ORGANIC LETTERS

2002
Vol. 4, No. 9
1459−1462

Alkylation of Nucleic Acids by the Antitumor Agent COMC

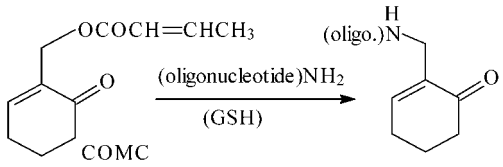
Qingrong Zhang,[†] Zhebo Ding,[‡] Donald J. Creighton,[†] Bruce Ganem,[‡] and Daniele Fabris*,[†]

Department of Chemistry and Biochemistry, University of Maryland, Baltimore County, Baltimore Maryland 21250, and Department of Chemistry and Chemical Biology, Baker Laboratory, Cornell University, Ithaca, New York 14853-1301

fabris@umbc.edu

Received January 24, 2002

ABSTRACT



Mass spectral data are presented indicating that the antitumor agent 2-crotonyloxymethyl-2-cyclohexenone (COMC) is capable of alkylating oligonucleotides via a mechanism involving an electrophilic exocyclic enone intermediate. Under physiological conditions, the exocyclic enone is likely the glutathionylated 2-exomethylenecyclohexenone. This supports a recent hypothesis that the antitumor activity of COMC arises from alkylation of nucleic acids and/or proteins critical to cell function and not from competitive inhibition of glyoxalase I by an adduct of COMC and glutathione.

2-Crotonyloxymethyl-2-cyclohexenone (COMC, **1**) is a synthetic analogue of the *Streptomyces* metabolite 2-crotonyloxymethyl-(4*R*,5*R*,6*R*)-4,5,6-trihydroxy-2-cyclohexenone (COTC).[1,2] Both compounds exhibit potent antitumor activity *in vitro* and *in vivo*.[3] Early investigators proposed that antitumor activity might arise from competitive inhibition of the methylglyoxal detoxifying enzyme glyoxalase I by the covalent adducts resulting from displacement of crotonate by intracellular glutathione (GSH).[1]

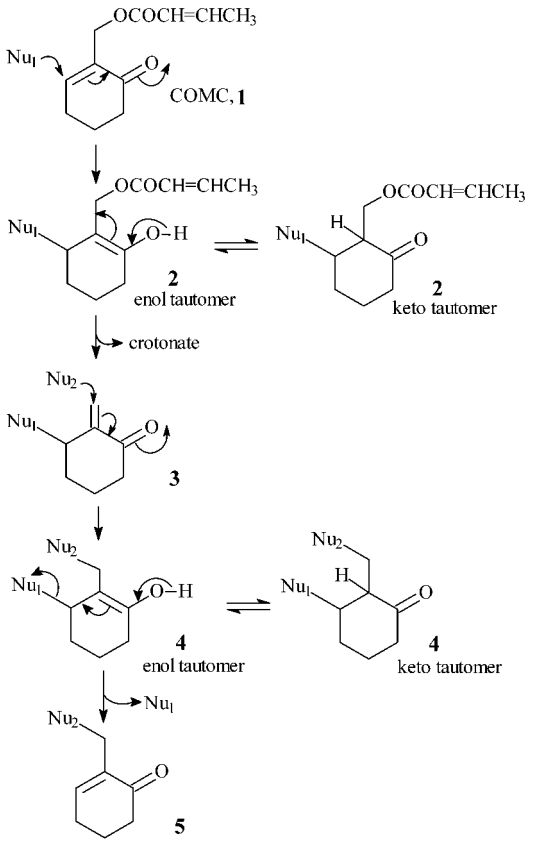
The recent development of simple, high-yield synthetic routes to the GSH adducts of COMC/COTC allowed a careful quantitative assessment of their ability to inhibit human glyoxalase I.[4,5] Surprisingly, the adducts proved to be relatively poor inhibitors of human glyoxalase I, casting serious doubts on the involvement of the enzyme in the antitumor activity of COTC/COMC. Kinetic measurements and intermediate trapping experiments also indicated that the mechanism of formation of the GSH adducts involves a multistep mechanism (Scheme 1) in which COMC (**1**) undergoes an initial Michael addition by 1 equiv of GSH to give a highly reactive exocyclic enone (**3**), a process that is catalyzed by human GSH transferase (hGST).[4] The intermediate then reacts with a second equivalent of GSH to give the final product (**5**). This chemistry might also account for the cytotoxicity of COMC, as the exocyclic enone is likely to react with nucleophilic groups on nucleic acids and proteins inside cells. Indeed, DNA alkylation, in particular, provides a plausible mechanism of cytotoxic activity, which is shared by a large number of chemical carcinogens and cross-linking antitumor agents.[6,7] Here we present direct evidence in support of this chemical mechanism in the case

[†] University of Maryland, Baltimore County.
[‡] Cornell University.

(1) Takeuchi, T.; Chimura, H.; Hamada, M.; Umezawa, H.; Yoshka, H.; Oguchi, N.; Takahashi, Y.; Matsuda, A. A. *J. Antibiot.* **1975**, *28*, 737−742.
(2) Chimura, H.; Nakamura, H.; Takita, T.; Takeuchi, T.; Umezawa, M.; Kato, K.; Saito, S.; Tomisawa, T.; Iitaka, Y. *J. Antibiot.* **1975**, *28*, 743−748.
(3) Aghil, O.; Bibby, M. C.; Carrington, S. J.; Double, J.; Douglas, K. T.; Phillips, R. M.; Shing, T. K. M. *Anti-Cancer Drug Des.* **1992**, *7*, 67.
(4) Huntley, C. F.; Hamilton, D. S.; Creighton, D. J.; Ganem, B. *Org. Lett.* **2000**, *2*, 3143−3144.
(5) Hamilton, D. S.; Ding, Z.; Ganem, B.; Creighton, D. J. *Org. Lett.*, in press.
(6) Rajski, S. R.; Williams, R. M. *Chem. Rev.* **1998**, *98*, 2723−2795.
(7) *Chemical Carcinogens and DNA*; Searle, C. E., Ed.; Monograph 173, American Chemical Society: Washington, DC, 1979.

10.1021/ol025612y CCC: $22.00
Published on Web 04/04/2002

Scheme 1



of COMC. The formation *in vitro* of a reactive glutathionyl exocyclic enone intermediate and its ability to produce adducts with nucleic acids has been confirmed by direct mass spectrometric analysis of the reaction mixtures.

Direct infusion electrospray ionization with an ion cyclotron resonance (ICR) analyzer[8,9] was employed to identify the products of the *in vitro* reaction of COMC with GSH and with dinucleotides and oligonucleotides.[10] Generally, reaction mixtures were made up in 10 mM ammonium acetate buffer (pH 7.0) containing 3.3 mM COMC, 1.3 mM GSH, and 1.3 mM dinucleotide or oligonucleotide. Reaction mixtures were incubated at room temperature for approximately 1 h, quenched by the addition of one volume of methanol, and stored at −20 °C.

For a reaction mixture containing COMC and GSH, protonated ions corresponding to the species shown in Scheme 1 (Nu_1 = Nu_2 = GSH) were detected in positive ion mode (Figure 1). The same spectrum was obtained in the presence of 1.2 units of hGST.[11] For each signal, the experimental mass over charge (*m*/*z*) value was in excellent agreement with the calculated monoisotopic mass (Table 1).

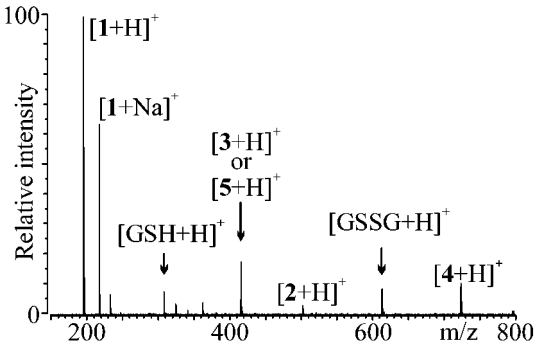


Figure 1. Positive ion mode ESI FT-MS spectrum of the product mixture initially composed of COMC and GSH in the presence of 1.2 units human glutathione-S-transferase, under the conditions given in the text.

Table 1. Summary of the Molecular Mass Determination of Reaction Products of COMC with GSH and Guanosylyl (3′−5′)Adenosine (GA)

product	elemental composition (neutral)	expected monoisotopic mass	observed monoisotopic mass[a]
COMC, **1**	$C_{11}H_{14}O_3$	195.1021[b]	195.1007[b]
GSH	$C_{10}H_{17}N_3O_6S$	308.0916[b]	308.0913[b]
species **3**,**5**	$C_{17}H_{25}N_3O_7S$	416.1491[b]	416.1486[b]
species **2**	$C_{21}H_{31}N_3O_9S$	502.1859[b]	502.1856[b]
GA	$C_{20}H_{25}N_{10}O_{11}P$	611.1364[c]	611.1140[c]
GSSG	$C_{20}H_{32}N_6O_{12}S_2$	613.1598[b]	613.1593[b]
GA+hex[d]	$C_{27}H_{33}N_{10}O_{12}P$	719.1939[c]	719.1888[c]
species **4**	$C_{27}H_{42}N_6O_{13}S_2$	723.2330[b]	723.2343[b]
GA+COMC	$C_{31}H_{39}N_{10}O_{14}P$	807.2463[b]	807.2472[b]
GA+GSH+hex[d]	$C_{37}H_{50}N_{13}O_{18}PS$	1026.278[c]	1026.240[c]
2GA+hex[d]	$C_{47}H_{58}N_{20}O_{23}P_2$	1331.338[c]	1331.258[c]

[a] The average mass accuracy was determined to be 12 ppm. [b] Protonated ($[M + H]^+$) species observed in positive ion mode. [c] Deprotonated ($[M - H]^-$) species observed in negative ion mode. [d] 2-Methylene-2-cyclohexenone.

Ions were detected for starting reagents COMC and GSH, together with oxidized glutathione (GSSG), which was also present in the initial stock solution of GSH. Adduct **2**, either as the enol- or keto-tautomer, gave a weak but recognizable signal. The 1 h reaction time is sufficient for the reaction to go nearly to completion but is probably short enough to allow for the detection of some residual keto-tautomer arising from

(8) Comisarow, M. B.; Marshall, A. G. *Chem. Phys. Lett.* **1974a**, *25*, 282−283.

(9) Marshall, A. G.; Hendrickson, C. L.; Jackson, G. S. *Mass Spectrom. Rev.* **1998**, *17*, 1−35.

(10) Mass spectrometric analyses were performed on a Bruker Daltonics (Billerica, MA) Apex III Fourier Transform mass spectrometer (FT-MS) equipped with a 7T actively shielded superconductive magnet and Apollo electrospray ionization (ESI) source. Positive ion determinations were obtained in water, methanol, glacial acetic acid (49:49:2); negative ion determinations in methanol and 10 mM ammonium acetate (1:1). Solutions were continuously infused through a syringe pump at a flow rate of 2μL/min. Tandem experiments were performed by sustained off-resonance irradiation (SORI) of selected precursor ions. Intensity and duration of off-resonance irradiation, type and pressure of collision gas (Ar), and duration of the collisional step were kept constant.

(11) hGST was purchased from Sigma Chem. Co. and is composed primarily of the GSTP1-1 isozyme.

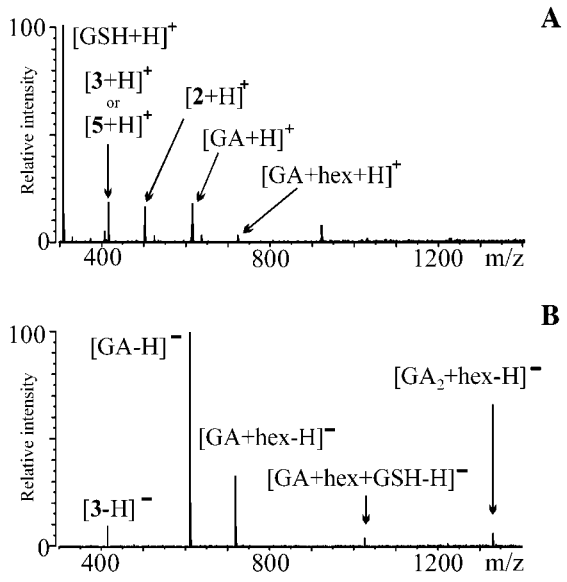


Figure 2. (A) Positive ion and (B) negative ion mode ESI FT-MS spectra of the product mixture initially composed of COMC, GA, and GSH. The abbreviation "hex" indicates the alkylating 2-methylene-2-cyclohexenone groups.

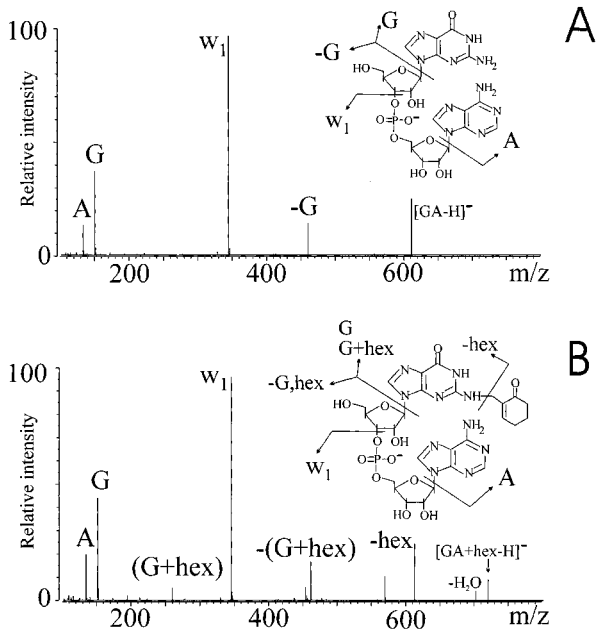


Figure 3. Collision-induced dissociation spectra of (A) deprotonated GA and (B) deprotonated COMC-GA adduct. To facilitate the interpretation, the putative structure with the proposed fragmentation pattern is included. The abbreviation "hex" indicates the 2-methylene-2-cyclohexenone group.

the unstable enol species formed along the reaction pathway. Similarly, the GSH bis-adduct **4** is most likely detected as the more stable keto-tautomer.

The isobaric (same mass) species **3** and **5**, having the same elemental composition, could not be distinguished on the basis of an accurate mass determination. In an analogous way, tandem mass spectrometry proved unsuccessful, as the ion corresponding to the **3**/**5** species gave a fragmentation pattern in reasonable agreement with either structural isomer. Nevertheless, the detection of ions corresponding to both **2** and **4** strongly argues for the Michael addition mechanism shown in Scheme 1. Therefore, a simple S_N2 mechanism seems inconsistent with the mass spectral data.

To assess the reactivity of 2-cyclohexenone derivatives with nucleic acids, COMC and GSH were incubated in the presence of ribo-dinucleotides[12] of different base composition. The products of the reaction with guanosylyl(3′-5′)-adenosine (GA) are shown in Figure 2 and summarized in Table 1. As expected, species **2**, **3**/**5**, and **4** (with Nu_1 = Nu_2 = GSH) are readily recognizable in the reaction mixture analyzed in positive ion mode (Figure 2A). However, only weak signals could be detected for protonated GA and GA-adducts. A more favorable signal-to-noise ratio was obtained in negative ion mode (Figure 2B), due to the negatively charged phosphate of the nucleotide function. The switch of polarity allowed the detection of abundant GA-COMC adducts (with Nu_1 = Nu_2 = GA) and bis-adducts, with and without GSH (**4**, $Nu_{1,2}$ = GSH and including either Nu_1 = Nu_2 = GA, or different permutations of $Nu_{2,1}$ = GA, respectively).

It is important to note that GSH is not a strict requirement for adduct formation with the dinucleotides, as the bis-adduct $[GA_2 + hex\text{-}H]^-$ is present in the spectrum shown in Figure 2B. Indeed, when the reaction is repeated in the absence of GSH, both the mono- and bis-adducts of the dinucleotides are observed (data not shown).

To identify the functional group(s) involved in the alkylation process, the dinucleotides AA, GG, CC, UU, and TT (as 2′-deoxy-ribonucleotides) were tested with COMC. All substrates produced adducts with the exception of UU and TT. Thus, the likely sites of alkylation are the exocyclic amino groups of the nitrogenous bases, which are known to be favorable targets of electrophilic attack by genotoxic carcinogens.[13]

As one of the leading techniques employed to identify nucleic acids modifications and to localize the sequence position of covalently modified bases,[14,15] tandem mass spectrometry was applied here to characterize the COMC-GA conjugate. Initially, collision-induced dissociation was performed on GA, which showed the normal fragmentation pattern provided by nucleic acids (Figure 3A). Typically, the nitrogenous base at the 5′-end is lost through facile cleavage of the N-glycosidic bond between C-1′ of the pentose and N-9 of the base.[16−18] Subsequently, the bond

(12) Di- and oligonucleotides were purchased from the Keck DNA Synthesis Facility of Yale University and used without further purification.

(13) Phillips, D. H. In *The Molecular Basis of Cancer*; Farmer, P. B., Walker, J. M., Eds.; Wiley-Interscience: London, UK, 1985; pp 133−179.

(14) Barry, J. P.; Vouros, P.; Van Schepdael, A.; Law, S.-J. *J. Mass Spectrom.* **1995**, *30*, 993−1006.

(15) Beck, J. L.; Colgrave, M. L.; Ralph, S. F.; Sheil, M. M *Mass Spectrom. Rev.* **2001**, *20*, 61−87.

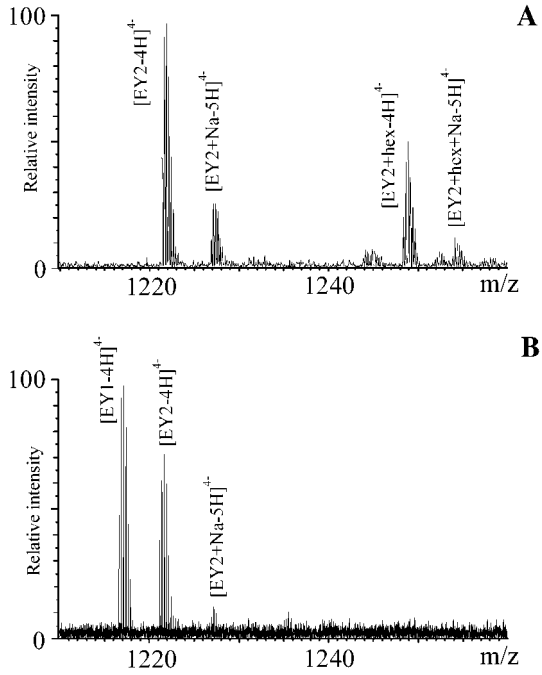


Figure 4. Negative ion mode ESI FT-MS spectra of the reaction of COMC with (A) the 16mer oligonucleotide EY2 (ACG GAC CGG CGT ACG C) and (B) a duplex formed after annealing with its complementary EY1 (GCG TAC GCC GGT CCG T). Only the region including the −4 charge state is shown for better comparison.

between C-3′ and O-3′ of the abasic pentose is cleaved, causing the scission of the phosphodiester backbone and producing the sequence ions employed for gas-phase sequencing of nucleic acids.[16−18]

In the case of GA, the initial fragmentation event resulted in the formation of the species indicated as G and its complementary ion −G (depending on which moiety retained the charge when scission was complete), and then the sequence ion $\mathbf{w_1}$ was formed by loss of the abasic pentose. The loss of adenine base was also observed, although with lower abundance than the loss of guanine, but the cleavage of the C−O bond at the 3′ end was not.

The fragmentation pattern provided by the COMC-GA adduct followed the same pattern, but the mass of characteristic fragments was shifted by the addition of COMC, helping to locate the 2-methylene-2-hexenone group on the guanine base (Figure 3B). In particular, guanine base was lost as alkylated guanine and detected as (G+hex) or as the complementary ion −(G+hex). The loss of nonalkylated base G was also observed and could be explained by consecutive cleavage reactions including the loss of COMC. To the contrary, adenine was only lost as unmodified base A. Moreover, the sequence ion $\mathbf{w_1}$ (encompassing the 3′-adenylyl moiety) afforded the same mass observed for COMC-free GA, thus excluding the possibility of alkylation on the 3′-nucleotide.

The specificity of COMC was also evaluated with oligonucleotides of different length and base composition, either in their single stranded form or annealed with their complementary oligonucleotide to form a duplex. For example, adduct formation was readily observed when the single stranded DNA 16mer EY2 (ACG GAC CGG CGT ACG C) was treated with COMC (Figure 4A). The molecular mass was determined to be 4889.815 Da (4889.850 Da calculated from sequence for the ^{12}C monoisotopic species) for the starting 16mer and 4997.901 Da (4997.907 Da from sequence) for the alkylated product. However, when the same reaction was carried out after annealing of EY2 with the complementary 16mer EY1 (GCG TAC GCC GGT CCG T), no alkylation was detected on either strand (Figure 4B). This finding suggests that the COMC function is attached to an exocyclic amino group, which is sterically protected by base pairing in the duplex oligonucleotide, and effectively rules out the purines C8 position as a possible reactive site for cyclohexenone addition.

In conclusion, COMC forms stable adducts *in vitro* with GSH and with nucleic acids. Adducts can form by either enzymatic or nonenzymatic pathways, following the addition−elimination mechanism shown in Scheme 1. This mechanism is strongly supported by the observation of key intermediates along the reaction pathway.

Alkylation of nucleic acids occurs both in the presence and in the absence of GSH/hGST and follows the same general reaction pathway. The site of alkylation appears to be the exocyclic amino groups of adenine, cytosine, and guanine. Future confirmation by other means of structural determination (*e.g.*, NMR) would be desirable. Conceivably, 2-hexenone derivatives may exhibit sequence specificity, on the basis of the observation that COMC preferentially modifies guanine in GA. Indeed, genotoxic alkylating agents have previously been reported to exhibit specificity toward GC-rich regions in oligonucleotides.[19] This observation suggests the possible existence of hypersensitive motifs toward alkylation by COMC and its derivatives.

Finally, the observations reported here clearly suggest that the cytotoxic activity of COMC and its derivatives may well arise from alkylation of polynucleic acids critical to cell function. Given the high concentration of GSH in cells under physiological conditions, GSH is likely to be involved in the initial activation step (Scheme 1). However, a clear assessment of GSH/GST activation *vs* direct COMC alkylation will require kinetics investigations.

Acknowledgment. This work was supported by UMBC institutional funding (to D.F.), by the NIH (GM 24054 to B.G.), and by the U.S. Army Medical Research and Materiel Command (to D.J.C.). We thank Dr. Diana S. Hamilton for helpful discussions.

OL025612Y

(16) McLuckey, S. A.; Habibi-Goudarzi, S. *J. Am. Chem. Soc.* **1993**, *115*, 12085−12095.

(17) Little, D. P.; Chorush, R. A.; Spier, J. P.; Senko, M. W.; Kelleher, N. L.; McLafferty, F. W. *J. Am. Chem. Soc.* **1994**, *116*, 4893−4897.

(18) Ni, J.; Pomerantz, C.; Rozenski, J.; Zhang, Y.; McCloskey J. A. *Anal. Chem.* **1996**, *68*, 1989−1999.

(19) Mattes, W. B.; Hartley, J. A.; Kohn, K. W.; Matheson, D. W. *Carcinogenesis* **1988**, *9*, 2065−2072.