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205. Keitaro Kato, Kazuo Yoshida, Kiyoshi Tatsumi, and Hisao Tsukamoto: Metabolism of Drugs. XXXIII.*1 Studies on the Ring Structure of *p*-Aminobenzoyl Glucuronide isolated from the Urine of Dog administered with *p*-Aminobenzoic Acid. (1).*2

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p-Aminobenzoyl glucuronide (PABA-GA) was first isolated by Bray, *et al.*[1] from the urine of rabbits administered with *p*-aminobenzoic acid (PABA). Nakao, *et al.*[2] isolated this glucuronide from the dog urine according to the method of Bray, and concluded that PABA-GA might possess a furanose configuration from the results of the infrared spectrum and periodate oxidation.

It is known fact that uridine diphosphate glucuronic acid acts as a glucuronyl donor in the synthesis of 'ester' glucuronide,[3] and has a pyranose configuration.[4] Therefore the 'ester' glucuronides isolated from the animal urine were believed to be a pyranose type. It is interesting to note that the furanose type of glucuronide would be formed in nature, but nothing is known of the mechanisms by which it is synthesized. Therefore, the authors reinvestigated Nakao's work leading to his postulation of the furanose structure of PABA-GA, and established the fact that this glucuronide had instead a pyranose configuration.

PABA-GA used for this study was isolated from the urine of dog administered with PABA according to the method of Bray and the physical constants of this compound were essentially the same as those described in Bray's paper. The ring structure of the 'ester' glucuronides isolated from the animal urine was first established by Pryde and Williams.[5] The ring size of the glucuronides was determined by methylation and oxidation procedures. However, a complete methylation of the 'ester' glucuronide was unsuccessful.[6] Thus, one of the most convenient methods to determine the structure of the 'ester' glucuronides was to compare the methyl acetyl derivatives of glucuronide with synthetic compounds prepared by condensation of methyl (1-bromo-2,3,4-tri-O-acetyl-α-D-glucopyranosid)uronate and aglicons. The compound obtained from PABA-GA by esterification with diazomethane and acetylation with pyridine-acetic anhydride was found to be identical with synthetic methyl (*p*-acetamidobenzoyl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate prepared by the condensation of silver *p*-acetamidobenzoate and methyl (1-bromo-2,3,4-tri-O-acetyl-α-D-glucopyranosid)uronate in acetonitrile by mixed fusion method and comparison of infrared spectra. Therefore, the methyl acetyl derivative of PABA-GA isolated from the dog urine had a pyranose configuration. However this conclusion has not been extended beyond the methyl acetyl derivative. It appeared necessary to investigate further a ring conversion, namely a conversion of furanose to pyranose, during the course of methylation and acetylation.

*1 Part XXXII. H. Tsukamoto, H. Yoshimura, H. Ide: This Bulletin

*2 Presented at the Kyushv Brancd Meeting of the Pharmaceutical Socity of Japan, at Fukuoka, October 21, 1960.

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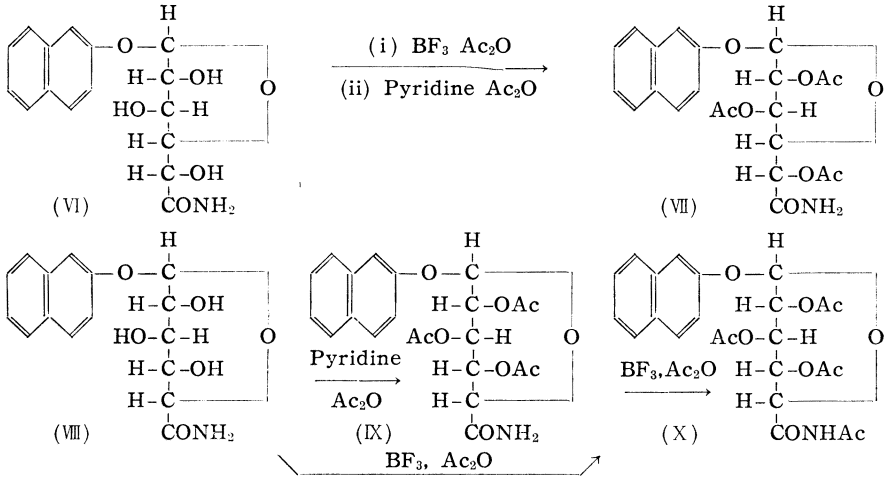
1) H.G. Bray, *et al.*: Biochem. J., **42**, 434 (1948).
2) T. Nakao, M. Nakao, T. Nakajima: J. Biochem. (Japan), **45**, 207 (1958).
3) G.J. Dutton: Biochem. J., **64**, 693 (1956).
4) M. Honjo, *et al.*: This Bulletin, **8**, 750 (1960).
5) J. Pryde, R.T. Williams: Biochem. J., **27**, 1197 (1933).
6) *Idem.*: *Ibid.*, **27**, 1210 (1933).

Since a furanose type of free glucuronide has not yet been synthesized, 2-naphthyl-β-D-glucofuranosiduronamide (VI) prepared previously by Tsou and Seligman[7] appeares to be suitable for this purpose. Acetylation of (VI) with pyridine-acetic anhydride or boron trifluoride-acetic anhydride gave the same compounds. This compound was not identical with the acetyl derivative (IX) of 2-naphthyl-β-D-glucopyranosiduronamide (VIII) prepared by the pyridine method. (IX) was converted to (X) by acetylation with boron



trifluoride-acetic anhydride and (X) was also obtained from (VIII) by the same acetylation method. In this case an acid amide group was acetylated. In the furanosiduronamide preparation, acetylation of an acid amide group did not occur using the boron trifluoride method.

The result of these experiments left no doubt that the ring conversion was not possible during the course of the acetylation and they led to the conclusion that the acetylation of PABA-GA followed by esterification with diazomethane produces a compound which retains its initial ring structure.

Since an attempt to acetylate the free glucuronide by the pyridine method was unsuccessful, PABA-GA was acetylated with boron trifluoride-acetic anhydride. Then a methyl-ester was prepared by esterification with diazomethane and this methyl acetyl derivative was identified with authentic methyl (*p*-acetamidobenzoyl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate by mixed fusion and comparison of infrared spectra. In view of above facts, one might conclude that PABA-GA isolated from the dog urine has a pyranose configuration.

PABA-GA used in the foregoing experiments was hydrolyzed by β-glucuronidase, prepared from the rabbit-liver, and this hydrolysis was inhibited by saccharo-1,4-lactone. Moreover, hydrolysis of *p*-nitrophenyl-β-D-glucuronide was inhibited competitively by PABA-GA for the same enzyme. But experiments dealing with the action of β-glucuronidase on β-D-glucofuranosiduronic acid have been criticized by Levvy and Marsh,[8] and the specificity of β-glucuronidase for β-D-glucofuranosiduronic acid is not certain at the present. So the ring structure of the glucuronide could not be confirmed by the action of β-glucuronidase.

Experimental*4

Isolation of PABA-GA (I) from Dog Urine——The animal used was a female dog weighing about 15 kg. 10 g. of PABA suspended in 50 cc. of H_2O with 5 g. of gum arabic was administered via

*4 All melting points are uncorrected.

7) K.C. Tsou, A.M. Seligman: J. Am. Chem. Soc., **74**, 5605 (1952).

8) G.A. Levvy, C.A. Marsh: Advances in Carbohydrate Chemistry, **14**, 423 (1959).

stomach tube. The 24-hr. urine collected from the dog was filtered, adjusted to about pH 4 with glacial AcOH and then treated with saturated lead acetate solution until precipitation was complete. The precipitate was removed by filtration. The filtrate was brought to pH 7.0 with NH_4OH and to which saturated basic lead acetate solution was added in excess. The basic lead precipitate was filtered off, washed with H_2O made into a fine suspension in MeOH, and the lead was removed by saturation with H_2S. After removal of PbS, MeOH solution was concentrated to a small volume at 20~25° in a reduced pressure.

An addition of a small amount of EtOH to the residue induced crystallization. The collected crystalline product (7 g.) was dissolved in 150 cc. of H_2O. The solution was extracted with Et_2O successively for 12 hr. to remove PABA and the aqueous layer was concentrated at 25~30° in a reduced pressure. After addition of EtOH to the concentrate, a substance crystallizing as fine white needles was obtained which upon recrystallization from MeOH-EtOH melted at 208°(decomp.). $[\alpha]_D^{14}$ −13.5° (c=1.0, H_2O). Yield, 3.5 g. *Anal.* Calcd. for $C_{13}H_{15}O_8N$: C, 49.80; H, 4.87; N, 4.47. Found : C, 49.85; H, 5.02; N, 4.29.

Synthesis of Methyl (*p*-Acetamidobenzoyl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate from PABA-GA (II)——To a solution of 0.29 g. of PABA-GA dissolved in a small volume of MeOH 35 cc. of Et_2O solution of CH_2N_2 was added. The mixture, after standing overnight in a refrigerator, was evaporated to dryness in a reduced pressure. The residue, after drying over P_2O_5 *in vacuo*, was dissolved in 5 cc. of pyridine and to the resulting solution 3.5 cc. of Ac_2O was added. The mixture was allowed to stand overnight at room temperature and then it was poured into 80 cc. of ice-water with stirring. The crystalline product was collected and recrystallized from EtOH to fine white needles, m.p. 197~199° : $[\alpha]_D^{10}$ −42.0°(c=1.0, $CHCl_3$). Yield, 0.37 g.(86%). *Anal.* Calcd. for $C_{22}H_{25}O_{12}N$: C, 53.33; H, 5.08; N, 2.87. Found : C, 52.90; H, 5.36; N, 2.84. There was no depression of melting point when it was mixed with authentic methyl (*p*-acetamidobenzoyl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (III) and its infrared spectrum was identical with that of the authentic sample.

Methyl (*p*-Acetamidobenzoyl-2,3,4-tri-O-acetyl-β-D-glucoypranosid)uronate (III)——To a solution of 1 g. of methyl (1-bromo-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate in 5 cc. of dehyd. MeOH, 1.4 g. of silver *p*-acetamidobenzoate was added and the mixture was shaken for 30 min. After removal of precipitate, the filtrate was concentrated in a reduced pressure. The residue was extracted with hot EtOH, the extract was treated with carbon, and filtered. The filtrate was then concentrated. After cool, a crystalline product was obtained and then recrystallized from EtOH to fine white needles, m.p. 202~203°; $[\alpha]_D^{10}$ −43.0°(c=1.0, $CHCl_3$). Yield, 0.5 g.(40 %). *Anal.* Calcd. for $C_{22}H_{25}O_{12}N$: C, 53.33; H, 5.08; N, 2.87. Found : C, 53.20; H, 5.23; N, 3.13.

Acetylation of PABA-GA with Boron Trifluoride-Acetic Anhydride(IV)——To 1 g. of PABA-GA suspended in 5 cc. of Ac_2O 1 cc. of BF_3 in Et_2O(47%) was added in small portions. The mixture, after standing at room temperature for 1 hr., was poured into ice-water with stirring. The crystalline precipitate was collected and recrystallized from EtOH to fine white needles, m.p. 148°(decomp.); $[\alpha]_D^{10}$ −30.4° (c=0.92, EtOH). Yield, 1.2 g.(80 %). Its IR spectrum indicated a carboxyl band. *Anal.* Calcd. for $C_{21}H_{23}O_{12}N\cdot H_2O$: C, 50.50; H, 5.01; N, 2.80. Found : C, 50.56; H, 5.05; N, 3.01.

Methylation of (IV) with Diazomethane (V)——When 20 cc. of Et_2O solution of CH_2N_2 was added to 0.3 g. of (IV) dissolved in a small volume of MeOH, crystallization occurred immediately. The mixture was allowed to stand overnight in a refrigerator. The crystalline products was collected and recrystallized from EtOH to fine white needles, m.p. 199~200°; $[\alpha]_D^{10}$ −41.0°(c=1.0, $CHCl_3$). Yield, 0.25 g.(84 %). *Anal.* Calcd. for $C_{22}H_{25}O_{12}N$: C, 53.33; H, 5.08; N, 2.87. Found : C, 52.91; H, 5.33; N, 2.92.

There was no depression of melting point when it was mixed with authentic methyl (*p*-acetamidobenzoyl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (III) and its IR spectrum was identical with that of the authentic sample.

Acetylation of 2-Naphthyl-β-D-glucofuranosiduronamide (VI) with Pyridine-Acetic Anhydride (VII)——To 0.12 g. of (VI) dissolved in 5 cc. of pyridine 3.5 cc. of Ac_2O was added. The mixture, after standing overnight at room temperature, was poured into ice-water with stirring. The powdered precipitate was collected and recrystallized from EtOH-H_2O to fine white needles, m.p. 164~165°; $[\alpha]_D^{19}$ −111°(c=1.0, $CHCl_3$). Yield, 0.13 g. *Anal.* Calcd. for $C_{22}H_{23}O_9N$: C, 59.33; H, 5.17; N, 3.15. Found : C, 59.24; H, 5.19; N, 3.37. This compound when mixed with (VIII) showed a marked depression of melting point and its IR spectrum was not identical with that of (VIII).

Acetylation of 2-Naphthyl-β-D-glucofuranosiduronamide (VI) with Boron Trifluoride-Acetic Anhydride(VII')——To 0.7 g. of (VI) suspended in 7 cc. of Ac_2O 0.7 cc. of BF_3 in Et_2O(47 %)was added in small portions. The mixture, after standing at room temperature for 40 min., was poured into ice-water with stirring. The precipitated brown gum was triturated with water. The resulting powder was collected and dissolved in hot EtOH. After standing, the crystalline product was obtained from EtOH solution and recrystallized from EtOH-H_2O to fine white needles, m.p. 163~164°. Yield, 0.1 g. This compound when mixed with (VII) showed no depression of melting point and its IR spectrum was identical with that of (VII).

2-Naphthyl-β-D-glucopyranosiduronamide (VIII)——2 g. of methyl(2-naphthyl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate was added to 60 cc. of dehyd. MeOH saturated with dry NH_3 gas. The mixture was stirred at 0~8°. The crystals disappeared after about 1 hr. and the solution was colored pale yellow. Stirring was continued for additional 20 min. then crystallization occurred in the solution. The resulting mixture was evaporated to dryness in a reduced pressure. The dried crystalline residure was recrystallized from 75% EtOH to fine white needles, m.p. 245~246°; $[\alpha]_D^{21}$ −145° (c=0.02, dioxane). Yield, 0.8 g. *Anal.* Calcd. for $C_{16}H_{17}O_6N$: C, 60.18; H, 5.37; N, 4.39. Found : C, 60.24; H, 5.48; N, 4.43.

Acetylation of 2-Naphthyl-β-D-glucopyranosiduronamide (VIII) with Pyridine-Acetic Anhydride (IX)——To 0.15 g. of (VIII) dissolved in 5 cc. of pyridine 3.5 cc. of Ac_2O was added. The mixture, after standing overnight at room temperature, was poured into ice-water with stirring. The crystalline precipitate was collected and recrystallized from EtOH-H_2O to fine white needles, m.p. 168~170°; $[\alpha]_D^{17}$ −17°(c=1.0, $CHCl_3$). Yield, 0.09 g. *Anal.* Calcd. for $C_{22}H_{23}O_9N$: C, 59.33; H, 5.17; N, 3.15. Found : C, 59.08. H, 5.17; N, 3.14.

Acetylation of 2-Naphthyl-β-D-glucopyranosiduronamide (VIII) with Boron Trifluoride-Acetic Anhydride (X)——To 0.7 g. of (VIII) suspended in 7 cc. of Ac_2O 1.7 cc. of BF_3 in Et_2O (47 %) was added in small portions. The mixture, after standing at room temperature for 1 hr., was poured into ice-water with stirring. The precipitated white gum was triturated with water. The resulting powder was collected and recrystallized from EtOH to fine white needles, m.p. 155~157°; $[\alpha]_D^{12}$ −58° (c=1.0, $CHCl_3$). Yield, 0.5 g. *Anal.* Calcd. for $C_{24}H_{25}O_{10}N$: C, 59.19; H, 5.13; N, 2.87. Found : C, 58.90; H, 5.24; N, 2.80.

Acetylation of 2-Naphthyl-tri-O-acetyl-β-D-glucopyranosiduronamide (IX) with Boron Trifluoride-Acetic Anhydride (X′)——To 0.05 g. of (IX) suspended in 1 cc. of Ac_2O 0.3 cc. of BF_3 in Et_2O (47 %) was added. The mixture, after standing at room temperature for 2 hr., was poured into ice-water with stirring. The precipitated orange gum was triturated with water. The resulting powder was collected and recrystallized from EtOH to fine white needles, m.p. 155~157°. Yield, 0.018 g. This compound when mixed with (X) showed no depression of melting point and its IR spectrum was identical with that of (X).

Hydrolysis of PABA-GA by Rabbit-liver β-Glucuronidase——The enzyme was partially purified according to method described by Talaley, *et al.* PABA liberated was separated from PABA-GA by extraction with Et_2O and determined with the Tsuda reagent.[9] The absorbancy at 550 mμ was measured in Hitachi photoelectric spectrophotometer. Results in Table I show that PABA-GA was rapidly hydrolyzed by rabbit-liver β-glucuronidase and the hydrolysis was inhibited by saccharo-1,4-lactone.[10] In a concentration of $4\times10^{-6}M$, saccharo-1,4-lactone caused 43.2% inhibition with $1\times10^{-3}M$ substrate.

TABLE I.

Liberation of PABA from PABA-GA by rabbit-liver β-glucuronidase. Hydrolysis mixture incubated for 100-min. at 38° and pH 4.5 in acetate final buffer, concentration 0.07 *M*.

substrats	Inhibition	PABA	liberated (γ/100 cc)	Inhibition (%)
$1\times10^{-3}M$ PABA-GA	none		660	—
$1\times10^{-3}M$ PABA-GA	$4\times10^{-6}M$	Saccharate	375	43.2
$1\times1o^{-3}M$ PABA-GA	$2\times10^{-6}M$	Saccharate	450	31.9

Inhibition of the Hydrolysis of *p*-Nitrophenyl-β-D-glucuronide——PABA-GA was tested as a competing substrate in the hydrolysis of *p*-nitrophenyl-β-D-glucuronide by rabbit-liver β-glucuronidase. For the enzyme assay, the method described in an earlier paper from this laboratory[11] was adopted. Incubation was for 1 hr. at 38° in acetate buffer at pH 3.8 and final concentration was 0.14*M*. The *p*-nitrophenyl-β-D-glucuronide concentration ranged from 0.01 to 0.001*M*. PABA-GA depressed the release of *p*-nitrophenol and acted competitively. The value of Ki determined by the method of Dixon[12] was $5\times10^{-4}M$.

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Summary

1. The ring structure of *p*-aminobenzoyl glucuronide isolated from the dog urine was confirmed through the synthetic experiment on methyl (*p*-acetamidobenzoyl-2,3,4-

9) K. Tsuda, S. Matsunaga : Yakugaku Zasshi, **62**, 362 (1942).
10) G. A. Levvy : Biochem. J., **52**, 464 (1952).
11) K. Kato, K. Yoshida, H. Tsukamoto, M. Nobunaga, T. Masuya, T. Sawada : This Bulletin, 8, 239 (1960).
12) M. Dixon : Biochem. J., **55**, 170 (1953).

tri-O-acetyl-β-D-glucopyranosid)uronate. It was concluded that *p*-aminobenzoyl glucuronide has a pyranose configuration.

2. *p*-Aminobenzoyl glucuronide isolated from the dog urine was rapidly hydrolyzed by a rabbit-liver β-glucuronidase preparation.

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206. Keitaro Kato, Kazuo Yoshida, and Hisao Tsukamoto : Metabolism of Drugs. XXXIV.*1 Studies on the Ring Structure of *p*-Aminobenzoyl Glucuronide isolated from the Urine of Dog administered with *p*-Aminobenzoic Acid. (2).*2

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In the preceding paper,[1] the present authors isolated from the urine of dog administered with *p*-aminobenzoic acid (PABA), *p*-aminobenzoyl glucuronide (PABA-GA) compound which was shown to have a pyranose configuration by comparing its acetylated and methylated derivatives with that synthesized by another method. On the other hand, Nakao, *et al.* surmised that this glucuronide might have a furanose configuration from the results obtained from their study by periodate oxidation and infrared spectra examination.[2] It was the purpose of this investigation to establish conclusively the pyranose structure of PABA-GA by actual application of Nakao's periodate oxidation procedure.

Nakao, *et al.* oxidized PABA-GA with periodic acid in aqueous solution to a dialdehyde, which was hydrolyzed in alkaline solution and then oxidized to two different products (Samples A and B) with bromine and with yellow mercuric oxide, respectively. They examined both samples by paper chromatography using Methyl Red as a spray reagent and indicated the presence of mesotartaric acid from the Rf value. According to this method, it is assumed that pyranose and furanose types of glucuronide yield tartronic acid and mesotartaric acid respectively. Therefore, the authors repeated this procedure and carefully examined Samples A and B by paper chromatography and paper electrophoresis but could not detect the presence of either mesotartaric acid or tartronic acid. Instead, it was demonstrated that hydrobromic acid and an unknown

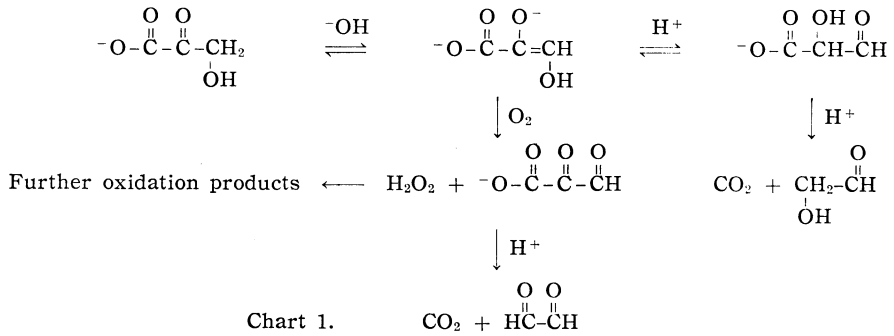

Chart 1.

*1 Part XXXIII. K. Kato, K. Yoshida, K. Tatsumi, H. Tsukamoto : This Bulletin, **10**, 1226 (1962).

*2 Presented at the Kyushu Branch Meeting of the Pharmaceutical Society of Japan, at Kumamoto, October, 1961.

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1) K. Kato, K. Yoshida, K. Tatsumi, H. Tsukamoto : This Bulletin, **10**, 1226(1962).

2) T. Nakao, M. Nakao, T. Nakajima : J. Biochem. (Japan), **45**, 207 (1958).