Reaction of Ferulic Acid with Nitrite: Formation of 7-Hydroxy-6-methoxy-1,2(4*H*)-benzoxazin-4-one

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Ferulic acid was reacted with nitrite under acidic conditions to give complex mixtures of products. Chromatographic purification afforded products that were characterized by ^{1}H- and ^{13}C-NMR spectral analyses. The major fluorescent product was identified as 7-hydroxy-6-methoxy-1,2(4*H*)-benzoxazin-4-one along with 3-methoxy-4-hydroxybenzaldehyde (vanillin) and 2-methoxy-4,6-dinitrophenol. The structure of the unusual benzoxazinone was confirmed by its chemical degradation in base to methyl-2,4-dihydroxy-5-methoxybenzoic acid.

Keywords: *Ferulic acid; 7-hydroxy-6-methoxy-1,2(4H)-benzoxazin-4-one; 2-methoxy-4,6-dinitrophenol; vanillin*

INTRODUCTION

Ferulic acid (**1**) is an extremely abundant plant phenolic cinnamic acid of interest as a renewable resource for biocatalytic or chemical conversion to other useful aromatic chemicals (Rosazza et al., 1995). In nature, ferulic acid esters are usually found associated with glycosidic fractions (Herrrmann, 1993; Hosny and Rosazza, 1997). Ferulic acid (**1**) is also a dietary antioxidant (Graf, 1992; Kato et al., 1997; Chen and Ho, 1997) and chemoprotectant which is thought to deter the process of carcinogenesis by inhibiting the formation of *N*-nitroso compounds (Kuenzig et al., 1984; Dikun et al., 1991). For example, it has been shown that in rats receiving aminopyrine and nitrite, ferulic acid blocked the elevation of serum N-nitrosamines. There has been concern over the reaction of nitrites with secondary amines to form nitrosamines or with substituted amides to form nitrosamides, both of which are known to cause cancer in many animal species (NAS/NRC, 1981). In vitro experiments in simulated gastric juice also showed that ferulic acid reacts very rapidly and quantitatively with equimolar quantities of nitrite (Kuenzig et al., 1984). Ferulic acid prevents peroxynitrite mediated nitration of tyrosine residues in collagen, thus implicating a chemoprotectant role for this antioxidant in the prevention of tissue injury (Kato et al., 1997). To understand how ferulic acid functions in this regard, nitration reactions were conducted with ferulic acid at pH 3. The reaction gave a mixture of several compounds from which two minor products were isolated in less than 1% yield each (Kikugawa et al., 1983). One isolated compound was identified as a decarboxylated and dehydrated product derived from ferulic acid by nitro and nitroso addition to the double bond. The structures of this and a second fluorescent compound were neither established nor fully characterized.

In connection with our continuing interests in metabolic and chemical aspects of nitrations of natural phenolic compounds (Rousseau et al., 1997), we examined the nitration of ferulic acid. This paper describes the isolation and identification of products obtained by the reaction of ferulic acid with nitrite.

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MATERIALS AND METHODS

Chemicals. Ferulic acid and vanillin were purchased from Aldrich (Milwaukee, WI). The purities of all chemicals were confirmed by TLC, HPLC, melting points, mass spectrometry, and ^{1}H- and ^{13}C-NMR spectroscopy before use.

Chromatography. Thin-layer chromatography (TLC) was carried out on 0.25-mm layers of silica gel GF254 (Merck) prepared on glass plates with a Quickfit Industries (London, England) spreader. Plates were air-dried and activated at 120 °C for 1 h before use. HPLC was performed with a Shimadzu LC-10AD liquid chromatograph equipped with a photodiode array UV−vis detector (SPD-M6A), using a Versapack C18 column (300 × 4.6 mm, 10-mm particle size; Alltech). The mobile phase of MeOH/2% HCOOH 30:70 (v/v) was pumped at a flow rate of 1 mL/min.

Spectroscopy. Electron impact mass spectra (EIMS) were obtained with a Fisons Trio-1 MS; high-resolution, fast atom bombardment mass spectra (HRFABMS) were obtained with a Fisons ZAB−HF mass spectrometer. ^{1}H- and ^{13}C-NMR spectra were obtained by using a Bruker WM-360 spectrometer equipped with an IBM Aspect-2000 processor. Hetero multiple-bond quantum correlation (HMQC) and hetero multiple-bond correlation (HMBC) spectra were recorded with a Bruker AMX-600 spectrometer. Tetramethylsilane was used as an internal standard. Chemical shift values are reported in parts per million (ppm) and coupling constants are given in hertz. Abbreviations for NMR are as follows: s, singlet; d, doublet; t, triplet; dd, doublet of doublets; m, multiplet. UV spectra were recorded with a Shimadzu (UV160U) UV−visible recording spectrophotometer, while IR spectra were determined with a Nicolet 205 FT-IR spectrometer. Melting points were determined on a capillary Mel-temp apparatus and are uncorrected.

Reaction of Ferulic Acid (1) with Nitrite. Ferulic acid (**1**) (2 g, 0.01 mol) dissolved in 20 mL of MeOH was added to a solution of $NaNO_2$ (5.44 g, 0.08 mol) in 500 mL of distilled water acidified to pH 2 with 6 N HCl. The mixture was stirred at room temperature for 2 h, saturated with NaCl, and extracted with EtOAc (5 × 250 mL). Organic phases were combined, dried over Na_2SO_4, and concentrated under vacuum to yield 1.8 g of black residue. TLC analysis (CH_2Cl_2/MeOH/

S0021-8561(98)00130-7 CCC: $15.00
Published on Web 07/28/1998

HCOOH 98:2:1 v/v/v) showed a major, fluorescent spot (R_f 0.4), two yellow secondary spots (R_f 0.7 and 0.17), a number of trace spots (R_f 0.66−0.5, 0.4, 0.2), and a residual brown spot at the bottom of the plate. HPLC analysis showed a major peak at t_R 20.3 min and numerous minor accompanying peaks. The residue was chromatographed over 60 g of flash-column silica gel (Baker, 40 mm) (CH_2Cl_2/MeOH/HCOOH, 100:1:0.5 v/v/v) and four fractions were collected.

The first fraction (60 mg) afforded the yellow compound at R_f 0.7 contaminated with traces of a minor impurity (R_f 0.66). The compound was characterized as 2-methoxy-4,6-dinitrophenol (**9**): mp (CH_2Cl_2/hexane) 114−116 °C; UV (MeOH) λ_{max} 216, 264, 330 nm; IR ($CHCl_3$) (ν, cm^{-1}) 1560, 1345, 1270, 1200; ^1H NMR ($CDCl_3$, 360 MHz) δ 11.19 (1 H, s, OH), 8.69 (1 H, d, J = 2.4 Hz), 7.97 (1H, d, J = 2.4 Hz), 4.08 (3 H, s, CH_3O); ^{13}C NMR ($CDCl_3$, 90.5 MHz) δ 150.9, 150.6, 139.3, 132.5, 112.6, 111.0, 57.26; EIMS (70 eV) m/z 214 (M^+, 100), 197 (95), 184 (13), 166 (31), 151 (13), 121 (47); HREIMS (70 eV) 214.0222 for $C_7H_6N_2O_6$ (calcd 214.0225).

The second fraction (100 mg) contained a mixture of at least four compounds (R_f 0.66−0.50). After concentration a strong characteristic vanilla odor was detected. Vanillin was isolated by further chromatography as a crude orange solid and characterized by comparison with a commercial sample of 3-methoxy-4-hydroxybenzaldehyde (vanillin).

The third fraction (465 mg) afforded a brown residue containing mostly the fluorescent compound (R_f 0.40). Very yellow impurities (R_f 0.36 and 0.50) were also present. A pale yellow solid was obtained by crystallization of the residue with CH_2Cl_2/MeOH. The filtrate was concentrated and chromatographed again. Thus by repeated chromatography/crystallization, 240 mg of a yellow solid were obtained. The compound was characterized as 7-hydroxy-6-methoxy-1,2(4*H*)-benzoxazin-4-one (**3**): mp (water) 171−175 °C (decomposition); UV (MeOH) λ_{max} 214, 347 nm; IR ($CHCl_3$) (ν, cm^{-1}) 1650, 1505, 1475, 1445, 1290, 1200, 1140; ^1H NMR (Me_2CO-d_6, 360 MHz) δ 8.18 (1 H, s, H-3), 7.34 (1 H, s, H-5), 6.96 (1 H, s, H-8), 3.99 (3 H, s, CH_3O); ^{13}C NMR (Me_2CO-d_6, 90.5 MHz) δ 166.5 (s, C-4), 159.5 (s, C-8a), 155.9 (s, C-7), 150.9 (d, C-3), 148.4 (s, C-6), 114.3 (s, C-4a), 103.5 (d, C-5), 101.3 (d, C-8), 56.65 (q, MeO); EIMS (70 eV) m/z 193 (M^+, 60), 166 (98), 151 (47), 138 (14), 123 (58), 110 (13), 95 (35), 69 (100), 53 (43); HRFABMS (3-NBA) m/z $(M+H)^+$ found 194.0459 (calcd for $C_9H_8NO_4$ 194.0453).

The fourth fraction (290 mg) contained an intractable mixture of components that was impossible to resolve.

Methanolysis of Compound 3. A sample of 15 mg of **3** dissolved in 2 mL of MeOH was added to 10 mL of aqueous 1 N NaOH solution. The mixture was stirred at room temperature for 15 min, acidified to pH 2 with 6 N HCl, and extracted with EtOAc. Concentration of the organic phase yielded 10 mg of an off-white solid. The compound was analyzed without further purification and characterized as methyl-2,4-dihydroxy-5-methoxybenzoate (**4**): mp 127−129 °C; UV (MeOH) λ_{max} 224, 260, 317 nm; IR ($CHCl_3$) (ν, cm^{-1}) 1670, 1505, 1440, 1270, 1245, 1200, 1160; ^1H NMR ($CDCl_3$, 360 MHz) δ 10.68 (1 H, s, HO-4), 7.22 (1 H, s), 6.53 (1 H, s), 6.13 (1 H, s br, HO-2), 3.92 (3 H, s, CH_3O-5), 3.87 (3 H, s, CH_3OCO); ^{13}C NMR ($CDCl_3$, 90.5 MHz) δ 170.2 (s, CO), 158.5 (s), 152.8 (s), 139.9 (s), 109.9 (d), 103.3 (s), 103.1 (d), 56.36 (q, CH_3O-5), 51.97 (q, CH_3OCO); EIMS (70 eV) m/z 198 (M^+, 42), 166 (100), 151 (29), 138 (9), 123 (29), 95 (13), 69 (33); HREIMS (70 eV) m/z 198.0521 for $C_9H_{10}O_5$ (calcd 198.0528).

RESULTS AND DISCUSSION

Reaction of ferulic acid (**1**) with nitrite at pH 2 yielded a complex mixture of compounds. TLC and HPLC analyses showed the presence of a major, fluorescent product (**3**) which was isolated and purified by repeated chromatography and crystallization and characterized by spectroscopic analysis.

The HRMS for compound **3** gave $C_9H_8NO_4$ indicating a structure with one nitrogen atom and containing seven

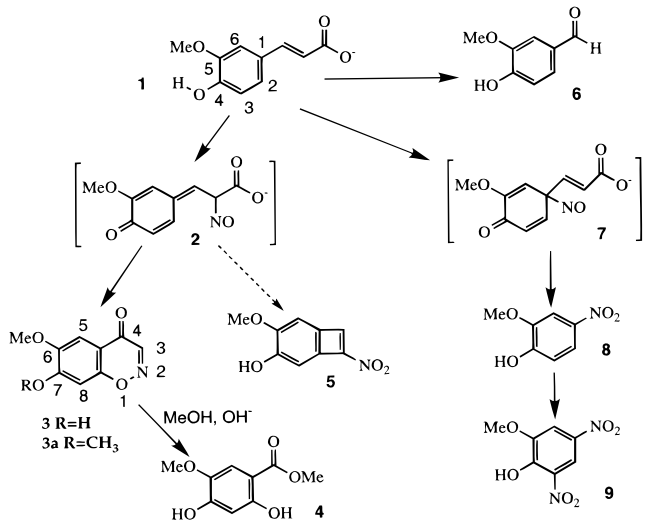

Figure 1. Products formed by the reaction of ferulic acid (**1**) with nitrite in acid.

rings or double bonds. The simple ^1H NMR spectrum contained signals for one methoxyl group (s, 3.99 ppm) and three uncoupled aromatic proton signals. The absence of coupling ruled out a position-6 ring substitution on ferulic acid and suggested that substitution had occurred on either position-2 or -3. The ^{13}C NMR spectrum contained nine carbon signals, all but the methoxyl being aromatic. The spectrum also revealed three methine-bearing carbons, three-oxygen bearing quaternary carbons, and one carbonyl carbon signal resonating at 166 ppm. HMBC and HMQC spectral analyses were used to assign the connectivities of protons and carbons to one another. Thus, H-3 was correlated with C-4a and C-4; H-5 with C-4, C-7, and C-8a; H-8 with C-4a and C-6; and the methoxyl group with C-6.

These connectivities initially caused us to consider two possible isomeric structures for the major ferulic acid product as either compound **3** or **5**. 3-Methoxy-7-nitrobicyclo[4,2,0]octan-4-ol (**5**) was ruled out because the MS required loss of an HCN equivalent to give a strong m/z 166 fragment ion. Furthermore, the IR spectrum contained strong absorption in the range 1530−1570 cm^{-1}, but none for a nitro group. These results suggested the presence of a conjugated carbonyl group in the ferulic acid product structure.

Studies of the reactivity of compound **3** toward acid and base were very informative. The compound was stable in acid. When compound **3** was stirred at room temperature in methanolic sodium hydroxide solution, a single, nonfluorescent compound was rapidly produced. ^1H-, ^{13}C-NMR, and EIMS data suggested that compound **4** was the methyl ester of 2,4-dihydroxy-5-methoxybenzoic acid. The EI mass spectra of compounds **3** and **4** were very similar except for their respective molecular ions. Thus, the structure 7-hydroxy-6-methoxy-1,2(4*H*)-benzoxazin-4-one could be assigned as compound **3** for the fluorescent product obtained by reaction of nitrite with ferulic acid (Figure 1).

1,2(4*H*)-Benzoxazin-4-one derivatives are not common. However, compatible spectroscopic data were reported for a dimethoxy substituted derivative (**3a**) (Sánchez-Viesca and Gómez, 1986). Furthermore hydrolysis of 5,6,7,8-tetrafluoro-2-phenyl-1,2(4*H*)benzoxazin-4-one either in acid or base at high temperature

gave a benzoic acid derivative similar to compound **3** (Shchegoleva et al., 1972). A fluorescent compound was reportedly obtained by nitrite oxidation of ferulic acid (Kikugawa et al., 1983). Although spectroscopic data (^{1}H NMR, UV, and MS) similar to that reported here were obtained, the structure of the fluorescent product was never determined. A second, minor product obtained by Kikugawa et al. was not observed in this work.

Two minor products of the nitration reaction were also isolated and identified as 2-methoxy-4,6-dinitrophenol (**9**) and 3-methoxy-4-hydroxybenzaldehyde (vanillin **6**). Compound **9** was yellow in color, and the ^{13}C-NMR spectrum exhibited signals for only seven carbon atoms. This indicated that the side chain of ferulic acid was lacking in the structure of the product. Three one-proton aromatic signals and a methoxyl group were evident in the ^{1}H NMR spectrum. Two of these signals were doublets (J = 2.4 Hz), indicating *meta*-coupled aromatic protons, and one was for a hydrogen-bonded phenolic hydroxyl group (11.19 ppm), indicating that positions *ortho* and *para* to the ferulic acid ring-hydroxyl group were substituted. The mass spectrum showed a molecular ion at *m*/*z* 214, indicating the presence of two nitrogen atoms and an empirical formula of $C_6H_6N_2O_6$. These spectral properties were in agreement with previously reported but incomplete data for this compound (Mende et al., 1994).

Vanillin was also isolated as a crude orange solid and was identified spectrally and chromatographically by comparison with authentic vanillin. Interestingly, when *p*-coumaric acid, a phenolic cinnamic acid, was reacted with nitrite, *p*-hydroxybenzaldehyde was identified as a major product (Kikugawa et al., 1983). However, the nature of intermediates or the pathway by which vanillin or *p*-hydroxybenzaldehyde are formed by nitrite oxidation remains unknown.

Para- or ortho-C-nitrosation of phenols with nitrite in acid is a well-known reaction which is assumed to occur through the protonated form of nitrous acid $(H_2ONO)^+$. Further oxidation of nitroso intermediates by nitrous acid leads to the corresponding nitro derivatives. With phenolic cinnamic acids such as ferulic acid or *p*-coumaric acid, a third reactive site for nitrosation is available on the side chain. Thus for the reaction of ferulic acid (**1**) with nitrite, three different intermediates (compounds **2**, **7** Figure 1) and a product of nitrosation at position-3 are theoretically conceivable.

2-Methoxy-4,6-dinitrophenol (**9**) requires two nitrations on the aromatic ring. This compound could arise by nitrosation para to the phenolic substituent of ferulic acid (**1**) to give compound **7** followed by elimination of the propenoic acid side-chain to afford 8 and subsequent further nitration to give compound **9**. Alternatively compound **9** may form through initial nitrosation of ferulic acid at position-3 to give an *o*-nitrophenol which then undergoes further transformation as with compounds **8** and **9** to eliminate the propenoic acid side chain. Nitrophenols **9** and **8** were reported as products obtained by acid-catalyzed nitrosation of capsaicin (Mende et al., 1994), and a similar dealkylation during nitrosation was used to explain the formation of compound **9**.

Mechanistically, the conversion of ferulic acid (**1**) to compound **3** (Figure 2) likely involves nitrosation of the ferulic acid side-chain to give intermediate compound **2**, which may either decarboxylate to compound **12** or, in concerted fashion, cyclize to benzoxazine **10**. Oxidation of compound **10**, addition of water at position-4, and subsequent oxidation of the alcohol would afford compound **3**. An alternative pathway based upon the formation of an intermediate α-oximino ketone (**16**) is plausible based on the reported formation of 1,2(4*H*)-benzoxazin-4-ones by cyclization of similar α-oximino ketones (Sánchez-Viesca and Gómez, 1986, and Shchegoleva et al., 1972). The possible involvement of vinylguaiacol, formed by ferulate decarboxylation (Huang et al., 1994; Peleg et al., 1992), as an intermediate in the formation of compound **3** is unlikely since this compound was never detected in our reaction mixtures.

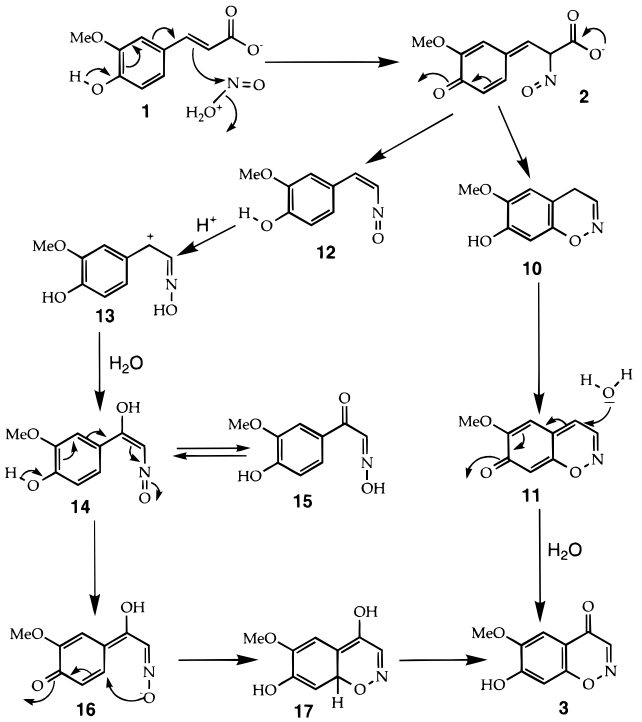

Figure 2. Proposed mechanisms for the conversion of ferulic acid (**1**) into 7-hydroxy-6-methoxy-1,2(4*H*)-benzoxazin-4-one (**3**) by reaction with nitrite in acidic medium.

We report the structure of a new benzoxazin-4-one (**3**) formed by reaction of ferulic acid with nitrite. The reaction yields numerous side products including vanillin and 2-methoxy-4,6-dinitrophenol (**9**). The nature of the products isolated and characterized suggests the basis by which ferulic acid may serve as an effective antioxidant or chemoprotective agent in the quenching of nitrosating species.

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Received for review February 13, 1998. Revised manuscript received June 9, 1998. Accepted June 10, 1998. Support for this work was provided through the USDA and the byproducts from Biotechnology Consortium.

JF980130F