# The Identification of o-Aminophenol and o-Aminophenyl Glucose in Wheat Bran

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**ABSTRACT**

Partial hydrolysis of wheat bran extracts (which also contained bound nicotinic acid) yielded several compounds that contained an aromatic amine. This amine was isolated and identified as *o*-aminophenol, by mass spectrometry, its ultraviolet spectrum, and chromatography. A second partial hydrolysis product was isolated and identified as *o*-aminophenyl glucose, by similar methods. *o*-Aminophenol is therefore present in wheat bran, contained in macromolecules of which *o*-aminophenyl glucose is a subunit.

Kodicek and Wilson (1) suggested that an aromatic amine, possibly *o*-aminophenol, was present in extracts of wheat bran that contained bound nicotinic acid (niacytin preparations). Recently it has been shown that this aromatic amine was not exclusively associated with bound nicotinic acid molecules (2). Moreover, if the amine was attached to the bound nicotinic acid molecules, it was not in close proximity to the nicotinic acid moiety (2), and thus was not involved directly by steric hindrance in the biological unavailability of bound nicotinic acid (3).

The aromatic amine is contained in macromolecules of molecular weight less than about 17,000 (2). In further studies, reported below, the identity of the aromatic amine and the nature of its linkage to macromolecules in wheat bran have been investigated. Micromethods have necessarily been used for identification because only very small amounts of the unknown compounds were available.

## MATERIALS AND METHODS

### Wheat Bran Extracts

The extracts of wheat bran used were the same as those that contained bound nicotinic acid (2). The preparations, referred to previously as niacytin and nondiffusible nicotinic acid preparations, had a content of *o*-aminophenol of about 10 $\gamma$ per mg. and 1 $\gamma$ per mg., respectively.

### Aminophenols

*o*-Aminophenol was obtained from Fison's Ltd., Loughborough, Leics; *m*-aminophenol from Hopkin & Williams Ltd., Chadwell Heath; *p*-aminophenol from Koch-Light Ltd., Colnbrook, Bucks. The *o*-aminophenol was recrystallized from heptane:ethanol (about 7:1, v./v.), and was stable when crystalline.

### Chromatographic Methods:

Thin-layer chromatography (TLC) was carried out on silica gel $GF_{254}$ thin-layers (E. Merck, AG, Damstadt, Germany). The solvent mixtures used were: system 1, butan-1-ol:acetic acid:water (4:1:1, by volume); system 2, chloroform:methanol (97:3, v./v.); system 3, benzene:methanol:acetone:acetic acid (14:4:1:1, by volume). Visualization was effected with the following reagents: Ehrlich's reagent (0.5%, w./v.) *p*-dimethylaminobenzaldehyde in ethanol containing 1% conc. HCl); nitrous acid (aqueous 1M sodium nitrite [4 vol.] mixed with 5M HCl [1 vol.] at 0°C.) followed by 1% $\beta$-naphthol in 2.5M NaOH; iodine vapor; and 50% aqueous (v./v.) sulfuric acid, followed by heating at 60°C. for 1 hr.

### Mass Spectrometry

The mass spectra of *o*-aminophenol and the trimethylsilyl (TMS) and acetyl derivatives of compound 3 (see below) were determined using an AEI MS9 mass spectrometer, with direct insertion. Acetylation was carried out in pyridine by addition of an equal volume of acetic anhydride for 3 hr. at 50°C. The reagents were removed by vacuum distillation.

## EXPERIMENTAL AND RESULTS

### Partial Hydrolysis

In preliminary experiments it was found that partial hydrolysis of extracts of wheat bran containing bound nicotinic acid in acid or alkali gave rise to at least four compounds having a positive reaction with Ehrlich's reagent, similar to that of *o*-aminophenol (see Methods). These compounds were detected by this reaction after separation by TLC in system 1, and $R_f$ values of the four compounds, designated compounds 1 to 4 with increasing $R_f$ values, are shown in Table I.

The conditions for formation of these compounds were investigated as follows. Samples of a niacytin preparation from wheat bran, later shown to contain 10 $\gamma$ *o*-aminophenol per mg., were hydrolyzed in 2.5M NaOH at 100°C. at a

TABLE I. $R_f$ VALUES OF AMINOPHENOLS AND RELATED COMPOUNDS ON TLC

| Compound[a] | System | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| o-Aminophenol | 0.64 | 0.25 | 0.45 |
| m-Aminophenol | 0.64 | 0.18 | 0.47 |
| p-Aminophenol | 0.52 | 0.14 | 0.19 |
| Compound 4 | 0.64 | 0.25 | 0.45 |
| Compound 3 | 0.48 | 0.00 | 0.11 |
| Compound 2 | 0.33 | 0.00 | 0.00 |
| Compound 1 | 0.18 | 0.00 | 0.00 |

[a]Compounds 1–4 were obtained by partial alkaline hydrolysis of wheat bran extracts.

concentration of 3.5 mg. per ml. and samples were taken at various times. After neutralization, reducing to dryness, and extraction of the residue with ethanol, aliquots of the hydrolysate equivalent to 0.1 mg. of the starting material were chromatographed on TLC in system 1. After spraying with Ehrlich's reagent, the relative proportions of the four hydrolysis products were determined using a Chromoscan densitometer (Joyce-Loebl & Co. Ltd., Gateshead, England) with a Wratten No. 47 filter. The results, shown in Fig. 1, determined the conditions used for formation of compounds 3 and 4. In view of the similarity of the color reactions of compounds 1–4 (see below) their decreasing polarity, and the time-course of their formation, it is likely that compound 4 was formed from the other compounds during hydrolysis. The identity of compound 4, being the least polar and hence possibly the simplest of the four compounds, was first studied.

**Preparation of Compound 4**

Compound 4 was prepared from samples of niacytin by the procedure in Fig. 2.

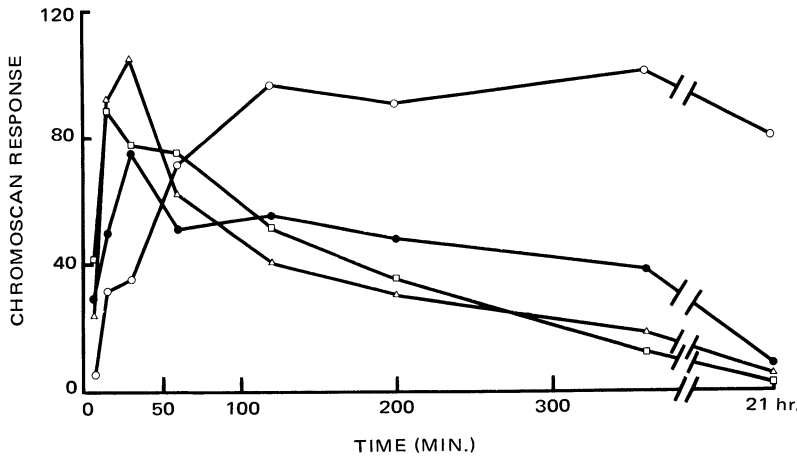


**Fig. 1. Samples of wheat bran extracts were hydrolyzed in 2.5M NaOH at 100°C. Aliquots were taken at the time intervals as shown, and the amounts of compounds 1–4 present determined as described in the text. ○, compound 4; □, compound 3; △, compound 2; ●, compound 1.**

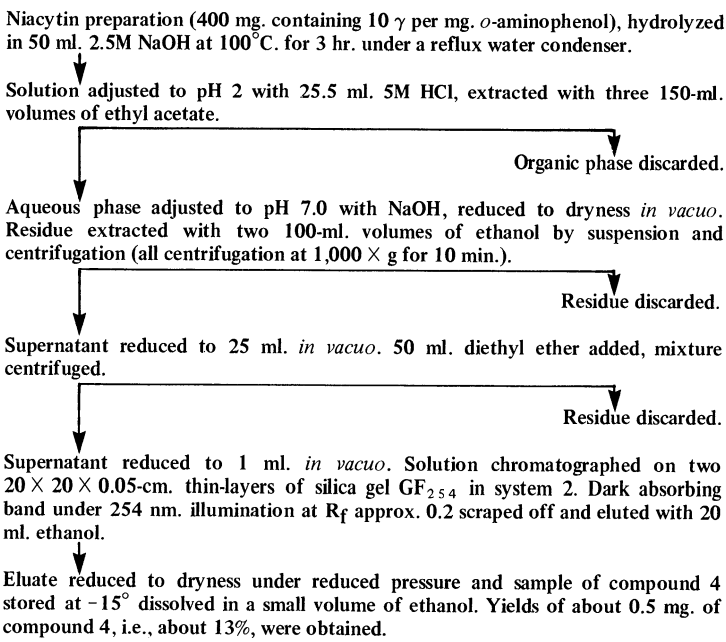


**Fig. 2. Preparation of compound 4 from wheat bran extracts.**

The final preparation of compound 4 gradually decomposed to form a nonpolar yellow compound. On rechromatography of freshly prepared samples in systems 1 and 2 and in ethyl acetate, a single spot was detected with iodine gas, sulfuric acid, and Ehrlich's reagent. (See Methods.)

#### Identification of Compound 4

*Mass Spectrometry.* The mass spectrum of compound 4 is shown in Fig. 3, compared with that of authentic *o*-aminophenol. The two spectra are essentially identical. The molecular ion, which is also the base peak, at *m/e* 109, corresponds to $C_6H_7NO$. The next most abundant ion in the spectrum is at *m/e* 80, and is probably $C_5H_6N$, the pyridinium cation formed by a rearrangement (4); the metastable peak for the transition *m/e* 109 to *m/e* 80 was observed at *m/e* 58.7. The ion at *m/e* 80 fragmented further by loss of $NH_2$ to form the ion at *m/e* 64, and by loss of HCN to form the ion at *m/e* 53. These results provided strong evidence that compound 4 was an aminophenol.

*Ultraviolet (UV) Spectrum.* The UV spectrum of compound 4 determined in ethanol showed $\lambda_{max}$ at 211, 234, and 287 nm. These maxima are the same as found in the spectrum of authentic *o*-aminophenol. The spectrum of *m*-aminophenol has $\lambda_{max}$ 213, 236, and 287 nm., and that of *p*-aminophenol has $\lambda_{max}$ at 206, 235, and 301 nm. The spectrum therefore confirmed that compound 4 was an aminophenol, and distinguished it from the *p*-isomer.

*Chromatography.* Compound 4 cochromatographed exactly with authentic *o*-aminophenol on TLC in systems 1, 2, and 3. Compound 4 was thus distinguished from the *m*- and *p*-isomers, particularly in system 2; the $R_f$ values are given in Table

I. On paper chromatography in systems B, E, and F of Reio (5), compound 4 again cochromatographed with authentic *o*-aminophenol; the $R_f$ values were 0.25, 0.93, and 0.68 in these three systems, respectively. These results showed that compound 4 was the *o*-isomer of aminophenol.

**Preparation of Compound 3**

Compound 3 was prepared from samples of niacytin by the procedure shown schematically in Fig. 4. Further purification was attempted by chromatography in a number of systems. The most successful method was as follows: the sample was applied to a 20 X 20 X 0.025-cm. thin-layer of silica

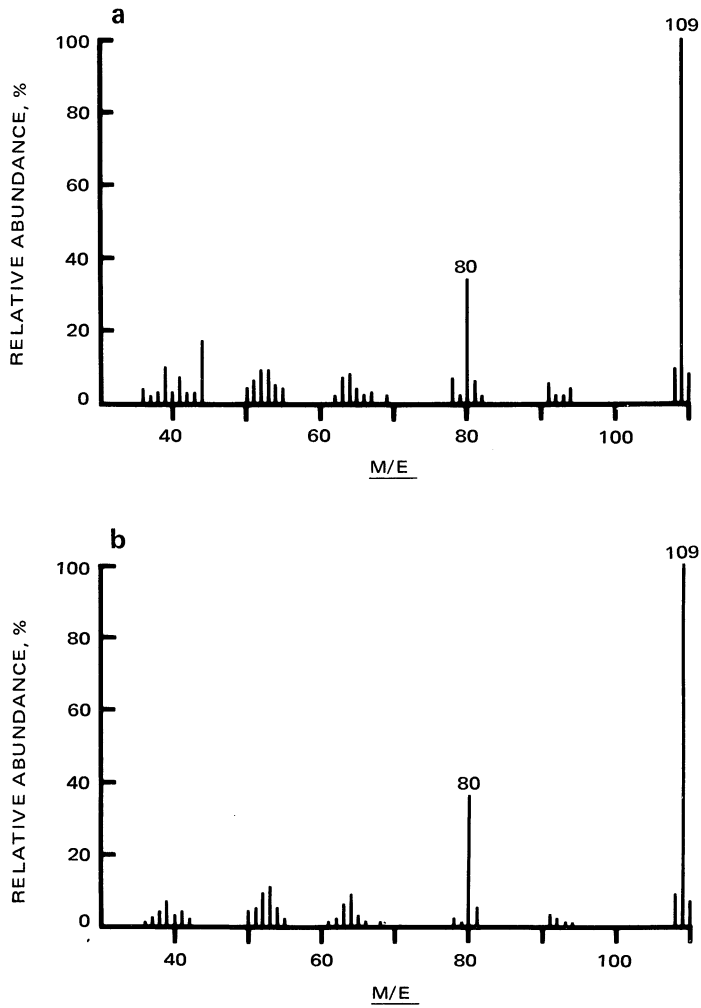


**Fig. 3. Mass spectra of a, compound 4 and b, *o*-aminophenol, obtained with a source temperature of 150°C.**

gel $GF_{254}$ and the chromatogram was developed in system 3 until the solvent front was about 2 cm. from the top of the plate; the tank was opened slightly, so that the solvent front remained in the same place because of evaporation of solvent from the top of the plate. After about 30 min. the $R_f$ of compound 3 had increased from 0.11 to 0.25. The band containing compound 3 was detected and eluted as before, and the sample stored in a small volume of ethanol at -15°C. Samples prepared in this way were chromatographically pure in systems 1 and 3, when visualized with iodine gas, sulfuric acid, and Ehrlich's reagent. (See Methods.) Yields of around 0.1 mg., i.e., around 4%, were obtained.

**Identification of Compound 3**

*Mass Spectrometry.* The TMS-ether of compound 3 was prepared by the method of Sweeley et al. (6). The mass spectrum of this derivative, obtained with a source temperature of 170°C., is shown in Fig. 5. The molecular ion was detected at *m/e* 559 (relative abundance 0.02%). This was confirmed by the presence of the M-15 ion at *m/e* 544 (relative abundance 0.28%), formed by loss of a methyl group, which is a typical first fragmentation of a TMS derivative (7). The molecular ion corresponds to the tetra-TMS derivative of aminophenyl hexose ($C_{24}H_{49}NO_6Si_4$). A fragment of mass 93, which was probably $C_6H_7N$ and contained the aromatic ring and nitrogen atom, was then lost to form the fragment at *m/e* 451; an ion of *m/e* 93 was also present in the spectrum. The *m/e* 451 ion then lost a

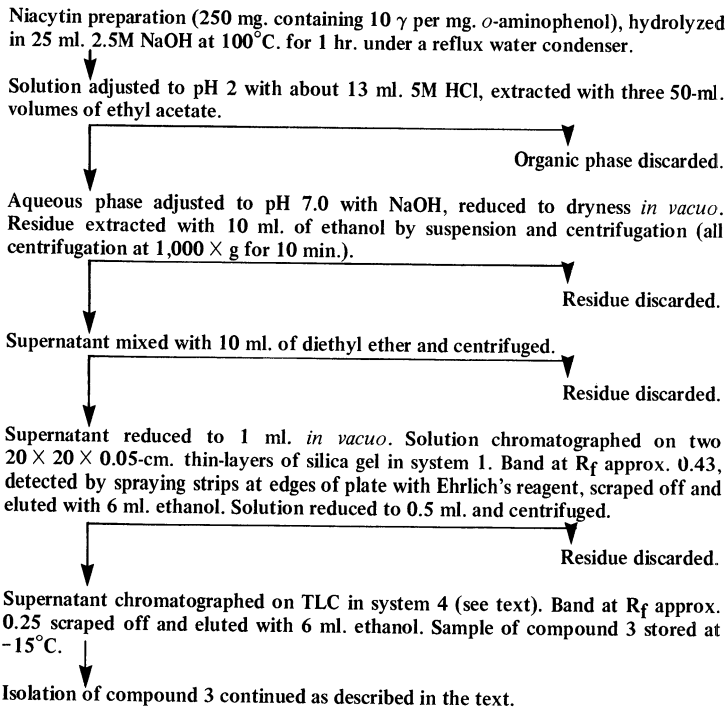


**Fig. 4. Preparation of compound 3 from wheat bran extracts.**

trimethylsilanol residue to form the *m/e* 361 ion. The base peak at *m/e* 275 probably contained the benzene ring, which was lost in forming the intense ion at *m/e* 199. The ions at *m/e* 204 and 217 were also present, and of high intensity, in the mass spectrum of TMS-glucose (8,9). The mass spectrum, therefore, provided evidence for the identity of compound 3 with an aminophenyl hexose.

The mass spectrum of the acetyl derivative of compound 3 (prepared as described in Methods) was also investigated. This derivative was relatively involatile, a temperature of 240°C. being required to obtain a spectrum. No molecular ion was detected. The ion of highest *m/e* value found was at *m/e* 331, which corresponds to the tetra-acetyl derivative of a hexose after loss of one oxygen atom ($C_{14}H_{19}O_9$). The spectrum below this consisted almost entirely of ions formed by the successive loss of fragments of mass 42 ($C_2H_2O$, ketene) and 60 ($C_2H_4O_2$, acetic acid) from the ion at *m/e* 331. An intense ion was present at *m/e* 109, which could have been either $C_6H_7NO$, corresponding to aminophenol, or possibly $C_6H_5O_2$, formed by loss of three acetic acid ($C_2H_4O_2$) and one ketene ($C_2H_2O$) moieties from the *m/e* 331 ion. This mass spectrum provided further evidence that compound 3 contained a hexose.

*UV Spectroscopy.* The UV spectrum of compound 3, determined in ethanol, had $\lambda_{max}$ 207, 227, and 285 nm. The spectrum was very similar to that of *o*-aminophenol, although the maxima were at slightly lower wavelengths. This indicated that compound 3 contained the *o*-aminophenol chromophore.

*Color Reactions.* Compound 3 reacted with Ehrlich's reagent (see Methods) to give a yellow fluorescent compound, and with nitrous acid followed by alkaline β-naphthol (see Methods) to give a pink color. These reactions occur with primary aromatic amino groups and show that in compound 3 the amine group was free. Thus in compound 3 the aminophenol was linked to the sugar by an ether bond. Compounds 1 and 2 (see above) gave identical color reactions to compound 3.

*Hydrolysis and Rechromatography.* Samples of compound 3 were hydrolyzed (2.5M NaOH at 100°C. for 3 hr.) and the hydrolysis products extracted with ethanol after neutralization and reduction to dryness. TLC of the hydrolysate in systems 1 and 2 showed the presence of a compound that cochromatographed with *o*-aminophenol.

To identify the sugar component of compound 3, samples were hydrolyzed in

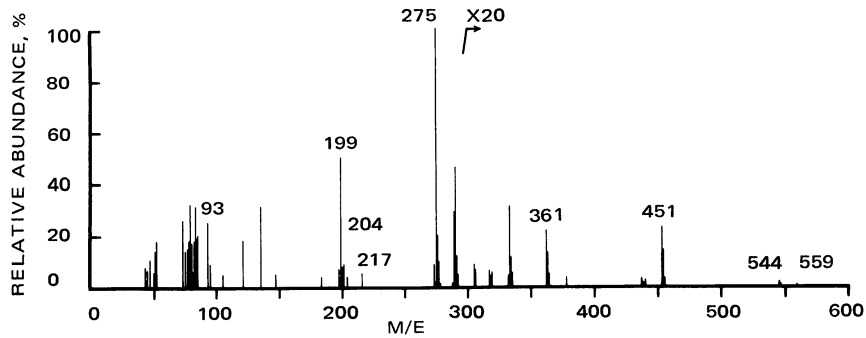


**Fig. 5. Mass spectrum of the TMS derivative of compound 3. The scale is expanded by a factor of 20 at *m/e* 300, as shown.**

acid (5M HCl at 100°C. for 30 min.). After drying the hydrolysate, the TMS derivative was prepared by the method of Sweeley et al. (6). The reagents were removed under nitrogen and the residue dissolved in carbon tetrachloride. Aliquots of this solution were chromatographed on gas-liquid chromatography (GLC) as described by Mason and Kodicek (9). The two peaks caused by the TMS-ethers of α- and β-glucose were detected at 13.3- and 20.2-min. retention time, respectively. The GLC system used resolved TMS-glucose from the TMS-ethers of other hexoses (fructose, galactose, mannose) as well as from methylpentoses and pentoses. Moreover, the peaks caused by TMS-glucose were not obtained from unhydrolyzed samples of compound 3. This result showed that the sugar moiety of compound 3 was glucose.

## DISCUSSION

Studies reported previously (2) have shown that extracts of wheat bran contain macromolecules to which an aromatic amine is linked. This amine has now been shown to be *o*-aminophenol, by isolation and identification of the least polar hydrolysis product that reacted with Ehrlich's reagent. The mass spectrum of this compound (referred to above as compound 4) was virtually identical to that of authentic *o*-aminophenol. The UV spectrum and chromatographic characteristics were also identical, and this data distinguished *o*-aminophenol from the *m*- and *p*-isomers.

A partial hydrolysis product from the wheat bran extracts, which reacted with Ehrlich's reagent, was identified as *o*-aminophenyl glucose. The mass spectrum of the TMS derivative of this compound (compound 3) showed that it was an aminophenol linked to a hexose. Chromatography of the hydrolysate of this substance on TLC showed that the aromatic moiety was *o*-aminophenol, and using GLC the sugar moiety was identified as glucose. Since the compound gave color reactions for a primary aromatic amine (with β-naphthol after diazotisation and with Ehrlich's reagent) the *o*-aminophenol was shown to be linked by an ether bond. Compounds 1 and 2, which had similar color reactions, and which could be shown also to give *o*-aminophenol on hydrolysis, were probably the di- and trisaccharides of *o*-aminophenol; their identity has not been investigated further. The position of substitution of glucose to which the *o*-aminophenol was linked has not been studied either, because sufficient quantities of compound 3 have not been available.

The occurrence of *o*-aminophenol in wheat bran, incorporated in macromolecules by an ether bond, has thus been demonstrated. Although the presence of phenolic glucosides in plants, including cereals, is well known (10), no report of the occurrence of *o*-aminophenyl glucose has been found. The occurrence of phenolic glucosides in macromolecules is also not well known (2). It may be that the presence of *o*-aminophenol incorporated in macromolecules is an example of a more general phenomenon, and that on investigation it would be found that macromolecules in plant material contain many phenolic compounds.

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