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Nitrite-Mediated Decarboxylative Conjugation of Caffeic Acid with Glutathione Under Mildly Acidic Conditions

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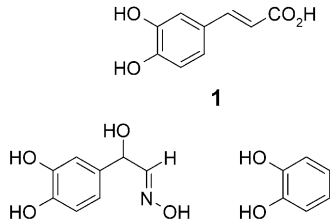
Abstract—In acetate buffer, pH 4, at room temperature, nitrite ions can mediate an unusual decarboxylative conjugation of caffeic acid with glutathione leading to novel isomeric 2-(3,4-dihydroxyphenyl)-2-*S*-glutathionylacetaldehyde oximes. These results hint at a possible role of endogenous and/or dietary glutathione in the mechanisms by which caffeic acid can affect the burden of carcinogenic *N*-nitroso compounds in the digestive tract.

Caffeic acid (**1**) belongs to a group of dietary polyphenolic micronutrients of plant origin endowed with antiinflammatory and chemopreventive properties against colorectal cancer and other tumours of the digestive tract.[1] Biochemical evidence suggested that part of the protective effects of **1** are due to the efficient inhibition of mutagenic and carcinogenic nitrosative reactions in the stomach.[2–5] Consistent with this view, **1** was found to react efficiently with nitrite under acidic conditions to afford mainly oximes **2** and **3**, as well as benzoxazinone and furoxan derivatives.[6,7]



1

2 **3**

Elucidation of this chemistry underscored nitrosative decarboxylation as a critical factor determining the superior antinitrosaminic properties of **1** with respect to its esters.[5–8]

In all these studies, however, little or no attention was paid to the possible involvement and role(s) of sulfhydryl compounds in the antinitrosaminic effects of **1**, although these compounds are widely distributed in food and have been implicated in the metabolic transformations of **1**.[9]

To address this issue, we investigated the reaction of **1** with nitrite ions in the presence of glutathione (GSH), a most important biological antioxidant and a potential anticarcinogen deputed to mobilization of xenobiotic toxicants. The results showed that under mildly acidic conditions mimicking those found in human gastric fluid[10] nitrite ions can mediate a remarkable decarboxylative coupling of **1** with GSH to afford unusual conjugates that could be isolated and structurally characterized.

In 0.05 M acetate buffer, pH 4/methanol (1%) and at 37 °C the reaction of **1** (2 mM) with nitrite ions (4 mM)[7] was affected by the presence of GSH in a dose-dependent fashion. With 10 mM GSH, average consumption of **1** after 2 h was about 25% and the product pattern was substantially modified, due to the formation of four main species which were eluted on reverse-phase HPLC (eluant HCOOH 0.1 M/CH_3CN 95:5 v/v) as a close group of peaks at 14.5, 15.4, 16.6 and 18.5 min (relative intensities ca. 1:1:2.5:2.5, in that order). In the absence of GSH formation of oximes **2** and **3** as well as of 3,4-dihydroxybenzaldehyde and 3,4-dihydroxybenzoic acid was observed.[7] On the other hand, **1** did not react with GSH in the absence of nitrite ions.

LC–MS analysis indicated for the four products the same molecular ion peak (*m*/*z* 472) and a main fragmentation

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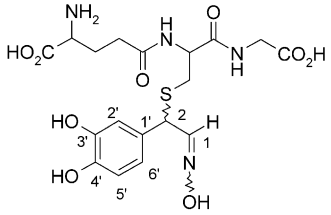
Table 1. NMR spectral data for isomeric adducts **4**[a]

	E[b]		*E*[c]		*Z*[b]		*Z*[c]	
	^{1}H (*J*, Hz)	^{13}C	^{1}H (*J*, Hz)	^{13}C	^{1}H (*J*, Hz)	^{13}C	^{1}H (*J*, Hz)	^{13}C
1	7.61 (d, 7.6)	152.8	7.62 (d, 7.6)	153.0	7.04 (d, 6.4)	151.8	7.06 (d, 6.4)	152.1
2	4.50 (m)	48.3	4.50 (m)	49.0	5.32 (d, 6.4)	41.8	5.34 (d, 6.4)	43.6
1′	—	130.2	—	130.2	—	130.2	—	130.2
2′	6.88 (d, 2.0)	116.8	6.88 (d, 2.0)	116.8	6.93 (d, 2.0)	116.8	6.93 (d, 2.0)	116.8
3′	—	145.8	—	145.8	—	145.8	—	145.8
4′	—	145.4	—	145.4	—	145.4	—	145.4
5′	6.85 (d, 8.4)	117.8	6.85 (d, 8.4)	117.8	6.86 (d, 8.4)	117.8	6.86 (d, 8.4)	117.8
6′	6.73 (dd, 8.4, 2.0)	121.6	6.73 (dd, 8.4, 2.0)	121.6	6.77 (dd, 8.4, 2.0)	121.6	6.77 (dd, 8.4, 2.0)	121.6
β-CH_2 glu	2.16 (m)	27.5	2.16 (m)	27.5	2.16 (m)	27.5	2.16 (m)	27.5
γ-CH_2 glu	2.50 (m)	32.7	2.50 (m)	32.7	2.50 (m)	32.7	2.50 (m)	32.7
CH_2 cys	2.79 (dd, 14.4, 9.6)	33.9	2.86 (dd, 14.0, 8.0)	33.9	2.79 (dd, 14.4, 9.6)	33.9	2.86 (dd, 14.0, 8.0)	33.9
	3.03 (dd, 14.4, 4.4)	33.9	2.95 (dd, 14.0, 4.8)	33.9	3.03 (dd, 14.4, 4.4)	33.9	2.95 (dd, 14.0, 4.8)	33.9
CH glu	3.81 (m)	55.5	3.81 (m)	55.5	3.81 (m)	55.5	3.81 (m)	55.5
CH_2 gly	3.84 (s)	44.2	3.84 (s)	44.2	3.84 (s)	44.2	3.84 (s)	44.2
CH cys	4.65 (m)	53.8	4.54 (m)	54.5	4.65 (m)	53.8	4.54 (m)	54.5
CO glu	—	176.0	—	176.0	—	176.0	—	176.0
CO cys	—	173.4	—	173.2	—	173.4	—	173.2
COOH glu	—	175.4	—	175.4	—	175.4	—	175.4
COOH gly	—	176.7	—	176.7	—	176.7	—	176.7

[a]Recorded at 400 MHz (^{1}H) and 100 MHz (^{13}C) at 25 °C in D_2O; chemical shifts (δ) are given as ppm.
[b]*R* or *S* at C-2.
[c]*S* or *R* at C-2.

peak at *m*/*z* 307, suggesting GSH adducts. Attempts to separate the products were defeated under a variety of conditions, due to the closely similar chromatographic behavior and their instability to acids and oxidants. Furthermore, some of them exhibited a marked tendency to interconvert following isolation. Accordingly, we resorted to isolate and characterize the products as a mixture and after several efforts a procedure was eventually developed. This involved extraction of the mixture with EtOAc, to remove unreacted **1**, and evaporation of the aqueous layer to dryness under carefully controlled conditions. The residue, taken up in water, was chromatographed on preparative HPLC (eluant HCOOH 0.02 M/CH_3OH 95:5 v/v) followed by Sephadex G-10 chromatography (eluant water). By this method, the four products could be obtained as a glassy oil [λ_{max} (H_2O) 281 nm], yield 40%.

^{1}H NMR analysis revealed in the low field region the signals for a 4-substituted catechol ring, as in **1**, the expected resonances for a GSH moiety and, as a most salient feature, two close pairs of doublets at δ 7.61/7.62 and 7.04/7.06 (relative areas 2.5:1). Each pair displayed one-bond correlations with two carbon signals at δ 152.8/153.0 and 151.8/152.1, respectively. The intense signals at δ 7.61/7.62 were coupled to signals at δ 4.50 which displayed cross peaks in the ^{1}H,^{13}C HMQC spectrum with two carbon resonances at δ 48.3/49.0, whereas the less intense signals at δ 7.04/7.06 were coupled to two doublets at δ 5.32/5.34 showing one-bond connectivities with two carbon signals at δ 41.8/43.6. The diastereotopic CH_2S–protons of GSH appeared as two distinct sets of double doublets, one at δ 2.86/2.95 and the other at δ 2.79/3.03. Close inspection of the COSY, ^{1}H,^{13}C HMQC and ^{1}H,^{13}C HMBC spectra eventually allowed formulation of the products as stereoisomers corresponding to the gross structure of 2-(3,4-dihydroxyphenyl)-2-*S*-glutathionylacetaldehyde oxime (**4**).



4

The main components of the mixture were assigned the *E* configuration at the oxime functionality, however the distinction between the *R* and *S* diastereoisomers at the stereogenic centre α to the oxime group was not straightforward based on NMR data (Table 1). Brief inspection of geometry optimized structures (MM+)[†] for *R* and *S* diastereoisomers of *E* oximes predicted a slightly lower energy for the *S* isomer. Since the integrated area of the CH_2S–resonances at δ 2.79/3.03 exceeded by ca. 20% that of the isomers at δ 2.86/2.95, it was argued that the former set pertained to the *S* isomers.

In separate experiments, the factors affecting the yield, stability and mode of decomposition of adducts **4** were investigated.

With 2 mM **1** and 10 mM GSH, increasing nitrite concentration from 4 to 8 mM resulted in a marked decrease in the overall yield of adducts **4** and the formation of **3** in about 9% yield. When substrate concentration was decreased to lower, more physiological values, for example, 500 μM, the reaction with equimolar concentrations of GSH and nitrite proceeded

[†]Molecular mechanics calculations were carried out with the Hyperchem 5.0 package produced by Hypercube Inc. (Waterloo, Ontario, Canada) 1997.

smoothly to give, after 1 h, adducts **4** (3% yield), oxime **2** (8% yield) along with 80% unreacted **1**.

At room temperature in acetate buffer at pH 4, isolated adducts **4** underwent slow decomposition leading to oxime **3** (estimated yield ca. 4%, after 24 h) as the main identifiable product. Under the same conditions **2** was not converted to **3**, ruling out its possible intermediacy in the acid-induced decomposition of **4** to **3**.

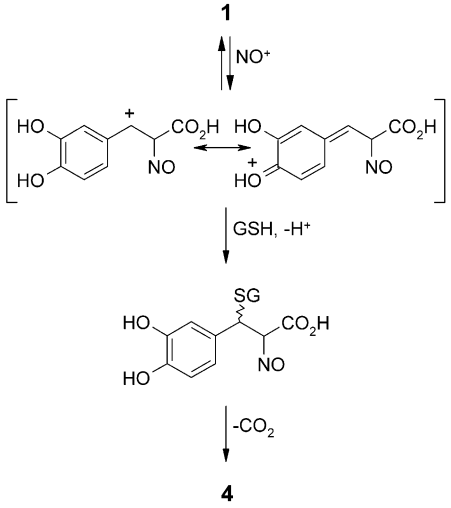
In neutral or alkaline medium adducts **4** proved likewise unstable and rapidly changed into polar species difficult to characterize.

Cysteine or *N*-acetylcysteine behaved similarly as GSH to give apparently addition products (HPLC evidence) which, however, proved unexpectedly unstable to isolation and rapidly degraded to give mainly oxime **3** along with ill characterized material. The reasons for such instability are not apparent and deserve further investigation.

A plausible mechanism of formation of **4** is depicted in Scheme 1.

In this scheme, nitrosation of **1** at C-2[7] would result in the generation of a transient β-nitroso cation susceptible to nucleophilic attack by the SH group of GSH to give 3-(3,4-dihydroxyphenyl)-2-nitroso-3-*S*-glutathionylpropanoic acid. This would suffer facile decarboxylation with concomitant tautomerization of the nitroso group to afford eventually the oxime conjugate.

Alternatively, decarboxylation of the primary nitroso cation might precede nucleophilic addition of GSH.[11] Though the mechanisms are not mutually exclusive, we would favor the pathway in Scheme 1 because of the expectedly higher proclivity of the putative nitroso cation to nucleophilic addition in the presence of excess GSH rather than to decarboxylation.[7]



Scheme 1. Proposed mechanism of formation of **4**.

Conclusions and Biological Implications

Catechol compounds, including **1**, are known to be susceptible to oxidative conjugation with GSH or related sulfhydryl compounds at neutral pH via the intermediacy of quinone species,[9,12,13] and this represents an established detoxification mechanism. However, to the best of our knowledge, no side-chain directed conjugation reaction between caffeic acid and sulfhydryl compounds has been reported under acidic conditions of physiological relevance. Besides the intrinsic chemical interest, the results of this study provide a useful background to inquire into the possible role of GSH and other sulfhydryl compounds in the antinitrosaminic effects of **1** upon nitrosation in the gastric fluid.

GSH is abundantly distributed in the mucosal cells of the gastrointestinal tract[14] and its levels are affected by several factors, including diet and alcohol ingestion. Dietary sources of GSH[15] include raw meats, fresh fruits and freshly cooked vegetables (ca. 40–150 mg/kg). Since in the gastric compartment pH varies from 2.5 to 4.5 during digestion, conditions may exist for the reported reaction to occur in vivo following high nitrite intake, for example with cured/pickled meats or vegetables.

In these circumstances, GSH-adduct formation would not interfere with the nitrite-scavenging properties of **1**. On the contrary, it may have the utilitarian effect of enhancing mobilization of nitrosation products of **1**, thus decreasing the risk of detrimental effects due to their accumulation in the lipophilic compartments of the gastric mucosa.

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