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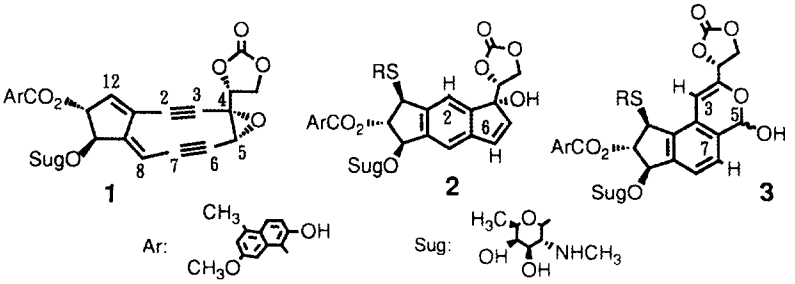
Abnormal Cycloaromatization of Neocarzinostatin Induced by Sugar Thiols.

Der-Hang Chin*, and Mei-Ching Tseng

Department of Chemistry, National Changhua University of Education,
Paisa Village, Changhua 50058, Taiwan, Republic of China

Abstract: After screening various thiols in succession, hexose thiols were found to be able to induce abnormal cycloaromatization of the protein bound neocarzinostatin chromophore. Evidence is provided that such abnormal cycloaromatization is neither due to dissociation of the chromophore nor from changes of binding conformation. © 1997 Elsevier Science Ltd.

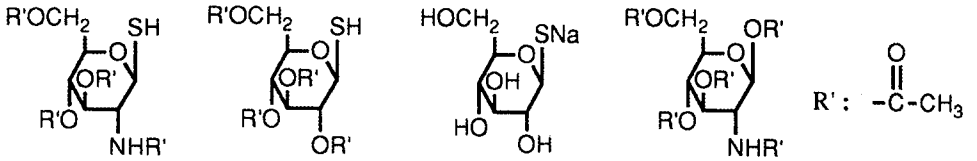
Cycloaromatization of enediyne compounds has attracted great attention lately. Neocarzinostatin (NCS)[1] is a potent enediyne containing chromoprotein antitumor antibiotic. The carrier apoprotein binds noncovalently to NCS chromophore **1** whose cycloaromatization is a key step for DNA lesions. NCS apoprotein shows an interesting unique behavior in that it can change the cycloaromatization route of the enediyne chromophore to form a distinct product. How the protein alters the cyclization mechanism remains as a mystery. Here we present a novel phenomenon of abnormal cycloaromatization induced by sugar thiols. The protein loses its strong chemical influence and the chromophore cyclizes as if it were not bound to the protein.



The thiol-induced activation of **1** without protein involves abstraction of hydrogens from various sources[2] into C2 and C6 to form **2**.[3] The absolute stereochemistry of **2** has been identified as one of C12-epimers that is only trans to the naphthoate.[3] A distinct cycloaromatization was discovered by Sugiyama et. al. from the protein bound **1** induced by 2-mercaptoethanol (BME).[4,5] Contrary to **2**, the product was characterized to be a C12-

epimeric mixture. Earlier, Goldberg and Chin[6] have questioned the assigned structure. Myers et. al.[7] recently revised the structure to hydroxyisochromene **3** based on new NMR evidence. The diastereoisomerism center was reassigned at the hemiacetal C5. Although the precursor of **3** is not responsible for the drug activity of cleaving DNA,[6] Sugiyama et. al.'s finding is still very significant. The formation of **3** is considered as a major inactivation process in holo-NCS. The finding provides first direct evidence that NCS protein is very chemically influential towards **1**. NCS protein has drawn increased attention lately due to its vital protecting and regulating functions as well as its significant proteolytic activity.[8]

Since thiol is the only cofactor in thiol-induced cycloaromatization of NCS, varying thiol structure could reveal some insight about the manipulative function of the protein. After screening more than fifty different thiols in succession, we have found that **2** is regularly produced from protein unbound **1** despite great structural variation of the applied thiol. Due to either low solubility in aqueous condition or shielding factor of NCS protein, about half of the tested thiols are unable to induce reaction from protein bound **1**. Other half of the tested thiols were found to be active towards protein bound **1**. Among them, except sugar thiols **4**, **5** and **6**, all regularly produce major product **3**. Active thiol in this category consists various functional groups including alcohols (dithioerythritol, 3-mercapto-2-butanol, etc.), amines (cysteamine, 2-diethylaminoethanethiol, etc.), esters (methyl thioglycolate, methyl-3-mercaptopropionate, etc.), aminoacids (L-cysteine (Cys)), and aminoesters (L-cysteine methyl and ethyl esters), etc. Protonated (2-aminoethylisothiouronium, etc.), zwitterionic (Cys, etc.), near neutral (1-mercapto-2-propanol, etc.), or unsaturated (allyl mercaptan) species can all produce **3**. None of functional properties of the employed thiol can substantially change the chemical directing role of the protein. Only bulky hexose thiols with the thiol group hidden on an anomeric position behave differently from all other thiols.



4 **5** **6** **7**

HPLC analyses have shown that the retention of **2**'s and **3**'s changes greatly with the thiolate structure at C12.[9] However, by the gradient method we have derived the elution of **3** is consistently 10 min. after its counterpart **2** from the same thiol.[9] When sugar thiols were applied, we have found that the major product abnormally eluted at the same time whether **1** had been bound or not to NCS protein (Figure 1A). When **4** was applied to the extracted **1** in the absence of NCS apoprotein in methanol, the result was the same.

We have found earlier that the **2**'s and **3**'s exhibit distinct spectroscopic fingerprints which are independent to thiol structures but associate only with the fused aromatic moiety cyclized from the enediyne nucleus.[9] The fingerprints are reliable criteria to distinguish **2** from **3** in micro scale studies. Figure 1B shows the spectroscopic properties of the isolated major product from the cycloaromatization of holo-NCS induced by **4**. It exhibits a single fluorescent emission at 440 nm. The excitation has a blunt valley at 300 nm between two maximums 260 and 330 nm whose intensity ratio is about 10 to 7. The 3-D fluorogram shows that two contour loops are crossed at excitation wavelength of 295 nm and emission of 440 nm. The UV absorption shows a valley near 300 nm and a shoulder at 282 nm with λmax at 272 nm (ε, 9.7 x 10^3), 335 nm (ε, 5.2 x 10^3) and 226

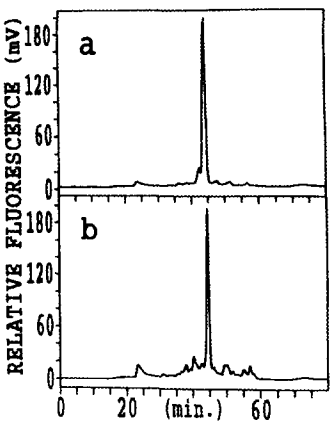


FIGURE 1A: HPLC analyses of 50 μl of the drug reaction solutions that contained 50 μM of holo-NCS with 5 mM of **4** and 50 mM of Tris HCl, pH 7.0 in: (a) 80% IPA at 0°C for 30 min.; and (b) aqueous solution at 0°C for 48 hours.

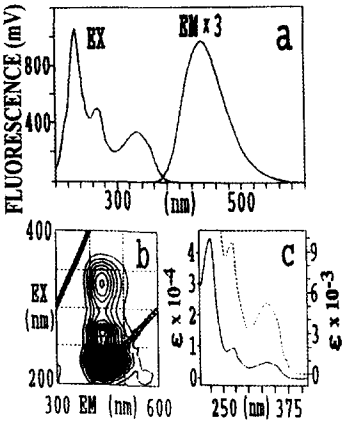


FIGURE 1B: (a) Uncorrected fluorescence EX (EM at 440 nm) and EM (EX at 338 nm) spectra of the isolated major product from the reaction of **4** with holo-NCS. The EM scan is triply enlarged. (b) The top view of 3-D fluorogram. The scattered radiation and Rayleigh scattering were not subtracted. (c) The UV absorption of the same product extracted from PDA in HPLC analyses for a reaction of 2.5 nmole of **1**. The expanded right side scale is indicated by the dotted line.

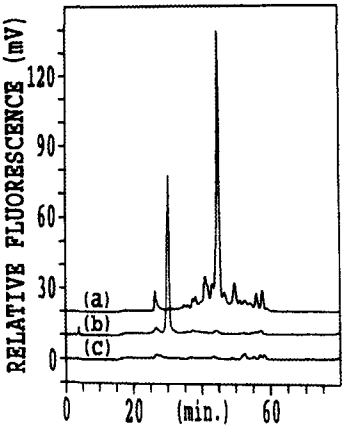


FIGURE 1C: HPLC profiles. Line (a) was from 50 μl of 27 μM of holo-NCS incubated with 5 mM of **4** and 5 mM of GSH at pH 7.0, 37°C for 30 min. Line (b) and (c) were from the same condition except that **4** was replaced by **7**. The solution was divided by two after the incubation. One half was converted into 80% IPA and incubated for an additional 30 min. at 0°C (line (b)); and the other half was analyzed without further treatment (line (c)).

nm (ε, 4.45 x 10^4). They are in agreement with the characteristic properties of **2**'s but different from that of **3**'s.[9] The major product from the same reaction without NCS protein exhibits indistinguishable spectroscopic properties from the above.

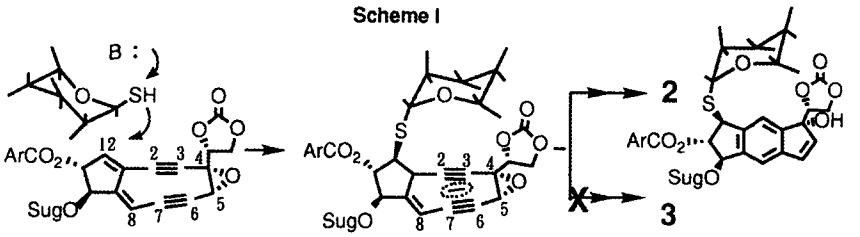
The abnormal cycloaromatization is further confirmed by positive FAB mass spectrometry. The measured MH^+ of the isolated thiol-drug adduct from **4** without protein is 1025 as expected for **2**. Under protein bound conditions, the isolated product also gives MH^+ of 1025 instead of 1041, which is the calculated MH^+ for **3**. All of these data suggest that sugar thiols can only produce **2** in holo-NCS and none of **3** can be detected.

During screening tests, **2** was either undetectable or remained as a minor product for a normal thiol reaction of holo-NCS. Only sugar thiols induce overwhelmed **2** as if **1** were not bound to its protein. One could not rule out the possibility that **1** has been freed from holo protein by the stimulation of hexose thiols before its cyclization. It has been shown that as little as 10 mM of D-galactosamine can cause 6% release of **1** from holo-NCS.[10] To examine the possibility of the dissociation of **1**, we tested with glutathione (GSH) which is very reactive to the extracted **1** but fails to participate in the reaction with the protein bound **1**.[6] Examining the formation of **2** from GSH thus becomes a good criterion to judge the extent of releasing of **1** from NCS protein cleft. By incubating protein bound **1** (1.3 nmole) with a mixture of **4** and GSH, only **2** from **4** (45 min.) but none of **2** from GSH (30 min.) could be detected (Figure 1C, line (a)). When a non-thiol analog **7** was introduced to replace **4** at the same condition, no product could be observed (line (c)). When half of the latter solution was converted into 80% of isopropanol to denature the protein, **2** of GSH was fully developed, indicating that **1** had been well preserved during the incubation (line (b)). The results demonstrate that the abnormal cycloaromatization induced by **4** is not due to dissociation factor and it occurs inside protein environment.

D-galactose and its derivative are known to be effective to interfere with binding of **1** with NCS protein.[10]

Abnormal cycloaromatization could be a result of an altered protein binding geometry induced by hexoses. We have examined such a possibility with BME which is effective in inducing normal cycloaromatization under a normal binding situation. When **4** was mixed with BME in a holo-NCS solution, we have found that **3** was still the major product from BME as usual and the minor proportion of **2** from BME did not increase by the presence of **4**. Furthermore, formation of **2** from **4** is at the expense of **3** from BME. The same competition phenomena can also be seen by mixing **4** with methyl-3-mercaptopropionate which is also active in producing **3** under normally bound conditions. The data indicate that the presence of **4** would only compete but not alter the protein directed normal cyclization path induced by BME or other thiols. When holo-NCS was incubated with a mixture of **7** and BME, we found that the ratio of the minor to major BME adducts, i.e., **2** to **3**, was unaffected. The presence of free molecules of **4** or **7** in solution apparently would not distort the protein binding conformation to favor producing **2** via its intermolecular interaction towards the protein cluster. The abnormal cycloaromatization induced by sugar thiols has to be contributed from intramolecular interactions.

Scheme I



Both cycloaromatization paths have been proposed to be initiated by a thiolate attack at C12 of **1**.[3,7] It is reasonable to postulate a common intermediate after the thiolate attack, which may undergo either epoxide ring opening to cascade down to **2** or irreversible cyclization to form **3**. The covalently attached thiolate at C12 on such a common intermediate could exert its structural effect to the cycloaromatization path (Scheme I). The sugar thiol is noticeably different from other employed thiols only by its bulky size. The steric hindrance from intramolecular interaction between the bonded sugar thiolate and the transformed epoxy dienediyne nucleus is probably the major cause to prevent a normal cyclization path that is directed by NCS protein.

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