Identification of *p*-Nitrophenyl Glucoside as a Urinary Metabolite

Keyphrases ☐ *p*-Nitrophenol-2,6-^{14}C urinary metabolites—determination ☐ Paper chromatography—separation, identification ☐ Rechromatography procedure—separation, identification

Sir:

In 1969, Gessner and Vollmer (1) reported that mouse liver microsomes fortified with uridine diphosphoglucose (UDPG) can effect glucosylation of *p*-nitrophenol (PNP) to *p*-nitrophenyl glucoside (PNPG). The pathway appeared to be about 5 times less active than glucuronidation of PNP when carried out by the same microsomal preparations fortified with uridine diphosphoglucuronic acid (UDPGA). In view of the fact that glucosides of phenols have not been previously detected among mammalian metabolites, it was of interest to test whether the pathway manifests itself at all *in vivo*. We found PNPG in the urine of PNP-treated mice. The metabolite was characterized chromatographically by employing a novel technique, which permits a chromatographic sample to be subjected to consecutive chromatography in several solvent systems without elution of the material. The method is described below.

Male, Albany Swiss mice, weighing 23–30 g., were injected intraperitoneally with a 50 mg./kg., 0.145 mc./kg., dose of radioactive PNP[1] in a quantity of isotonic saline which was equivalent to 25 ml./kg. per dose. Urine from six mice was pooled and subjected to lyophilization. The residue of lyophilization was dissolved in distilled water; the concentrate thus obtained was used for chromatography. Descending chromatography was carried out on Whatman No. 1 paper strips in the solvents listed in Table I. Authentic reference compounds, PNP, PNPG, *p*-nitrophenylglucuronic acid (PNPGA), and *p*-nitrophenylsulfate (PNPS) were chromatographed contemporaneously for comparison of the R_f values of metabolites with those of authentic compounds. The compounds were detected on chromatograms with the aid of UV light; they appeared as quenching spots. Radioactive metabolites were located by scanning chromatographic strips with the aid of the Nuclear Chicago 4 pi chromatogram scanner, model 1002. In this manner, R_f values of the metabolites were located and compared to the R_f values of the authentic compounds. The presence of a radioactive peak at the R_f of PNPG on the initial chromatogram was interpreted as an indication of the possible presence of PNPG. The hypothesis was further tested by subjecting the chromatographically separated radioactive metabolite to chromatography in other solvents.

A novel method of rechromatography was developed, one that does not require elution of the substance under investigation. Verbal report of the method was made in 1969 in conjunction with the identification of PNPG as an *in vitro* metabolite (1). The method is suitable for consecutive chromatography in several solvents and permits purification and characterization of an unknown substance by its chromatographic mobility. The paper chromatogram is developed in the usual manner, and the region with the R_f range under investigation is cut out as a strip. The cutout strip is then pinned at two opposite ends (using rustless pins) to two strips of chromatographic paper; a short leader-strip is for dipping into the solvent, and a long strip is for the development of the chromatogram. Thus assembled, the chromatogram is developed in the usual manner. Again the region with the R_f value of interest can be cut out from the chromatogram and subjected to chromatography in another solvent. The process can be repeated several times with little loss of the original material, and data from several solvents can be obtained from experiments on a single chromatographic sample of a substance.

Table I—Comparison of the Chromatographic Mobility of a Urinary Metabolite with that of Authentic *p*-Nitrophenylglucoside

Solvent System	R_f[a] of PNPG	R_f[a] of Metabolite
I *n*-Butanol–pyridine–water (14:3:3)	0.63	0.63
II Benzene–acetic acid–water (125:72:3)	0.48	0.44
III *n*-Propanol–ethyl acetate–water (7:1:2)	0.73	0.74
IV Methanol–formic acid–water (16:3:1)	0.68	0.66
V 1 *N* Sodium formate–formic acid (200:1)	0.77	0.77

[a] Chromatography was carried out on Whatman No. 1 paper strips in a consecutive manner as described in the text.

The method of consecutive chromatography was used for the detection of PNPG in the urine of PNP-treated mice as follows. The concentrate described previously was chromatographed in Solvent I, *n*-butanol–pyridine–water (14:3:3), and the radioactive peak with the R_f of PNPG was cut out and subjected to chromatography in Solvent II, benzene–acetic acid–water (125:72:3). Again the chromatogram that was developed in Solvent II was cut up, and the radioactive peak with R_f of PNPG was subjected to chromatography in Solvent III, *n*-propanol–ethyl acetate–water (7:1:2). The process was repeated with the chromatogram from each solvent in turn. Chromatography in Solvent III was followed by chromatography in Solvent IV, methanol–formic acid–water (16:3:1), and then by chromatography in Solvent V, 1 *N* sodium formate–formic acid (200:1). The R_f values of the reference PNPG and radioactive metabolite were noted in each solvent, and the results are summarized in Table I.

Thus, the presence of PNPG in the urine of PNP-treated mice was established by consecutive chromatography in five solvent systems. Data in the table show that in each solvent system the radioactive peak of metabolite behaved like PNPG. Our experiments showed that in the first solvent system, PNPG overlapped with PNPS. However, the two components separated out in the second solvent system and, thereafter, a single radioactive peak with the mobility of PNPG was detectable on chromatograms. The chroma-

[1] *p*-Nitrophenol-2,6-^{14}C from Tracerlabs, Waltham, Mass.

tographically pure PNPG was present in a small quantity and accounted for about 1–2% of the urinary metabolites. We also noted from our experiments that 24–50% of the administered PNP was excreted in urine in the form of metabolites of PNP in the first 5 hr., and that PNPGA and PNPS were the major products detectable on chromatograms at that time. The two metabolites accounted for about 95% of the urinary metabolites. This observation is in accord with an earlier work of Robinson *et al.* (2). In the 5-hr. urine, PNPGA and PNPS appeared to be present in equal amounts.

The fact that PNPG was present only in small amounts in the urine explains why the metabolite eluded detection in the past. However, low urinary excretion need not necessarily imply low production of this metabolite at all times in the body, but may be related to the fact that PNPG is not sufficiently acidic for extensive renal excretion. *In vitro*, the glucosylation pathway appears quite active (1). In this context perhaps it is significant that *N*-acetylglucosamine conjugation of steroids is also a pathway that appears to be very active *in vitro* (3), but such metabolites are detected with difficulty in mammalian urine (4).

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(4) M. Arcos and S. Lieberman, *Biochem. J.*, **66**, 2032(1967).

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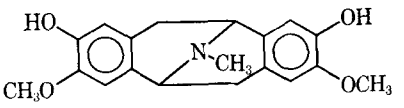
Total Synthesis of (±)-Bisnorargemonine

Keyphrases ☐ (±)-Bisnorargemonine—synthesis ☐ IR spectrophotometry—identification ☐ UV spectrophotometry—identification ☐ NMR spectroscopy—identification ☐ Mass spectroscopy—identification

Sir:

We had previously (1) postulated Structure I for bisnorargemonine based on its unambiguous NMR spectrum, in which the chemical shifts of the aromatic and methoxyl protons appeared to be unequivocal for the structural assignment. Nevertheless, since a structure based on spectral evidence alone should be substantiated by synthesis, we have completed the necessary synthesis



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by a 14-step procedure and an alternate 16-step procedure.

In the shorter synthesis, we started with the preparation of *O*-benzylvanillin, which was condensed with hippuric acid to yield 5-keto-2-phenyl-4-(4′-benzyloxy-3′-methoxybenzylidene)-4,5-dihydroöxazole. The latter then was hydrolyzed with barium hydroxide to give 3-methoxy-4-benzyloxyphenyl-pyruvic acid, which was readily oxidized with hydrogen peroxide to provide the desired 3-methoxy-4-benzyloxyphenylacetic acid, m.p. 114–116° [lit. (2) m.p. 116°]. This acid was converted to its acid chloride with thionyl chloride prior to reaction with β-methoxy-β-(3-benzyloxy-4-methoxyphenyl)ethylamine, which was obtained by treating the nitromethane adduct of *O*-benzylisovanillin with sodium methoxide, according to Rosenmund *et al.* (3), to give 1-methoxy-1-(3-benzyloxy-4-methoxyphenyl)-2-nitroethane,[1] m.p. 100–102°, which was then reduced with lithium aluminum hydride. The resulting amide, m.p. 96.5–98.5°, was then submitted to Bischler–Napieralski cyclization (4) with phosphorus oxychloride to give 1-(3′-methoxy-4′-benzyloxybenzyl)-6-benzyloxy-7-methoxyisoquinoline, m.p. 146–147°.

Another route to this isoquinoline was achieved by dehydrogenation of the known 1-(3′-methoxy-4′-benzyloxybenzyl)-6-benzyloxy-7-methoxy-1,2,3,4-tetrahydroisoquinoline (5) with 10% palladium-on-carbon and rebenzylation of the resulting phenolic isoquinoline with benzyl chloride according to Lee and Soine (6). This route also served to confirm the identity of the isoquinoline from the first method. The tetrahydroisoquinoline was prepared, according to Tomita and Kunimoto (5), by condensation of 3-methoxy-4-benzyloxyphenylacetyl chloride with β-(3-benzyloxy-4-methoxyphenyl)-ethylamine. Subsequent Bischler–Napieralski cyclization with phosphorus pentachloride gave 1-(3′-methoxy-4′-benzyloxybenzyl)-6-benzyloxy-7-methoxy-3,4-dihydroisoquinoline hydrochloride. The latter, on reduction with sodium borohydride in aqueous methanol, provided the desired tetrahydroisoquinoline.

The isoquinoline obtained by either of the two methods then was quaternized with methyl iodide and reduced with sodium borohydride in pyridine, according to Barton *et al.* (7), to yield 1-(3′-methoxy-4′-benzyloxybenzyl)-2-methyl-6-benzyloxy-7-methoxy-1,2-dihydroisoquinoline, m.p. 61–64°. Acid cyclization of this product, by the method of Battersby and Binks (8), gave (±)-bisnorargemonine, m.p. 231.5–232.5°. Except for melting point and rotational differences, the natural and synthetic compounds were shown to be identical. The identity of natural and synthetic materials was established by comparative UV, IR, NMR, and mass

[1] This compound has been prepared but not described by E. Brochmann-Hanssen and K. Hirai, *J. Pharm. Sci.*, **57**, 940(1968). Private communication with the authors indicates that the compound was obtained as a pale-yellow oil with suitable chromatographic and NMR characteristics. Our solid product meets similar criteria and is assumed to be identical to their product.