydrolysis was substantially inhibited by saccharo-1,4-lactone at 10^{-4} M, indicating that the conjugates were un-

doubtedly glucuronides.

In other experiments using ¹⁴C-450191-S, we found that only a small percent of the dose administered was excreted these glucuronides in rat bile. Though these glucuronides are minor metabolites in rat, their existence may be important in 450191-S metabolism because not only M-1 but also M-2 and M-A exist in a ring-opened form in the body. 191DG, a ring-opened form of M-1, aids in the reduction of extensive hepatic extraction resulting in the increase of the area under the plasma concentration-time curve (*AUC*) of the active metabolite M-1.⁴⁾ Similarly, the ring-opened forms of M-2 and M-A may also increase *AUC*s of M-2 and M-A. The glucuronides are also found in monkey and human urine (data not shown), which indicates that the ring-opened precursors also exist in monkey and human plasma, where they may act to increase the *AUC*s.

In conclusion, we have assigned the chemical structures of the three novel conjugates that released the 1,4-benzodiazepine metabolites after β -glucuronidase hydrolysis as *N*-glucuronides of the ring-opened form of 1,4-benzodiazepine in rat bile after 450191-S administration.

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