ORGANIC LETTERS

2002
Vol. 4, No. 7
1209−1212

Glutathionyl Transferase Catalyzed Addition of Glutathione to COMC: A New Hypothesis for Antitumor Activity

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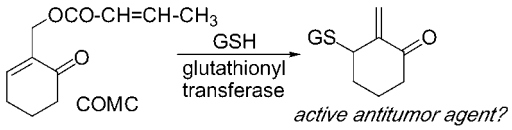
Received January 31, 2002

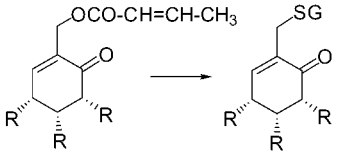
ABSTRACT



Data are presented indicating that the potent antitumor activity of 2-crotonyloxymethyl-(4*R*,5*R*,6*R*)-4,5,6-trihydroxy-2-cyclohexenone (COTC) and 2-crotonyloxymethyl-2-cyclohexenone (COMC) is not likely the result of glyoxalase I inhibition, as has long been assumed. An alternative hypothesis is presented, based on the finding that COMC is a substrate for human glutathionyl transferase, which produces a transient, highly electrophilic glutathionylated 2-exomethylenecyclohexanone that can covalently modify proteins and nucleic acids.

The *Streptomyces* metabolite 2-crotonyloxymethyl-(4*R*,5*R*,6*R*)-4,5,6-trihydroxy-2-cyclohexenone (COTC, **1a,** Scheme 1)[1,2]

Scheme 1



1a R = OH
1b R = H

2a R = OH
2b R = H

and its simpler synthetic analogue 2-crotonyloxymethyl-2-cyclohexenone (COMC, **1b**)[3] both exhibit potent antitumor activity against murine and human tumors in culture. Compound **1a** is somewhat more active (IC_{50} = 0.5−19 μM) than **1b** (IC_{50} = 3−44 μM).[3] In 1975, Takeuchi et al. attributed the antitumor activity of **1a** to its putative glutathione (GSH) adduct **2a**, which was proposed to inhibit the enzyme glyoxalase I (GlxI).[1]

Glyoxalase I plays a pivotal role in detoxifying intracellular methylglyoxal, which is formed during normal carbohydrate metabolism.[4] Indeed, certain inhibitors of human GlxI, the most potent of which are enediol transition state analogue inhibitors, have been shown to retard the growth of both murine and human tumors in culture and in tumor-bearing mice by causing the accumulation of intracellular methylglyoxal.[5,6]

While a priori quite plausible, the "prodrug" hypothesis of Takeuchi et al. for the antitumor action of COTC and

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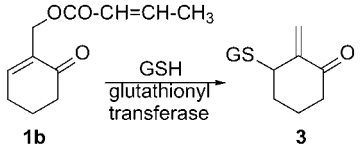
10.1021/ol025650h CCC: $22.00
Published on Web 03/03/2002

COMC has never been tested. Recently, an authentic sample of **2a** was prepared by independent synthesis and shown to be a modest inhibitor of GlxI.[7]

We have now prepared and tested **2b** against human erythrocyte GlxI and report that, like **2a**, it is several orders of magnitude weaker than other, mechanism-based, GlxI inhibitors that display potent antitumor activity.[5,6] To establish an alternative mode of tumor toxicity for COTC and COMC, we have developed a short and efficient synthesis of **1b** and have now demonstrated that it is a substrate for glutathionyl transferase, an enzyme that is widely distributed in mammalian tissue.

Here we show that glutathionyl transferase catalyzes a conjugate addition of GSH to **1b** with concomitant elimination of crotonic acid leading to 3-glutathionyl-2-exomethylenecyclohexanone **3** (Scheme 2), a reactive intermediate that

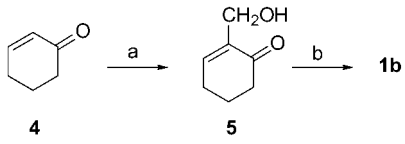
Scheme 2



has now been isolated and characterized for the first time. Trapping studies with amino acids and nucleotides support the hypothesis that **3** can react with and covalently modify reactive groups on proteins and DNA that may be critical to cell viability.

Scheme 3 depicts a simple and efficient synthesis of **1b** that is superior to the earlier reported seven-step procedure.[3] Baylis−Hillman reaction of 2-cyclohexenone **4** with formaldehyde afforded 2-hydroxymethyl-2-cyclohexenone **5**,[8] which was then crotonylated to **1b** following the literature procedure.[3]

Scheme 3



(a) DMAP, CH_2O, THF, rt 65%; (b) crotonic anhydride, pyridine, DMAP, rt 92%.

The GSH conjugate **2b** was prepared from **1b** by adapting the procedure for making **2a** from **1a**.[7] The NMR spectrum of **2b** featured the expected glutathionyl resonances and the downfield resonance (δ 7.12, triplet) characteristic of H3 in β,γ-unsubstituted 2-cyclohexenones.

Kinetic studies with human erythrocyte GlxI (sodium phosphate buffer, pH 7.0, 25 °C) indicated that **2b** was a competitive inhibitor of the enzyme, with a dissociation constant $K_i = 107 \pm 1$ μM. We have previously reported that **2a** competitively inhibited human erythrocyte GlxI, with a dissociation constant $K_i = 183 \pm 6$ μM.[7]

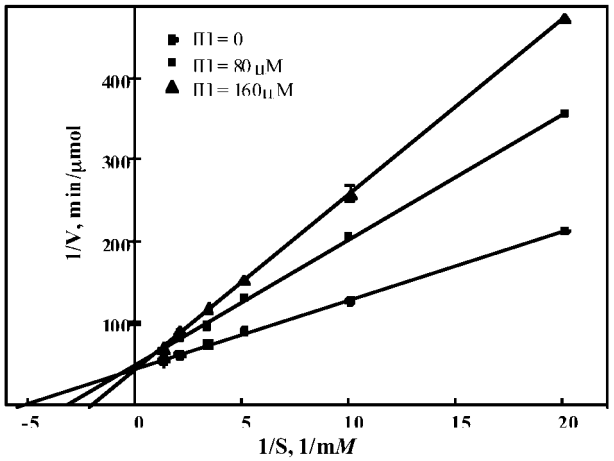


Figure 1. Reciprocal plot of the velocity of the glyoxalase I reaction (ΔOD_{240}) versus the concentration of GSH-methylglyoxal-thiohemiacetal (S) in the absence and presence of different concentrations of **2b**. In each kinetic run, the concentration of free GSH was maintained at 0.2 mM by varying the total concentration of GSH and methylglyoxal on the basis of the dissociation constant of the hemithioacetal ($K_{diss} = 2.2$ mM).[9] Conditions: 50 mM phosphate buffer, pH 7.0, 25 °C.

To investigate the mechanism of formation of **2b**, the nonenzymatic reaction of **1b** with GSH was monitored spectrophotometrically and followed a simple first-order decay (Figure 2, trace A). No intermediate species was detectable. However, in the presence of human placental glutathione transferase (GSTP1-1)[10] the reaction rate profile

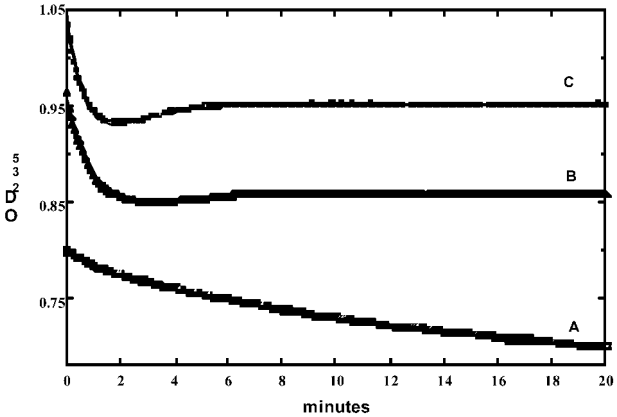
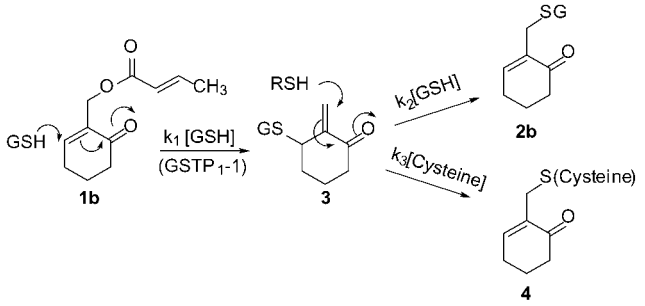


Figure 2. Spectrophotometrically determined rates of reaction of GSH (1.03 mM) with **1b** (0.05 mM) (A) in the absence of GSTP1-1 ($k = 0.070 \pm 0.0002$ min^{-1}); (B) in the presence of 1.8 units of GSTP1-1 ($k_1 = 0.882 \pm 0.055$ min^{-1}; $k_2 = 0.633 \pm 0.053$ min^{-1}); and (C) in the presence of 2.4 units of GSTP1-1 ($k_1 = 1.33 \pm 0.037$ min^{-1}; $k_2 = 0.685 \pm 0.024$ min^{-1}). Conditions: 100 mM phosphate buffer, 0.05 mM EDTA, pH 6.5, 25 °C.

conformed to a double exponential decay, composed of a rapid, enzyme-dependent, initial phase involving **1b** followed by a slower enzyme-independent first-order phase (Figure 2, traces B and C).

This finding was consistent with the mechanism shown in Scheme 4, whereby an initial, enzyme-catalyzed Michael addition of GSH to **1b** afforded the exocyclic enone **3**. Once dissociated from the enzyme, free **3** reacted with GSH nonenzymatically to form **2b**.

Scheme 4



When **1b** (0.1 mM) was incubated with cysteine (0.5 mM) and GSH (0.5 mM) in the presence of GSTP1-1 (1.5 units) for 30 min and the reaction mixture was fractionated by reverse-phase HPLC, thiol adducts **2b** and **4** were isolated. On the basis of the integrated intensities of the well-resolved peaks corresponding to **4** (~9.5 min) and **2b** (~11.5 min), the product ratio in the presence of GSTP1-1 was identical to that obtained from a nonenzymatic incubation of **1b** with the same concentrations of cysteine and GSH (Figure 3).

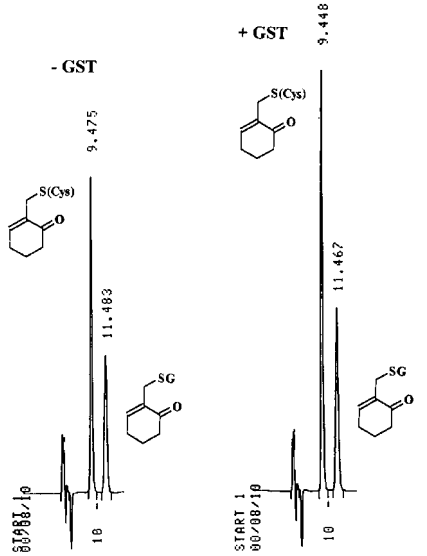


Figure 3. Fractionation of product mixtures in the absence and presence of GSTP1-1 by reverse-phase HPLC (Waters, μ-Bondapak C_{18}, 0.78 × 30 cm).

That finding indicated that **3** dissociated from the enzyme prior to reacting with the free thiols in solution and was consistent with the mechanism in Scheme 4. Adduct **2b** (0.5 mM) was stable under the reaction conditions, undergoing less than 1% conversion to **4** in the presence of cysteine (2.5 mM) over 27 h, as determined by HPLC.

Initial rates of reaction of **1a** and **1b** with GSTP1-1 were determined from reciprocal plots of initial velocities (ΔOD_{235}/min), versus [substrate] in buffered solution at pH 6.5, with [GSH] = 1 mM (25 °C). Under those conditions, the enzyme-catalyzed GSH addition became rate determining (<0.01 units of transferase in the assay cuvettes). For **1b,** k_{cat} = 1.2 ± 0.2 s^{-1} and K_m = 52 ± 10 μM. For **1a**, the individual kinetic constants could not be accurately determined, although k_{cat}/K_m was estimated to be 8.3-fold lower than that of **1b**.

Brief incubation of a mixture of **1b**, GSH, and GSTP1-1 gave rise to a transient intermediate that could be isolated by reverse-phase HPLC, with a retention time close to that of synthetic **2b**. The kinetic properties of this species suggest that it is an obligatory intermediate associated with the enzymatic conversion of **1b** to **2b**. When this species is combined with cysteine in buffered solution (pH 7), a new species is produced that comigrates with authentic **4**. Moreover, incubation of this intermediate species with excess GSH (1.04 mM) in potassium phosphate buffer (0.1 M, pH 6.5) at 25 °C results in a first order *increase* in OD_{235} with a rate constant of 0.87 ± 0.08 min^{-1} similar in magnitude to that associated with the second phase of the transferase-catalyzed conversion of **1b** to **2b**, Figure 2. The absorptivity of the intermediate (ϵ_{235} = 4300 cm^{-1} M^{-1}) is significantly less than that of the final product **2b** (ϵ_{235} = 7500 cm^{-1} M^{-1}), which accounts for the overall shape of the reaction-rate profile in the presence of transferase (Figure 2).

The 600-MHz ^{1}NMR spectrum of the intermediate was consistent with the structure of **3** (Figure 4). The vinyl proton resonances at 5.76 and 5.29 ppm were characteristic of

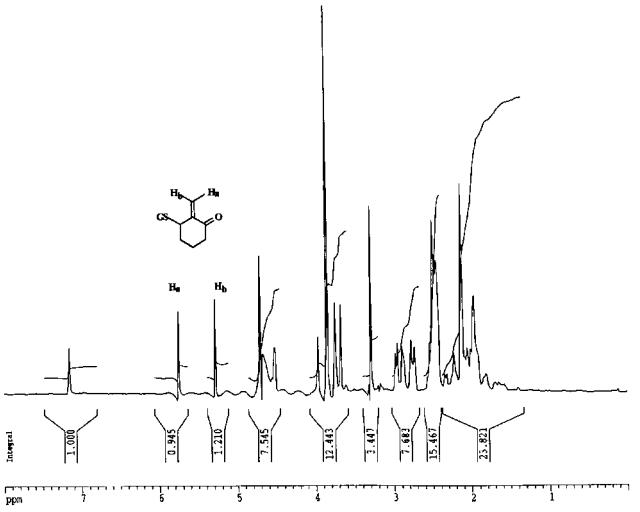


Figure 4. The 600-MHz ^{1}H NMR spectrum of intermediate **3**.

geminal vinylic hydrogens and consistent with published NMR spectra of several closely related 2-methylenecyclohexanones.[11] Other resonances in the spectrum corresponded to those expected for the tripeptide moiety.[12] The resonance at δ 7.12 indicated the presence of **2b**, which was formed in the transferase-independent addition of GSH to **3**.

Comparative data analysis suggested that the potent antitumor activities of COTC (**1a**) and COMC (**1b**) could not be rationalized by the biological activity of **2a** and **2b**, respectively, which are weak competitive inhibitors of human erythrocyte GlxI. Earlier in vitro studies on enediol analogue inhibitors of GlxI that inhibited the growth of L1210 and B16 melanotic melanoma as ester prodrugs revealed K_i values against GlxI in the submicromolar range.[6] In fact, a direct correlation was observed between the K_i values of the prodrugs against GlxI and their corresponding antitumor activities, as measured by IC_{50} values. For example, the weakest GlxI inhibitor in that study ($K_i = 0.16\ \mu M$) displayed an IC_{50} value $>100\ \mu M$. On the basis of that correlation, the potent antitumor activity of **1b** ($IC_{50} = 0.5-19\ \mu M$) is inconsistent with the hypothesis of Takeuchi et al. attributing antitumor activity to the action of **2b** as GlxI inhibitor ($K_i = 107\ \mu M$).[1]

Besides providing insight into the mechanism of addition of GSH to COMC, the formation of **3** enzymatically from **1b** also offers an attractive alternative biological mechanism for the tumoricidal activity of both COTC and COMC. It may now be hypothesized that **1a** and **1b** are enzyme-activated prodrugs in which the crotonate ester serves as a leaving group, in a process triggered by glutathionyl transferase. Methylenecyclohexanones such as **3** are known to be highly reactive Michael acceptors[11] and can function as carcinostatic agents by reacting with proteins and nucleic acids critical to cell function. Besides the thiol adducts documented here, covalent adducts between **1b** and model polynucleic acids in the presence of GSTP1-1 have been detected by mass spectrometry.[13]

Acknowledgment. This work was supported in part by grants from the NIH (GM 24054 to B.G.; CA 59612 to D.J.C.) and the U.S. Army Medical Research and Material Command (to D.J.C.). Support of the Cornell NMR Facility has been provided by NSF and NIH.

Note Added after Print Publication: Due to a production error, certain μM concentrations appeared as mM concentrations in the version published on the Web 03/03/2002 (ASAP) and in the April 4, 2002 issue (Vol. 4, No. 7, pp 1209−1212); the correct electronic version of the paper was published on 04/23/2002 and an Addition and Correction appears in the May 16, 2002 issue (Vol. 4, No. 10).

Supporting Information Available: Experimental procedures and ^1H and ^{13}C NMR data for the synthesis of **1b**. This material is available free of charge via the Internet at http://pubs.acs.org.

OL025650H

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