Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry 11 (2003) 4797–4805

BIOORGANIC & MEDICINAL CHEMISTRY

Oxidative Conjugation of Chlorogenic Acid with Glutathione: Structural Characterization of Addition Products and a New Nitrite-Promoted Pathway

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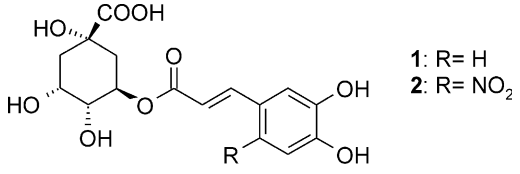
Received 25 April 2003; accepted 10 July 2003

Abstract—Chlorogenic acid (**1**), a cancer chemopreventive agent widely found in fruits, tea and coffee, undergoes efficient conjugation with glutathione (GSH), in the presence of horseradish peroxidase/H_2O_2 or tyrosinase at pH 7.4, to yield three main adducts that have been isolated and identified as 2-*S*-glutathionylchlorogenic acid (**3**), 2,5-di-*S*-glutathionylchlorogenic acid (**4**) and 2,5,6-tri-*S*-glutathionylchlorogenic acid (**5**) by extensive NMR analysis. The same pattern of products could be obtained by reaction of **1** with GSH in the presence of nitrite ions in acetate buffer at pH 4. Mechanistic experiments suggested that oxidative conjugation reactions proceed by sequential nucleophilic attack of GSH on *ortho*-quinone intermediates. Overall, these results provide the first complete spectral characterization of the adducts generated by biomimetic oxidation of **1** in the presence of GSH, and disclose a new possible nitrite-mediated conjugation pathway of **1** with GSH at acidic pH of physiological relevance.

Introduction

Epidemiological, biological and biochemical data concur to support a beneficial role of chlorogenic acid (5-caffeoyl-D-quinic acid, **1**)[1] and other dietary polyphenolic compounds in human health. Widely occuring in various agricultural products at relatively elevated levels, for example, 3.4–14 mg/100 g fresh weight in potatoes, 12–31 mg/100 mL of apple juice, 89 mg/100 g of dry tea shoots, and ca. 250 mg per cup of coffee,[2,3] **1** has been attributed anticarcinogenic,[4–6] antimutagenic[7] and antiinflammatory effects.[8] It contributes significantly to the total polyphenol intake and has been suggested to play a role in the apparent association between the regular consumption of polyphenol-rich food and beverages and the prevention of inflammatory and proliferative diseases. In particular, **1** has been reported to exert inhibitory effects on carcinogenesis in the large intestine, liver, and tongue,[9–11] and a protective action on oxidative stress in vivo.[12]

Recent research has also emphasized the antinitrosating properties of polyphenolic compounds such as **1**, which may serve as efficient nitrite scavengers and suppressors of nitrosamine formation in the gastric compartment.[2,13–15] Elucidation of the underlying chemistry has underscored a marked reactivity of **1** toward nitrous acid and reactive nitrogen species thereof, to give nitrochlorogenic acid **2** as the prevailing product.[16,17]



1: R= H
2: R= NO_2

Both antioxidant and antinitrosaminic properties of **1** stem from the oxidizable *ortho*-diphenolic functionality, which can act as an H-atom donor toward reactive oxygen species and other biological oxidants, as a transition metal chelator preventing Fenton-type processes,[18] and as an efficient trap for electrophilic nitrosating agents.[16,17] It can also account for inhibition of lipoxygenase activity,[19] retinoic acid epoxidation[20] and H_2O_2-induced erythrocyte haemolysis and lipid peroxidation.[21]

In spite of continuing advances, the oxidative metabolism of **1** and its interactions with potential biological

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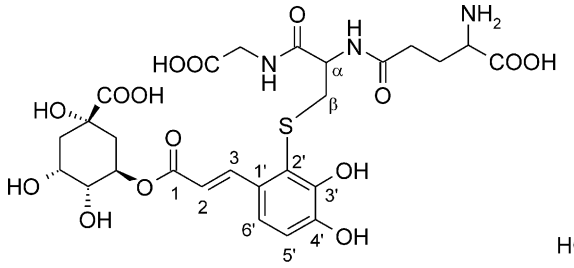
doi:10.1016/S0968-0896(03)00460-7



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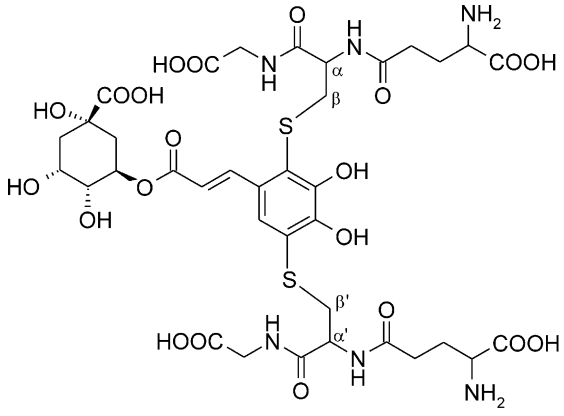

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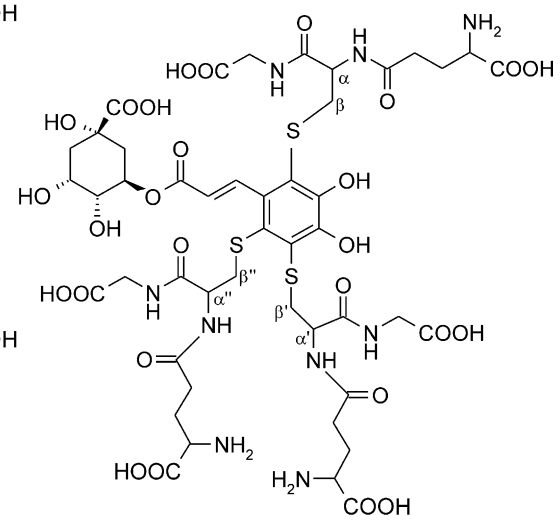

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targets in relation to cancer chemoprevention are still not entirely elucidated. In a relevant study,[22] it has been reported that **1** reacts with cysteine in the presence of apple polyphenol oxidase to give a main product identified as 2-*S*-cysteinylchlorogenic acid. More recently,[23] it has been shown that **1**, caffeic acid and dihydrocaffeic acid form quinonoid and hydroxylated products when oxidized by horseradish peroxidase (HRP)/H_2O_2 or tyrosinase/O_2. Mass spectrometric analyses of the metabolites formed by HRP/H_2O_2 oxidation in the presence of glutathione (GSH), the most prevalent intracellular thiol antioxidant and a potential anticarcinogen deputed to mobilization of xenobiotic toxicants, revealed formation of mono- and di-GSH conjugates for all three compounds. However, the chemical structures and properties of these latter adducts were not supported by detailed spectral analysis.

In this paper, we report the isolation and complete spectral characterization of the adducts formed by tyrosinase and HRP/H_2O_2 oxidation of **1** in the presence of GSH, including a hitherto unknown triadduct, and describe for the first time the ability of nitrite ions to promote the same oxidative conjugation processes under mildly acidic conditions simulating those within the gastric compartment.

Results

HRP/H_2O_2 or tyrosinase-promoted oxidation of chlorogenic acid (1) in the presence of GSH

Oxidation of **1** (50 μM) by HRP (1U/mL) and H_2O_2 (two molar equivalents) under the reported conditions[23] in 0.05 M phosphate buffer, pH 7.4, proceeded rapidly to give a yellow solution displaying no chromatographically isolable species under reverse phase HPLC conditions. When the reaction was carried out in the presence of excess GSH (200 μM), HPLC analysis of the oxidation mixture revealed a well defined pattern of products comprising a species eluting faster than **1** and two more abundant components eluting at higher retention times.

For preparative purposes the reaction was carried out using the substrate at 2 mM concentration with 4 U/mL of HRP and 4 mM H_2O_2 in the presence of 10 mM GSH. Preparative HPLC allowed isolation of the more retained products in pure form. One of these (32% isolated yield) was formulated as 2-*S*-glutathionylchlorogenic acid **3**[24] based on the $[M+H]^+$ peak at *m*/*z* 660 in the positive ion electrospray (ESI+)-MS spectrum and straightforward analysis of the ^1H NMR spectrum, showing two doublets in the aromatic region ($J=8.4$ Hz) ascribed to the *ortho*-coupled H-5 and H-6 protons and the expected resonances for a GSH unit. The *E* configuration at the double bond was maintained, as apparent from the large coupling constant ($J=16.0$ Hz). In agreement with previous data for 2-*S*-glutathionylcaftaric acid,[25] the vinyl α proton of **3** suffered a marked downfield shift compared to **1**. The integrity of the quinic acid unit was deduced by analysis of the ^1H and ^{13}C NMR spectra, in which relevant resonances did not differ appreciably from the chemical shift values of **1**. Moreover, the chromophoric features of **3** were consistent with those of catechol–thiol adducts.[26]

The other product, showing a pseudomolecular peak at *m*/*z* 965 in the ESI+-MS spectrum, was evidently a

diadduct (18% yield). The ^{1}H NMR spectrum consistently showed only a single proton resonance in the aromatic region (δ 7.38) appearing as a singlet, suggesting either the 2,5- or the 2,6-disubstituted isomer. That the former (**4**) was the correct structure was deduced from extensive 2D homonuclear and ^{1}H,^{13}C heteronuclear correlation experiments. As a most diagnostic feature, the singlet at δ 7.38 displayed a cross peak in the ^{1}H, ^{13}C heteronuclear multiple bond correlation spectrum with the vinyl α carbon resonance at δ 144.2.

The third, more polar product was usually formed in lower yields with respect to **3** and **4**. However, after several trials it was eventually obtained in satisfactory amounts using higher concentrations of H_2O_2, for example, 8 mM. Under such conditions, **1** was completely consumed and adducts **3** and **4** were formed in negligible amounts. Chromatography of the reaction mixture on a Sephadex G10 column, eluant water, yielded the product in practically pure form for spectral analysis (90% yield). The $[M+H]^+$ peak at m/z 1270 and the lack of signals for aromatic protons in the ^{1}H NMR spectrum, together with ^{13}C NMR data, concurred to assign the product the structure of the novel triadduct **5**.

Selected NMR resonances of compounds **3**, **4** and **5** and assignments based on 2D correlation experiments are listed in Table 1.

In another series of experiments the effect of various parameters on the kinetics and product distribution in the oxidation of **1** (50 μM) in the presence of GSH was investigated.

Figure 1 reports substrate consumption and formation yields of adducts **3**–**5** as a function of GSH equivalents in the reaction of **1** with HRP and one (plot A) or two (plot B) molar equivalents of H_2O_2. Analysis of the reaction mixtures was carried out 5 min after addition of the oxidant. In most of the mixtures examined, mass balance was higher than 80%. With 1 molar equivalent

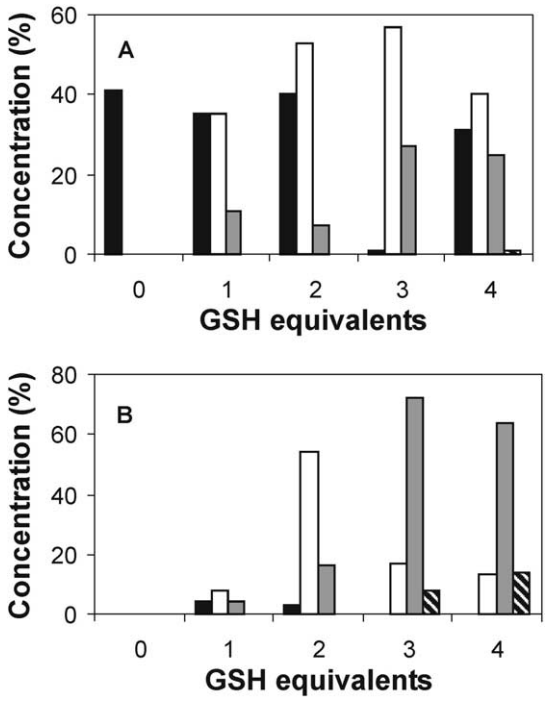

Figure 1. Substrate consumption and formation yields of adducts **3**–**5** as a function of molar equivalents of GSH in the HRP/H_2O_2 promoted oxidation of **1**. Reaction of **1** (50 μM) was run in 0.05 M phosphate buffer (pH 7.4), in the presence of HRP (1 U/mL) and one (plot A) or two (plot B) molar equivalents of H_2O_2, with GSH varying in the range of 0–200 μM. The reaction mixture was analyzed by HPLC 5 min after addition of the oxidant. Percent concentration of **1** (black bars), **3** (open bars), **4** (grey bars) and **5** (dashed bars) in the reaction mixture. Shown are the mean values for three separate experiments with SD ≤10%.

Table 1. Selected NMR spectral data for adducts **3**, **4** and **5**[a]

	3		**4**		**5**	
	^{1}H (J, Hz)	^{13}C	^{1}H (J, Hz)	^{13}C	^{1}H (J, Hz)	^{13}C
1[b]	—	170.0	—	169.6	—	169.1
2	6.27 (d, 16.0)	117.8	6.42 (d, 16.0)	118.6	6.38 (d, 16.0)	126.4
3	8.10 (d, 16.0)	145.2	8.17 (d, 16.0)	144.2	8.00 (d, 16.0)	144.5
1′	—	130.9	—	130.8	—	138.1
2′	—	121.2	—	121.2	—	120.9
3′	—	147.4	—	147.5	—	148.3[c]
4′	—	147.7	—	147.5	—	148.5[c]
5′	6.88 (d, 8.4)	118.2	—	122.9	—	127.1
6′	7.12 (d, 8.4)	121.2	7.38 (s)	124.1	—	129.9
CH_2 β	3.05 (dd, 14.4, 8.8)	36.7	3.06 (dd, 14.4, 8.8)	37.1	3.00 (dd, 14.4, 9.6)	37.4
	3.22 (dd, 14.4, 3.6)		3.27 (dd, 14.4, 3.6)		3.29 (dd, 14.4, 4.4)	
CH_2 β′	—	—	3.23 (dd, 14.4, 8.4)	35.8	3.28 (m)	38.3
			3.41 (dd, 14.4, 4.4)		3.46 (dd, 13.6, 4.8)	
CH_2 β″	—	—	—	—	3.09 (dd, 14.0, 9.2) 3.22 (m)	36.8
CH α	4.22 (dd, 8.8, 3.6)	54.8	4.20 (dd, 8.8, 3.6)	54.5	4.74 (m)	54.0[d]
CH α′	—	—	4.46 (dd, 8.4, 4.4)	54.2	4.24 (dd, 8.4, 4.8)	54.4
CH α″	—	—	—	—	4.17 (dd, 9.2, 4.8)	54.1[d]

[a]Spectra run in D_2O, chemical shift values given in ppm.
[b]Numbering as shown in structural formulas **3**, **4** and **5**.
[c]Interchangeable.
[d]Interchangeable.

of H_2O_2 (plot A), an increase in the number of molar equivalents of GSH affected product distribution, favouring the diadduct **4** at the expense of **3**. Substrate consumption, however, was not significantly affected by the concentration of GSH in the reaction mixture. Formation of triadduct **5** became relevant only in the reactions run with two molar equivalents of H_2O_2 (plot B): in this case, consumption of **1** was complete in most of the mixture analyzed and an increase in the number of molar equivalents of GSH shifted the product distribution in favour of the more substituted adducts. Surprisingly, with one molar equivalent of GSH the mass balance was very poor. This could be explained considering that other reaction pathways prevailed when GSH concentrations are too low for efficient coupling.

Addition of two molar equivalents of H_2O_2 in two portions at 10-min intervals rather than as a bolus resulted in comparable substrate consumption, but increased the yields of the more substituted adducts **4** and **5** (data not shown).

In separate experiments, **3** was allowed to react with HRP/H_2O_2 in the presence of GSH. HPLC analysis revealed quantitative conversion to **4**. Moreover, reaction of **4** with HRP/H_2O_2 in the presence of GSH was found to yield **5** as the sole product.

Using the glucose/glucose oxidase system as source of H_2O_2, oxidation of **1** (50 μM) in the presence of 200 μM GSH was complete in 1 h leading to formation of **3**, **4** and **5** in 8, 68 and 20% yields, in that order. As the reaction proceeded further, for example at 2 h, triadduct **5** gradually accumulated up to 80% yield.

When oxidation of **1** (50 μM) was carried out with tyrosinase in the presence of 200 μM GSH, substrate consumption was about 95% after 5 min and **3** was obtained in 72% yield, whereas after 3 h all of the conjugation products **3**, **4** and **5** were formed in 27, 54 and 10% yield, in that order. With **1** at 2 mM concentration and 5 molar equivalents of GSH the reaction was complete after about 50 min to give **3** as the only detectable adduct (76% yield). Consistently with previous data,[23,25] pure **3** or **4** were poor substrates of tyrosinase.

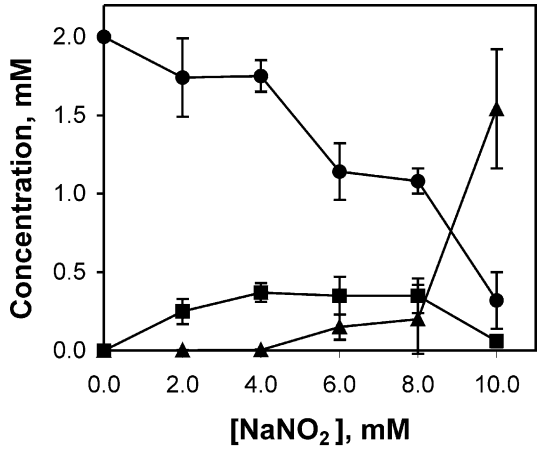

Figure 2. Consumption of **1** and product formation in the reaction with GSH and nitrite ions as a function of nitrite concentration. Reaction of **1** (2 mM) was run in the presence of GSH (1 mM) and varying amounts of $NaNO_2$ (0–10 mM) in 0.05 M acetate buffer (pH 4) and the mixture was analyzed by HPLC at 3 h. Concentration of **1** (●), **2** (▲), and **3** (■) in the reaction mixture. Shown are the mean ± SD values for three separate experiments.

Nitrite-mediated reaction of chlorogenic acid (1) with GSH under acidic conditions

In a recent paper,[27] we demonstrated that nitrite ions can mediate a remarkable decarboxylative conjugation of caffeic acid with GSH under mildly acidic conditions mimicking those within the stomach. As an extension of that study, we found that GSH can likewise affect the reaction of **1** with nitrite ions in acetate buffer at pH 4. Comparative HPLC analysis of reaction mixtures in the presence of even low GSH concentrations revealed suppression of the 6-nitroderivative **2** and formation of a main product which was identical with **3** by chromatographic, mass spectrometric and NMR analysis (Table 2). Formation of **2** was observed only with high nitrite/GSH molar ratios.

To investigate in more detail the competition between nitration and GSH adduct formation, the effect of increasing nitrite concentration in the reaction of 2 mM **1** with 1 mM GSH in acetate buffer, pH 4, was then examined. The results in Figure 2 showed that formation of the nitroderivative **2** becomes significant only when nitrite was ca. 10-fold with respect to GSH.

When the reaction was run at pH 4 with **1** at 2 mM concentration in the presence of 4 and 5 molar equivalents of nitrite and GSH, respectively, in addition to **3**, diadduct **4** and triadduct **5** were also formed in the reaction mixture. Relative yields after 2 h were 33, 12 and 6% in that order. No other adduct was detectable despite careful scrutiny of the reaction mixture. Analysis of the reaction mixture at various times indicated that all adducts were stable under the reaction conditions for at least 16 h. An abundant species eluting under a less retained peak was identified as *S*-nitrosoglutathione (GSNO) by comparison with an authentic sample prepared by a literature procedure.[28] As confirmed in control experiments, in the early stages of the reaction GSH was converted to GSNO which started to decompose over about 2 h to yield GSH disulphide (GSSG). Formation of GSSG was observed also in the late stages of the reaction of **1** with nitrite and GSH at pH 4.

Table 2. Fomation of **2** and **3** in the reaction mixture of **1** with GSH and $NaNO_2$ at pH 4

Concentration (mM)				
1	GSH	$NaNO_2$	**3**[a,b]	**2**[a,b]
1	0.1	1	0.046	—
1	0.1	5	—	0.75
1	5	5	0.017	—

[a]Concentration at 3-h reaction time.
[b]Reported are the mean values for three separate experiments with SD ≤10%.

To assess the possible role of GSNO in the nitrite-mediated formation of conjugates, in separate experiments GSNO was allowed to react with **1** at pH 4. HPLC analysis, however, did not reveal appreciable adduct formation. Similar reactions of **1** with nitrite in the presence of GSSG led to **2** as the main product, with no detectable GSH adducts.

Significant formation of **3** was observed when **1** was allowed to react at concentrations as low as 25 μM in the presence of comparable amounts of GSH and nitrite ions at pH 3, at 37 °C, that is under conditions mimicking those occurring in the gastric compartment[29] (Table 3).

Diode array spectrophotometric analysis was used to investigate the possible intermediacy of **1** quinone in the nitrite-mediated conjugation of **1** with GSH. Reaction of **1** (2 mM) with nitrite (1 mM) at pH 4 in the presence of 8 mM GSH led to the development of an intermediate species exhibiting a chromophore centred at 400 nm, apparently identical with that ascribed to the quinone of **1** or of caffeic acid,[23,30,31] whose intensity decreased when sodium borohydride was added. The possible contribution of **2** [λ_{max} (pH 4) 278, 340–360 (broad) nm] to the 400 nm chromophore was ruled out by HPLC analysis of the mixture. The same chromophore was obtained by oxidation of **1** with sodium nitrite at pH 4, but in the absence of GSH, as well as with periodate at pH 4 or 6.[23] Under both conditions, the chromophore disappeared by treatment with sodium borohydride or GSH. HPLC analysis of the oxidation mixture of **1** with acidic nitrite after addition of GSH revealed formation of **3** and the 6-nitroderivative **2** in comparable amounts.

Discussion

Despite considerable interest in the oxidation chemistry of **1**, the mechanisms and mode of conjugation with GSH have not yet been fully elucidated. Under at least three different conditions of physiological relevance, we have now shown that **1** is susceptible to sequential oxidative conjugation with GSH up to the hitherto unknown triadduct stage. Overall, these results expand current knowledge of oxidative conjugation of caffeic acid derivatives with GSH,[23,25,32] but raise a number of mechanistic issues.

In a previous paper,[23] it was speculated that GSH conjugation with **1** proceeded mainly via the 5-position of the catechol ring, based on the known reactivity of 4-substituted *ortho*-quinones with thiol compounds.[33–35] However, caftaric acid was reported to react with GSH to give exclusively the 2-*S* conjugate,[25] as did **1** with cysteine,[22] whereas similar reaction of caffeic acid with cysteine was shown to give also lower amounts of the 5-*S* isomer.[36] In line with these results was also the failure to observe appreciable formation of the 5-*S*-isomer in the oxidation reaction of **1** with GSH under all the conditions examined in the present study. On this basis it can be argued that the dominant mode of coupling of caffeic acid-derived quinones is via the 2-position, at variance with catecholamine quinones which react mainly at the 5-position.[33] A plausible explanation would envisage the electronwithdrawing effect of the unsaturated propenoate chain, which is lacking in those *ortho*-quinones, such as dopaquinone and dopamine-quinone, that react via the 5-position.[33,34] Inspection of canonical resonance structures for **1** quinone indicates that the propenoate chain decreases electron density at the 2-position with respect to the 5-position, thus subverting the usual regiochemistry of *S*-conjugation on 4-alkyl-*ortho*-quinones and rendering the 2-position more susceptible to nucleophilic attack. To substantiate this view, the total charge density and LUMO coefficients of methyl caffeate quinone as a model system were preliminarily investigated by semiempirical methods (AM1 and PM3). The results, summarized in Table 4, indicated a more positive charge density on the 6-position but a larger LUMO coefficient on the 2-position.

Considering that the SH group is a soft nucleophile,[37] it can be speculated that the regiochemistry of the nucleophilic attack is governed by frontier orbital interactions, which consistently predicted the higher reactivity for the 2-position.

The above discussion relied on a classical mechanism of conjugation involving nucleophilic addition of GSH to the quinone of **1** through the sulphydryl group. Such a mechanism notoriously operates in the case of tyrosinase, a copper enzyme which can catalyze two-electron oxidation of catechols, and was previously demonstrated for the HRP-promoted conjugation.[23] In the latter case, the quinone conceivably arises by disproportionation of the semiquinone produced by one-electron oxidation of **1**. A detailed description of the semiquinone radicals derived from **1** with enzyme

Table 3. Formation of **3** in the reaction mixture of **1** with GSH and $NaNO_2$ at pH 3 and 37 °C

Concentration (μM)			
1	GSH	$NaNO_2$	**3**[a]
25	25	25	4±0.7
500	25	25	16±1.8
500	500	500	13±1.2

[a]Determined at 1-h reaction time. Reported are mean values±SD for experiments run in triplicate.

Table 4. Total charge density (TCD) and LUMO coefficients of methyl caffeate quinone

	TCD		LUMO	
Atom[a]	AM1	PM3	AM1	PM3
C-1	−0.017	−0.023	0.372	0.375
C-2	−0.180	−0.174	0.437	0.449
C-3	0.201	0.254	0.295	0.279
C-4	0.205	0.253	0.246	0.233
C-5	−0.200	−0.183	0.312	0.327
C-6	−0.079	−0.047	0.272	0.273

[a]Numbering as indicated in ref. 24.

systems has been reported using the ESR spin stabilization technique.[38]

Based on the present study, we can propose a similar thiol–quinone interaction mechanism for the nitrite-promoted conjugation, in which the role of nitrite would be to generate the strong oxidant NO_2 ($E_o = 0.99$ V)[39] by decomposition of nitrous acid, according to the following equations:

$$NO_2^- + H^+ \rightleftharpoons NO_2$$
$$HNO_2 + H^+ \rightleftharpoons NO^+ + H_2O$$
$$2HNO_2 \rightleftharpoons N_2O_3 + H_2O$$
$$N_2O_3 \rightleftharpoons NO + NO_2$$
$$2NO + O_2 \rightarrow 2NO_2$$

Thus, one-electron oxidation of **1** by NO_2 would generate the semiquinone which would suffer disproportionation to give the *ortho*-quinone.

A schematic outline of the conjugation pathways of **1** with GSH described in the present study is shown in Figure 3.

In this scheme, formation of adducts **3–5** is envisaged as involving sequential addition of GSH to quinone intermediates. These latter may be produced by HRP/H_2O_2 or acidic nitrite-promoted oxidations, or by a redox cycling process brought about by **1** quinone.[40]

The demonstration that mildly acidic nitrite solutions can promote the efficient coupling of **1** with GSH is, to the best of our knowledge, unprecedented in the field of catechol-thiol conjugation chemistry. Besides the intrinsic chemical interest, the results of this study provide an improved background for studies aimed at clarify the mechanisms through which **1** exerts its biological effects. Dietary sources of GSH include raw meats, fresh fruits and freshly cooked vegetables (ca. 40–150 mg/kg).[41] 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. Exposure of humans to excess nitrite from diet is currently implicated as a potential contributory factor in the etiology of cancers of the gastrointestinal tract. In these circumstances, GSH-adduct formation, by preventing formation of **2**, would interfere with the nitrite-scavenging properties of **1**, at variance with the case of caffeic acid.[27] This would support the superior anti-nitrosaminic and chemopreventive properties of caffeic acid compared to **1**.[2,14,17] Moreover, GSH is found widespread in phenol-rich plant foods, e.g., in grapes, tomatoes and peaches, whereby conjugation reactions may occur during oxidation and browning, modifying both organoleptic properties and effects of phenols on human health.

In addition to the discovery of the nitrite-mediated conjugation of **1** with GSH, another major outcome of this study was the first characterization of a GSH triadduct with a caffeic acid derivative. Whereas catechol–thiol diadducts have been reported in several studies,[32,36] the only triadducts so far known were those

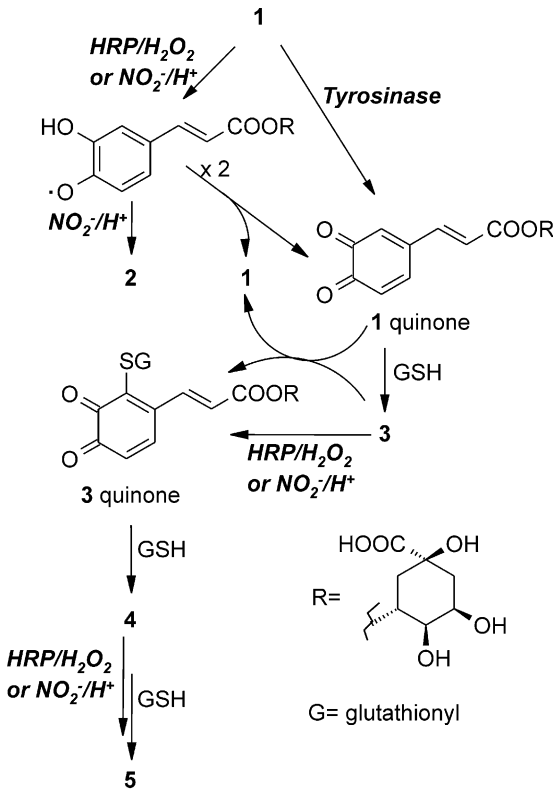

Figure 3. Oxidative conjugation pathways of **1** with GSH.

obtained by electrochemical oxidation of dopamine in the presence of GSH[35] as well as by HRP/H_2O_2-mediated reaction of GSH with the flavonoid quercetin.[42] This latter paper contains structural information for *ortho*-quinone GSH adducts in a case close to that presented in this study. Clearly, more work is required before the actual biological relevance of the reported chemistry is assessed.

Experimental

Materials and methods

Chlorogenic acid (**1**), glutathione (GSH), D-glucose, sodium nitrite and sodium borohydride were purchased from Aldrich and used as obtained. Hydrogen peroxide (30% solution) and sodium periodate were form Carlo Erba. Glutathione disulphide (GSSG) was obtained from Fluka. Horseradish peroxidase (HRP) (EC 1.11.1.7) type II, mushroom tyrosinase (EC 1.14.18.1) and glucose oxidase (EC 1.1.3.4) type II were from Sigma. *S*-nitrosoglutathione (GSNO) was prepared by treatment of GSH with sodium nitrite in 2 M HCl.[28] UV spectra were performed on a diode array spectrophotometer (Hewlett Packard model 8453E). ^1H and ^{13}C NMR spectra were recorded at 400.1 and 100.6 MHz using a Bruker DRX-400MHz instrument fitted with a 5-mm ^1H/broadband gradient probe with inverse geometry. Heteronuclear multiple quantum coherence, heteronuclear multiple bond correlation and ^1H,^1H correlation spectroscopy experiments were from Bruker library. Chemical shifts are reported in δ values

(ppm) downfield from TMS. D_2O was used as the solvent containing *t*-butanol as internal standard. Positive ion electrospray (ESI +)-MS spectra were obtained in 1:1 v/v acetonitrile–H_2O/1% acetic acid using a MicromassZQ Waters equipment. Main peaks are reported with their relative intensities (percent values are in parentheses). Gel filtration was performed using Sephadex G-10 (eluant water). HPLC analyses were performed with a Gilson instrument equipped with an UV detector set at 280 nm. Analyses were run on octadecylsilane coated columns, 250×4.6 mm, 5 μm particle size (Sphereclone, Phenomenex) using 0.1 M formic acid–methanol (80:20 v/v) at a 1.0 mL/min flow rate. For preparative purposes a 250×22 mm, 10 μm particle size (Econosil, Alltech) was used at a flow rate of 15.0 mL/min (eluant: 0.1 M formic acid–methanol 70:30 v/v). Semiempirical AM1/PM3 calculations were carried out with the Hyperchem 5.0 package produced by Hypercube Inc. (Waterloo, Ontario, Canada) 1997.

Oxidation of chlorogenic acid (1) in the presence of GSH

To a solution of **1** (50 μM) and GSH (200 μM) in 0.05 M phosphate buffer (pH 7.4), HRP (1 pyrogallol U/mL) was added followed by H_2O_2 in two portions up to 100 μM concentration at 10-min intervals while the mixture was taken under vigorous stirring at room temperature. The reaction course was followed by HPLC analysis.

In other experiments the reaction was run: (i) as above but with GSH varying in the range of 0–3 molar equivalents with respect to **1**, adding H_2O_2 (100 μM final concentration) in two portions at 10 min intervals or as a bolus; (ii) as above with glucose (0.56 mM)/glucose oxidase (0.1 U/mL) in place of H_2O_2.

In those experiments using tyrosinase, the substrate at 50 μM or 2.0 mM concentration in 0.05 M phosphate buffer, pH 7.4, was treated with the enzyme, 25 or 100 U/mL, respectively, in the presence of GSH 200 μM or 10 mM, respectively, and the mixture was taken under vigorous stirring. The reaction course was followed by HPLC analysis as above.

Isolation of 2-*S*-glutathionylchlorogenic acid (3) and 2,5-di-*S*-glutathionylchlorogenic Acid (4). For preparative purposes, the reaction of **1** with HRP/H_2O_2 and GSH was carried out using 200 mg of the starting material. To a solution of **1** (2 mM) in 0.05 M phosphate buffer (pH 7.4), GSH (10 mM) was added followed by HRP (4 U/mL) and H_2O_2 in two portions up to 4 mM concentration at 10 min intervals while the mixture was taken under vigorous stirring at room temperature. After 1 h, the reaction mixture was treated with $Na_2S_2O_5$ (20 mg), acidified with 4 M HCl to pH 3.0 and evaporated to dryness. The residue was dissolved in water and purified by preparative HPLC to give **3** (t_R 25.7 min, 120 mg, 32% yield) and **4** (t_R 39.4 min, 98 mg, 18% yield) in pure form as pale yellow glassy oils. Either **3** or **4** at 50 μM concentration were reacted separately with tyrosinase (25 U/mL) or with HRP (1 U/mL) and H_2O_2 (100 μM) added in two portions in the presence of GSH (200 μM), and the reaction course was followed by HPLC.

2-*S*-Glutathionylchlorogenic acid (3). UV: λ_{max} (water) 251, 327 nm; ^{1}H NMR (400 MHz, D_2O) δ (ppm): 2.05–2.16 (5H, m), 2.23 (1H, m), 2.41 (2H, t, J = 6.8 Hz), 3.05 (1H, dd, J = 14.4, 8.8 Hz), 3.22 (1H, dd, J = 14.4, 3.6 Hz), 3.68 (2H, s), 3.73 (1H, t, J = 6.4 Hz), 3.90 (1H, m), 4.22 (1H, dd, J = 8.8, 3.6 Hz), 4.27 (1H, m), 5.31 (1H, m), 6.27 (1H, d, J = 16.0 Hz), 6.88 (1H, d, J = 8.4 Hz), 7.12 (1H, d, J = 8.4 Hz), 8.10 (1H, d, J = 16.0 Hz); ^{13}C NMR (100 MHz, D_2O) δ (ppm): 27.3 (CH_2), 32.6 (CH_2), 36.7 (CH_2), 38.4 (CH_2), 39.3 (CH_2), 43.8 (CH_2), 54.8 (CH), 55.3 (CH), 71.6 (CH), 72.4 (CH), 73.8 (CH), 77.5 (C), 117.8 (CH), 118.2 (CH), 121.2 (C), 121.2 (CH), 130.9 (C), 145.2 (CH), 147.4 (C), 147.7 (C), 170.0 (C), 173.0 (C), 175.1(C), 175.8 (C), 176.1 (C), 180.8 (C); ESI + -MS: m/z 660 ($[M+H]^+$, 100), 682 ($[M+Na]^+$, 17), 698 ($[M+K]^+$, 9).

2,5-Di-*S*-glutathionylchlorogenic acid (4). UV: λ_{max} (water) 268, 379 nm; ^{1}H NMR (400 MHz, D_2O) δ (ppm): 2.04–2.11 (7H, m), 2.26 (1H, m), 2.44 (4H, m), 3.06 (1H, dd, J = 14.4, 8.8 Hz), 3.23 (1H, dd, J = 14.4, 8.4 Hz), 3.27 (1H, dd, J = 14.4, 3.6 Hz), 3.41 (1H, dd, J = 14.4, 4.4 Hz), 3.71–3.75 (6H, m), 3.92 (1H, dd, J = 9.2, 2.8 Hz), 4.20 (1H, dd, J = 8.8, 3.6 Hz), 4.26 (1H, d, J = 2.8 Hz), 4.46 (1H, dd, J = 8.4, 4.4 Hz), 5.33 (1H, m), 6.42 (1H, d, J = 16.0 Hz), 7.38 (1H, s), 8.17 (1H, d, J = 16.0 Hz); ^{13}C NMR (100 MHz, D_2O) δ (ppm): 27.2 (2×CH_2), 32.6 (2×CH_2), 35.8 (CH_2), 37.1 (CH_2), 38.3 (CH_2), 39.4 (CH_2), 43.8 (2×CH_2), 54.2 (CH), 54.5 (CH), 55.3 (2×CH), 71.5 (CH), 72.5 (CH), 73.6 (CH), 77.4 (C), 118.6 (CH), 121.2 (C), 122.9 (C), 124.1 (CH), 130.8 (C), 144.2 (CH), 147.5 (2×C), 169.6 (C), 173.0 (2×C), 175.2 (2×C), 175.8 (2×C), 176.2 (2×C), 180.9 (C); ESI + -MS: m/z 483 ($[M+2H]^{2+}$, 55), 965 ($[M+H]^+$, 47), 987 ($[M+Na]^+$, 24).

Isolation of 2,5,6-tri-*S*-glutathionylchlorogenic acid (5). The reaction of **1** with HRP/H_2O_2 and GSH was carried out as for the preparation of **3** and **4** using 50 mg of the starting material and adding four portions of H_2O_2 (1 molar equivalent each) at 50-min intervals. At complete consumption of **1**, **3** and **4** (HPLC analysis), the reaction mixture was worked up as above. The residue was dissolved in water and purified by Sephadex G-10 chromatography to give **5** (t_R 10.1 min, 161 mg, 90% yield) as a pale yellow glassy oil.

2,5,6-Tri-*S*-glutathionylchlorogenic acid (5). UV: λ_{max} (water) 279 nm; ^{1}H NMR (400 MHz, D_2O) δ (ppm): 2.08–2.23 (10H, m), 2.39–2.61 (6H, m), 3.00 (1H, dd, J = 14.4, 9.6 Hz), 3.09 (1H, dd, J = 14.0, 9.2 Hz), 3.22 (1H, m), 3.28 (1H, m) 3.29 (1H, dd, J = 14.4, 4.4 Hz), 3.46 (1H, dd, J = 13.6, 4.8 Hz), 3.80 (3H, m), 3.82 (6H, s), 3.91 (1H, m), 4.17 (1H, dd, J = 9.2, 4.8 Hz), 4.24 (1H, dd, J = 8.4, 4.8 Hz), 4.29 (1H, m), 4.74 (1H, m), 5.37 (1H, m), 6.38 (1H, d, J = 16.0 Hz), 8.00 (1H, d, J = 16.0 Hz); ^{13}C NMR (100 MHz, D_2O) δ (ppm): 27.3 (3×CH_2), 32.6 (3×CH_2), 36.8 (CH_2), 37.4 (CH_2), 38.3 (CH_2), 39.1 (CH_2), 39.5 (CH_2), 44.1 (3×CH_2), 54.0

(CH), 54.1 (CH), 54.4 (CH), 55.3 (3×CH), 71.6 (CH), 72.6 (CH), 73.7 (CH), 77.6 (C), 120.9 (C), 126.4 (CH), 127.1 (C), 129.9 (C), 138.1 (C), 144.5 (CH), 148.3 (C), 148.5 (C), 169.1 (C), 173.0 (C), 173.2 (C), 175.6(C), 176.1 (3×C), 176.3 (3×C), 176.5 (3×C), 181.1 (C); ESI +-MS: m/z 635 ($[M+2H]^{2+}$, 78), 1270 ($[M+H]^{+}$, 4).

Reaction of 1 with GSH and nitrite. A solution of **1** (200 mM) in methanol, was added to 0.05 M phosphate buffer (pH 4.0) up to a final concentration of 2 mM, followed by GSH (10 mM) and sodium nitrite (8 mM). The mixture was taken under vigorous stirring at room temperature and periodically analyzed by HPLC.

In other experiments the reaction of **1** was run: (i) as above, but in the presence of GSNO (5 molar equivalents) without addition of GSH and nitrite; (ii) using GSSG in place of GSH; (iii) using GSH and nitrite varying in the range 0.1–5 and 1–5 molar equivalents, respectively, with the substrate at 1.0 mM concentration; (iv) as in the general procedure but using 0.05 M acetate buffer (pH 3.0) as the reaction medium taken at 37 °C, with the substrate at 25 or 500 μM in the presence of GSH and nitrite varying in the range 0.05–1 molar equivalents. In other experiments, GSH was reacted with sodium nitrite under the conditions of the general procedure but in the absence of **1**.

Generation and identification of 1 quinone

A solution of **1** (20 mM) in methanol was added to 0.05 M acetate buffer (pH 4) up to a 2 mM concentration, followed by GSH (8 mM) and sodium nitrite (1 mM). The spectrum of the mixture was recorded within 2 s after addition of nitrite and then periodically over 10-min by a diode array spectrophotometer. Product analysis was carried by HPLC. In other experiments the reaction was run: (i) as above but with addition of $NaBH_4$ (2 mM) at 10 min reaction time; (ii) with GSH (8 mM) or $NaBH_4$ (2 mM) added 10 min after addition of sodium nitrite; 5 min after addition of GSH, the mixture was analyzed by HPLC. In control experiments the absorbance at 400 nm of a solution of sodium nitrite (1 mM) or of **3** (2 mM) in the presence of equimolar amounts of $NaBH_4$ at pH 4 was measured. In other experiments, oxidation of **1** by sodium periodate in 0.05M phosphate buffer (pH 6.0 or 4.0) was carried out as described,[23] with the substrate and the oxidant at 250 μM concentration, and the spectrum of the mixture was recorded at the addition of the oxidant. When required, $NaBH_4$ (250 μM) or GSH (250 μM) was added to the mixture 30 s after addition of the oxidant; 15 s after addition of GSH, the reaction mixture was acidified to pH 3.0 with 4 M HCl and analyzed by HPLC.

Acknowledgements

This work was carried out in the frame of a research program on antitumour agents sponsored by MURST (PRIN 2001). We thank the 'Centro Interdipartimentale di Metodologie Chimico-Fisiche' of Naples University 'Federico II' for NMR facilities and Prof. M. M. Corsaro (Naples University) for mass spectra and helpful discussions. Thanks are also due to Miss Silvana Corsani for excellent technical assistance.

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