A New and Unusual Pathway for the Reaction of Neocarzinostatin Chromophore with Thiols. Revised Structure of the Protein-Directed Thiol Adduct

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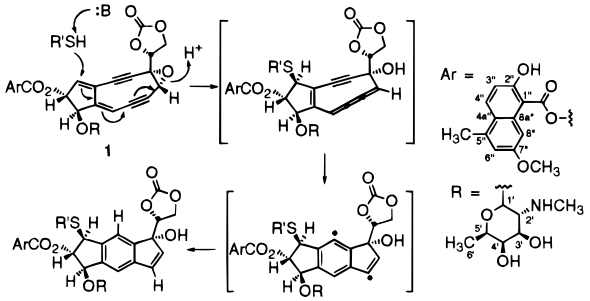
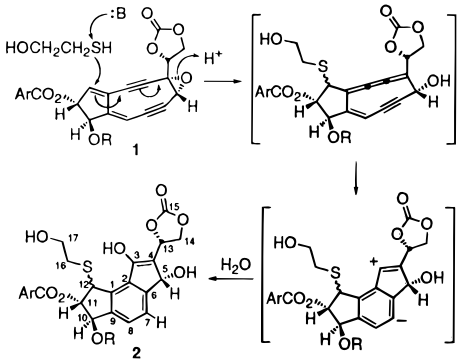
Neocarzinostatin (holo-NCS) is an antitumor antibiotic comprising a nonprotein chromophore component (**1**) and a 113-amino acid carrier protein (apo-NCS).[1] Goldberg and co-workers first demonstrated that the reaction of the isolated chromophore (**1**) with thiols in the presence of double-stranded DNA leads to DNA cleavage by a free-radical mechanism.[2] The pathway shown in Scheme 1 was later proposed to account for this activity,[3] a proposal that is now supported by a considerable body of evidence.[4] In 1992, Saito and co-workers showed that the reaction of holo-NCS with small thiols, such as β-mercaptoethanol (BME), takes a different course, to form a product that is formally a 1:1:1 adduct of thiol, **1**, and water.[5] Structure **2** was proposed for this adduct, along with the mechanistic pathway shown in Scheme 2. Complicating the analysis was the fact that **2** was an inseparable mixture of two components, present in equal parts.[5]

Several features of the structural assignment **2** caused us to question the proposal. One was the argument that an equal distribution of epimers at C12 accounted for the observed mixture. Not only was the probability of α-face attack by thiol viewed as low, but if such were to occur, the α- and β-oriented isomers would exhibit distinct coupling between H11 and H12, which was not the case. Also problematic was the pattern of chemical shift variations between the isomers in the ^{1}H and ^{13}C NMR spectra. The largest variations occurred in the right-hand portion of the molecule, in signals associated with the hydroxyl, enol, and carbonate groups; signals associated with the left-most ring were virtually superimposable for the two isomers. Most disturbing was the proposed stable enol functionality.[5] 1-Indanone itself shows no detectable enol content[6] and the rationalization of an unusually strong hydrogen bond between the enol proton and the carbonate group,[5] to us, was not compelling. In a study in which they showed that the pathway producing **2** does not lead to DNA cleavage, Goldberg and Chin also called into question the likelihood of the enol group when they discovered that the enol proton did not exchange with solvent water.[7] Later, Saito et al. demonstrated that the enol proton did exchange, given sufficient time, and rationalized the slow exchange, again, on the basis of an unusually strong hydrogen bond with the carbonate group.[5b]

Scheme 1. Mechanism of Reaction of **1** with Thiols in the Absence of NCS Apoprotein



Scheme 2. Proposal of Saito et al. for the Reaction of $HSCH_2CH_2OH$ with Holo-NCS



After careful reconsideration of all spectroscopic and chemical data, and on the basis of experimental data described herein, we propose that structure **2** should be revised as the hydroxyisochromene derivative **3** (see Scheme 3). This proposal resolves each of the problematic features of the prior assignment **2**. Most notably, the proposed structure **3** accounts for diastereoisomerism with the presence of the hemiacetal group at C5. Equilibration with the open-chain keto aldehyde isomer[8] allows for facile epimerization of the C5 hydroxyl group (thus precluding isolation of the individual diastereomers in pure form), provides a mechanism for slow exchange of the (carbon-bound) C3 proton, and is consistent with the observed patterns of chemical shift differences between the diastereomers. The stable enol functionality is not invoked. A single mode of thiol addition is proposed, consistent with the observed sense of thiol addition to **1** not bound to protein (Scheme 1).

A fundamental distinction between our proposed structure **3** and the prior assignment **2** is the distribution of quaternary and ternary carbons: structure **2** has 16 methines and 14 quaternary carbons, whereas **3** has 17 methines and 13 quaternary carbons. We therefore prepared an authentic sample of the protein-directed BME adduct, following literature precedent,[5b] and conducted a DEPT-90 NMR experiment in order to quantify the methine carbons. We observed 17 methine signals: 6 as single peaks integrating for ~1 carbon each and 11 as pairs of closely spaced $^1/_2$-carbon signals for the resolved diastereomers. In a key experiment, ^{1}H−^{13}C gradient-enhanced inverse detected HETCOR NMR analysis of the BME adduct showed a pair of $^1/_2$-carbon signals, at 119.7 and 122.6 ppm, to correlate with $^1/_2$-proton singlets at 7.23 and 7.27 ppm, respectively. The latter

(1) Isolation: (a) Shoji, J. *J. Antibiot.* **1961**, *14*, 27. (b) Ishida, N.; Miyazaki, K.; Kumagai, K.; Rikimaru, M. *J. Antibiot.* **1965**, *18*, 68. Characterization: (c) Napier, M. A.; Holmquist, B.; Strydom, D. J.; Goldberg, I. H. *Biochem. Biophys. Res. Commun.* **1979**, *89*, 635. (d) Koide, Y.; Ishi, F.; Hasuda, K.; Koyama, Y.; Edo, K.; Katamine, S.; Kitame, F.; Ishida, N. *J. Antibiot.* **1980**, *33*, 342. Chromophore structure: (e) Edo, K.; Mizugaki, M.; Koide, Y.; Seto, H.; Furihata, K.; Otake, N.; Ishida, N. *Tetrahedron Lett.* **1985**, *26*, 331. Chromophore stereochemistry: (f) Myers, A. G.; Proteau, P. J.; Handel, T. M. *J. Am. Chem.Soc.* **1988**, *110*, 7212. X-ray crystal structure of holo-NCS: (g) Kim, K.-H.; Kwon, B.-M.; Myers, A. G.; Rees, D. C. *Science* **1993**, *262*, 1042.

(2) (a) Poon, R.; Beerman, T. A.; Goldberg, I. H. *Biochemistry* **1977**, *16*, 486. (b) Hatayama, T.; Goldberg, I. H.; Takeshita, M.; Grollman, A. P. *Proc. Natl. Acad. Sci. USA* **1978**, *75*, 3603. (c) D'Andrea, A. D.; Haseltine, W. A. *Proc. Natl. Acad. Sci. USA* **1978**, *75*, 3608.

(3) Myers, A. G. *Tetrahedron Lett.* **1987**, *28*, 4493.

(4) Myers, A. G.; Cohen, S. B.; Kwon, B.-M. *J. Am. Chem. Soc.* **1994**, *116*, 1670 and references therein.

(5) (a) Sugiyama, H.; Yamashita, K.; Nishi, M.; Saito, I. *Tetrahedron Lett.* **1992**, *33*, 515. (b) Sugiyama, H.; Yamashita, K.; Fujiwara, T.; Saito, I. *Tetrahedron* **1994**, *50*, 1311.

(6) >95% keto form by ^{1}H and ^{13}C NMR at 400 and 100 MHz, respectively (DMSO-d_6).

(7) Chin, D.-H.; Goldberg, I. H. *J. Am. Chem. Soc.* **1993**, *115*, 9341.

(8) (a) Padwa, A.; Au, A. *J. Am. Chem. Soc.* **1976**, *98*, 5581. (b) Baer, H. H.; Kienzle, F. *J. Org. Chem.* **1968**, *33*, 1823. (c) Parisot, D.; Devys, M.; Barbier, M. *J. Antibiot.* **1989**, *42*, 1189.

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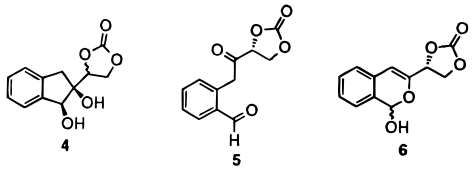
$^1/_2$-proton singlets correspond to the slowly exchanging "enol" signals in the assignment of Saito et al.[5] This result conclusively establishes that these protons are carbon-bound, corroborates structure **3**, and removes from further consideration assignment **2**.

To provide further evidence for the proposed structure **3**, we synthesized the model hydroxyisochromene **6** (see supporting information). In the penultimate step, the diol **4** was cleaved with sodium metaperiodate in aqueous tetrahydrofuran; ^{1}H NMR analysis of the crude reaction product showed it to exist almost

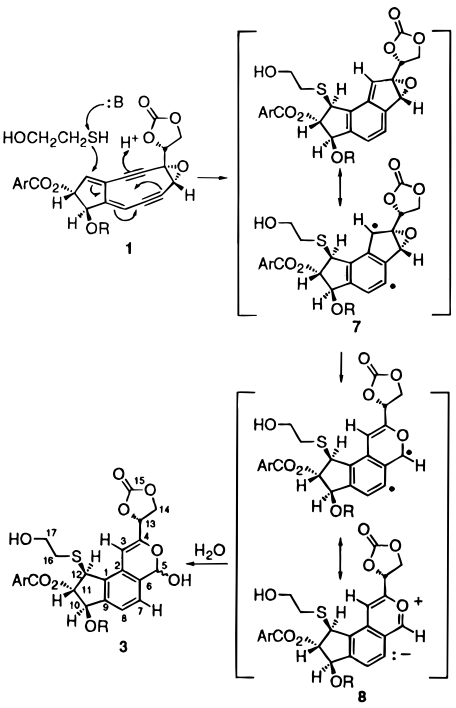


entirely in the keto aldehyde form (**5**, 89% yield). When **5** was treated with acetic acid in DMSO-d_6, it was converted smoothly and quantitatively into the hydroxyisochromene **6**, a 1:1 mixture of diastereomers at the hemiacetal center. Importantly, when **6** was treated with excess deuterium oxide in DMSO-d_6, rapid exchange of the hydroxyl proton was observed ($t_{1/2}$ < 5 min, ^{1}H NMR analysis) while the olefinic proton exchanged much more slowly ($t_{1/2}$ = 13 h). Saito et al. report that the protein-directed BME adduct undergoes rapid ($t_{1/2}$ < 1 min) exchange of the 5OH proton and slow ($t_{1/2}$ > 2 h) exchange of the "3OH" proton under similar conditions.[5b] The sum of spectroscopic and chemical data provides a compelling case in support of structure **3**.

We presently favor the pathway shown in Scheme 3 to account for the formation of **3** from **1**. We propose that the epoxide of **1** in holo-NCS is not disposed toward nucleophilic opening because it lies within a hydrophobic pocket of the protein.[1g] Thus, as shown in Scheme 3, following "conventional" attack of thiolate at C12, trans to the naphthoate, the pathway of Scheme 1 is diverted by transannular nucleophilic addition to the triple bond, followed by protonation of the resultant vinyl carbanion. This sequence produces the substituted α,3-dehydrotoluene biradical **7**.[9] Fragmentation of the α-epoxy radical within **7** then exchanges one benzylic radical for another to form **8**, presumably driven by the release of strain within the epoxide ring and by the greater stability of the benzylic radical in the product. The net transformation represents the rearrangement of an α,3-dehydrotoluene biradical to an α,2-dehydrotoluene biradical.[10] Reaction of the latter (**8**) with water in a polar process then produces hydroxyisochromenes **3**.[11]

It has been recognized that the addition of small thiols to holo-NCS provides a mechanism for inactivation of the chromophore.[5b] Importantly, glutathione does not react with

Scheme 3. Proposed Mechanism of Reaction of $HOCH_2CH_2SH$ with Holo-NCS, This Work



holo-NCS.[7] It is perhaps significant that many prokaryotes utilize small thiols such as hydrogen sulfide or cysteine for metabolism, in contrast to eukaryotes, which typically employ glutathione.[12] The protein-directed addition reaction of small thiols may provide a self-protective mechanism for the organism that produces NCS (*Streptomyces carzinostaticus*, a prokaryote). It could also provide the basis for species-selective toxicity. These questions touch upon the fascinating issues of why and how microorganisms have evolved to produce toxic metabolites, such as neocarzinostatin, and whether an observed activity confers an evolutionary advantage upon the organism, represents an archeological remnant, or is simply an artifact.

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Supporting Information Available: Details of the preparation of **6**, tables of comparative ^{13}C NMR data for **3** from ref 5 and this work, DEPT-90, and ^{1}H−^{13}C gradient-enhanced inverse detected HETCOR NMR spectra of **3** (9 pages). Ordering information is given on any current masthead page.

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(9) Myers, A. G.; Dragovich, P. S.; Kuo, E. Y. *J. Am. Chem. Soc.* **1992**, *114*, 9369 and references therein.

(10) (a) Closs, G. L.; Kaplan, L. R.; Bendall, V. I. *J. Am. Chem. Soc.* **1967**, *89*, 3376. (b) Wenthold, P. G.; Wierschke, S. G.; Nash, J. J.; Squires, R. R. *J. Am. Chem. Soc.* **1994**, *116*, 7378.

(11) In contrast to σ,σ-biradicals (such as that in Scheme 1, or those produced in the Bergman reaction), the σ,π-biradical α,3-dehydrotoluene is known to engage in polar addition reactions.[9] Presumably, the alkoxy-substituted α,2-dehydrotoluene biradical of Scheme 3 can react in the same capacity.

(12) Newton, G. L.; Fahey, R. C. Glutathione In Prokaryotes In *Glutathione: Metabolism and Physiological Functions*; Viña, J., Ed.; CRC Press: Boca Raton, FL, 1990; pp 69−77.