MECHANISM OF ACTIVATION OF THE ANTITUMOR ANTIBIOTIC NEOCARZINOSTATIN BY MERCAPTAN AND SODIUM BOROHYDRIDE

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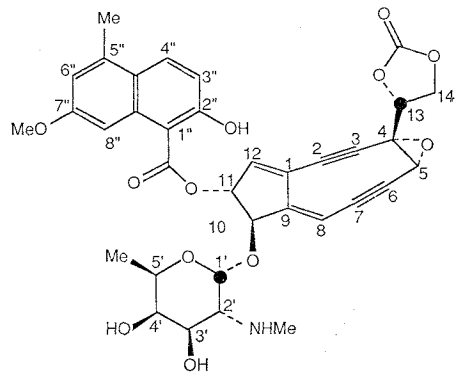
The structures of mercaptan and sodium borohydride reaction products of neocarzinostatin chromophore A (NCS Chrom A) are compared. Implications on the mechanism of activation of NCS are discussed.

Neocarzinostatin (NCS) is an antitumor and DNA-damaging antibiotic consisting of a 1 : 1 complex of a separable protein and a non-protein chromophore fraction[1~3] which consists principally of NCS chromophore A (Chrom A) possessing all the biological activity of NCS.[4] The novel structure **1** has recently been determined[5,6] for the chromophore as well as its absolute stereochemistry[7] and features a unique epoxybicyclo[7.3.0]dodecadienediyne ring system.[5~7] Its activity involves single-strand breaks in linear duplex or superhelical DNA *in vitro* in an oxygen-dependent reaction which is greatly stimulated by mercaptans.[8] The cleavage reaction mainly (>80%) generates a 5′-aldehyde of deoxythymidine and deoxyadenosine residues of DNA selectively[9] and involves hydrogen atom transfer from C-5′ to a covalently bound carbon in the rearranged chromophore[10] as well as transfer of ^{18}O from dioxygen to C-5′.[11] Less than 20% of the strand breaks result from formation of a labile 3′-formylphosphate ended-DNA fragment.[12] Although chromophore inactivated by pre-incubation with thiol, failed to abstract 5′-[^{3}H] from DNA or to produce DNA damage,[13] it produced the same amount of UV-absorbing and fluorescing material with the same elution time as in a parallel reaction containing active drug.[10] The implication of similar structures for activated and inactivated species, therefore, provided the rationale for investigation of the mode of action by determining the structures of mercaptan and borohydride treated chromophore end-products. Very recently MYERS[14] proposed an elegant mechanism for the thiol nucleophilic activation of NCS and suggested **4a** (Scheme 1) as the NCS Chrom A-methyl thioglycolate adduct based on our previously reported spectroscopic data, in particular, ^{1}H NMR.[5] The reaction with $NaBH_4$ is also of interest as the requirement for mercaptan in the *in vitro* DNA scission activity of NCS Chrom A can be replaced by borohydride as was reported



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for native NCS.[8] Relatively minor changes in the absorption and fluorescent spectral properties[15] suggested that comparison of the two reactions with borohydride and mercaptan may therefore shed further light on the activation pathway.

Results

Table 1 lists the ^{1}H NMR assignments of the major methyl thioglycolate and $NaBH_4$ or $NaBD_4$ reaction products of NCS Chrom A obtained under conditions previously reported.[5] Only one stereoisomer of the thiol adduct was obtained resulting from β face attack at C-12.[7] Comparison of the spectra indicate a remarkable similarity and it is evident that the naphthoate, amino sugar, and cyclic carbonate moieties remain relatively unchanged. It is clear that the spectra contain at least two extra resonances comprising a sharp singlet (δ 7.83/7.66) and sharp doublet (δ 7.01/6.97) which were pivotal in assignment of 2-H and 6-H of the indene structure **4a**.[14] These are absent in spectra when $NaBD_4$ is substituted for $NaBH_4$ resulting in collapse of the doublet for 5-H at δ 6.35 to a singlet. These two resonances are still present in the deuteriothioglycolate spectra with apparently only slightly reduced intensity.

Previously, we showed[5] that in the reaction with $NaBH_4$, four protons are added to the C_{12} substructure of NCS Chrom A, one of which is active and three of which are carbon-bound. This is

Table 1. ^{1}H NMR assignments of methyl thioglycolate **4a** and $NaBH_4$ **4b** rearrangement products of NCS Chrom A (CD_3COOD) (J in Hz).

Assignment	**4a**	**4b**[a]
5′-CH_3	1.23 (3H, d, J=6.5)	1.27 (3H, d, J=6.5)
5″-CH_3	2.56 (3H, s)	2.56 (3H, s)
2′-NHCH_3	3.06 (3H, s)	3.00 (3H, s)
7″-OCH_3	3.27 (3H, s)	3.35 (3H, s)
2′-H	obsc	3.79 (1H, m)
$COOCH_3$	3.78 (3H, s)	—
4′-H	3.85 (1H, br s)	3.86 (1H, br s)
5′-H	3.96 (1H, q, J=6.5)	4.00 (1H, q, J=6.5)
3′-H	4.33 (1H, m)	4.29 (1H, dd, J=3, 9.5)
14-H_a	4.44 (1H, dd, J=6.5, 8.5)	4.37 (1H, dd, J=6.5, 8.5)
14-H_b	4.56 (1H, t, J=8.5)	4.52 (1H, t, J=8.5)
12-H_a	4.75 (1H, br s)	~3.90 (2H, v br m, obsc)[a]
12-H_b	—	
13-H	4.83 (1H, dd, J=6.5, 8.5)	4.82 (1H, dd, J=6.5, 8.5)
10-H	5.46 (1H, br s)	5.47 (1H, br s)
1′-H	5.88 (1H, v br s)	5.81 (1H, v br s)
11-H	6.08 (1H, br s)	6.03 (1H, br m)
5-H	6.42 (1H, d, J=5.5)	6.35 (1H, d, J=5.5)[a]
6″-H	6.82 (1H, br s)	6.82 (1H, br s)
6-H	7.01 (1H, d, J=5.5)	6.97 (1H, d, J=5.5)[a]
3″-H	7.05 (1H, d, J=9)	7.04 (1H, d, J=9)
8-H	7.52 (1H, s)	7.42 (1H, s)
8″-H	7.58 (1H, br s)	7.75 (1H, br s)
2-H	7.83 (1H, s)	7.66 (1H, s)[a]
4″-H	8.10 (1H, d, J=9)	8.09 (1H, d, J=9)

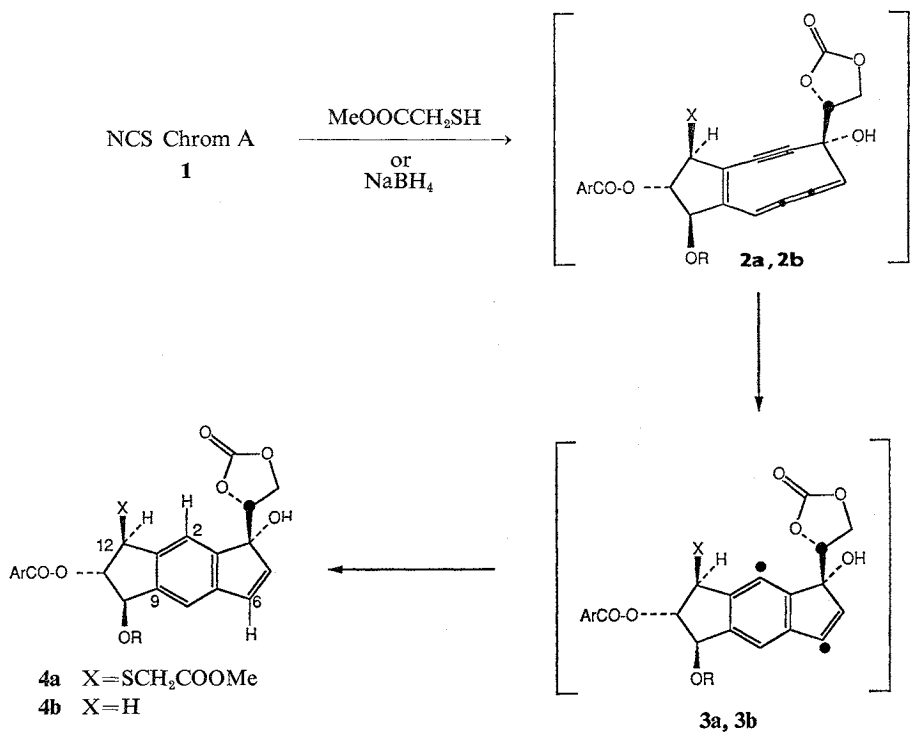
[a] In the $NaBD_4$ spectrum, 2-H and 6-H are absent; 5-H is a sharp singlet, and the very broad singlet of 11-H and the obscured methylene multiplet of C-12 have sharpened considerably.

obsc: Obscured, v: very.

formally consistent with the reduction of a double/triple bond and opening of the epoxide ring to generate one silylatable OH function. The additional singlet at δ 4.75 in the methyl thioglycolate spectra and assigned to 12-H in **4a**, is no longer present in the $NaBH_4$ or $NaBD_4$ spectra whereas 10-H and 11-H in the thioglycolate spectrum appear as singlets at slightly shifted positions at δ 5.46 and 6.08 from those in the chromophore. By contrast, 11-H in the $NaBH_4$ spectrum now appears as a very broad singlet which sharpens to a singlet in the $NaBD_4$ spectrum. Moreover, in the overlapping region of the spectrum between δ 3.6~4.1, the 2H multiplet centered near δ 3.90, sharpens appreciably, similar to the effect observed on irradiation of 11-H, and can be assigned to the methylene protons at C-12. Therefore, besides incorporation of deuterium at C-2 and C-6 in the proposed $NaBH_4$-derived structure **4b**, the third and remaining deuterium is incorporated at C-12. The incoming proton at C-12 appears to be from the less hindered β-face of the molecule as evident from the observation that incorporation with deuterium removes the larger *cis* coupling of 12-H with 11-H, which leaves the small *trans* coupling ($J_{H11,12} \leq 1$ Hz) as found in the thioglycolate product **4a**. Reaction with thioglycolate and $NaBH_4$ therefore both involve β-face attack at C-12, *trans* to the naphthoic acid moiety at C-11.

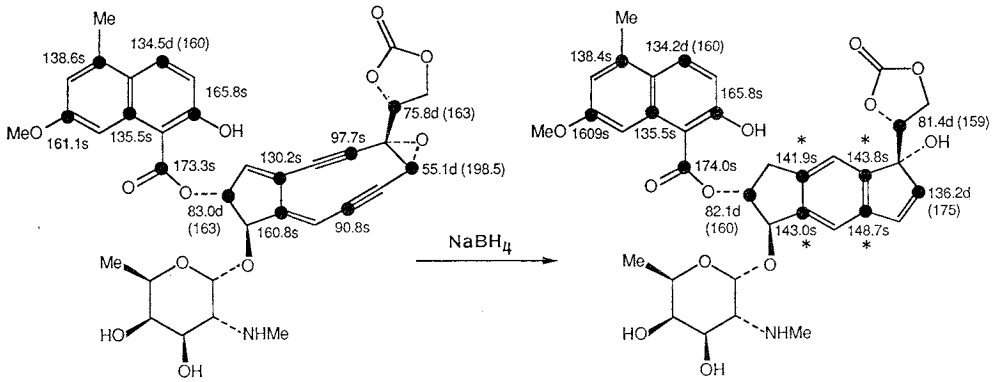
Corroboration of the $NaBH_4$ derived structure **4b** was obtained from ^{13}C NMR evidence. Lack of adequate sample quantities and sample instability precluded studies at the natural abundance level and hence we examined the reduction product of NCS Chrom A which had been labeled by [1-^{13}C]-acetate. Previous studies[16] have shown that the C_{14} cyclic carbonate/bicyclic dienediyne and naphthoic acid ring skeletons are totally derived from acetate. ^1H decoupled and 'gated' coupled ^{13}C

Scheme 1. Proposed mechanism of action of NCS Chrom A with methyl thioglycolate and $NaBH_4$.



Ar and R denote the naphthoate and *N*-methylfucosamine moieties as in **1** respectively.

Fig. 1. ^{13}C NMR assignments of [1-^{13}C]acetate labeled NCS Chrom A (**1**)[16] and $NaBH_4$ product **4b** in CD_3COOD at ~5 and 25°C respectively.



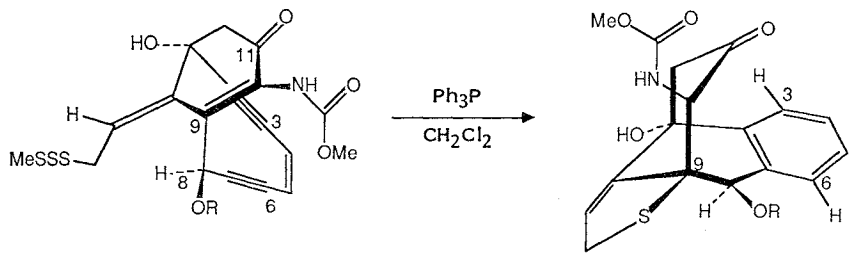
The circled carbons are derived from [1-^{13}C]acetate. $^1J_{CH}$ (Hz) values are given in parentheses. Assignments of carbons marked with an asterisk (*) may be interchanged.

NMR spectra of the labeled borohydride product confirmed that the naphthoic acid ring remained unchanged (see Fig. 1) but that the C_{14} substructure was dramatically modified consistent with the proposed structure **4b**. Noteworthy is the conversion of the epoxide C-5 to an sp^2 methine carbon at 136.2 ppm with a ^{13}C-^1H coupling constant (175 Hz), higher than that observed in unsubstituted indene (165 Hz) itself (see Experimental) and consistent with increased ring strain resulting from the second fused five membered ring. The four quaternary carbons C-1, C-3, C-7 and C-9 remain quaternary and the chemical shift changes are consistent with the proposed aromatization rearrangement[17] (see Fig. 1). Inadequate sample quantities precluded similar experiments with [2-^{13}C]- and [1,2-$^{13}C_2$]-acetate labeled NCS Chrom A.

Discussion

Having demonstrated the similarity of structures for the thiol adduct **4a** and $NaBH_4$ reduction product **4b** of NCS Chrom A (Scheme 1), we wish to make some observations on the mechanism of the reaction as well as its implications in the activation process of NCS. KAPPEN and GOLDBERG[10] have proposed the involvement of a biradical species in the activation of NCS which was recently defined in molecular terms by MYERS[14] on the basis of our ^1H NMR data[5] (see Scheme 1). The process involves nucleophilic attack at C-12 with concomitant epoxide ring opening resulting in the cumulene **2a** which can undergo a modified Bergman reaction[18~23] to form the biradical **3a**. In the absence of DNA, hydrogen atom abstraction from solvent or excess reagent can thus give rise to the thiol adduct **4a**. It must be pointed out that when the thiol reaction was carried out in the presence of deuterated solvent (CH_3OD), no apparent incorporation of deuterium was observed. Only a slight reduction (within experimental error) in the intensities of 2-H and 6-H was noted. This is consistent with the initial determination of the molecular formula for NCS Chrom A which required pretreatment with a mercaptan before trimethylsilylation in order to yield a vaporisable product for electron impact (EI)-MS analysis.[2,3] The molecular formula had to be revised downward by two hydrogens due to the incorrect assumption that reduction by thiol had not occurred, as protonated or deuterated solvent made no difference to the observed molecular weight.[5] The implication, therefore, of hydrogen atom abstraction from carbon provides strong support for the intermediacy of a biradical species **3a**.[24] Contrary to our findings, MYERS *et al.*[7] have very recently demonstrated that with greatly increased thiol concentrations (300 compared to 2~5 equivalents), significant deuterium in-

Fig. 2. Reaction of calicheamicin γ_1, **5** with triphenylphosphine in CH_2Cl_2.[19]



5 **6**

R denotes four glycosidic units and a hexasubstituted benzene moiety.

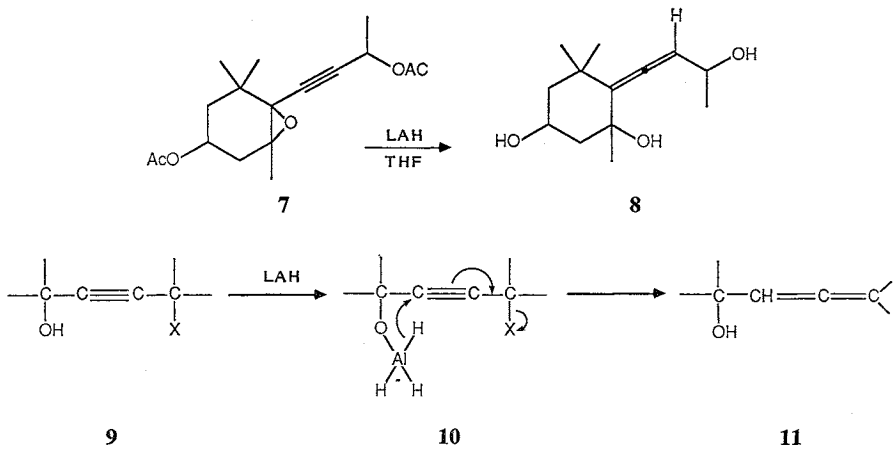
corporation is in fact observed at C-2 and C-6 in addition to the formation of a 6,12-bisthiol adduct which is consistent with the well known behavior of mercaptans to act as radical scavengers.

Mercaptans, like methanol, are capable of reaction in either a dipolar or radical fashion.[18] The stereospecificity with which thiol nucleophilic substitution at C-12 occurs, suggests involvement of an ionic thiolate species in triggering the reaction *i.e.* in formation of the initial intermediate cumulene adduct **2a**. This is consistent with the observation that DNA strand scission activity increases with increasing pH[25,26] and that $NaBH_4$ can substitute for thiol. By analogy, the recently reported enediyne→1,4-benzenediyl rearrangement in calicheamicin γ_1, **5** was shown to involve deuterium incorporation from CD_2Cl_2 at the 1,4-benzene positions C-3 and C-6 in **6** (by radical abstraction) but not at the site of Michael addition C-10 (see Fig. 2) of the *in situ* generated thiolate species.[19] In support of the subsequent biradical abstraction mechanism, deuterium incorporation into the drug was found only in CD_2Cl_2 - CD_3OD but not in CH_2Cl_2 - CD_3OD.[19] Both ionic and radical mechanisms are implicated in the reaction of sodium borohydride with NCS Chrom A as evidenced by deuterium incorporation at C-2, C-6 and C-12 from $NaBD_4$. Apparently, hydride ion attack at C-12 is followed by hydrogen radical transfer from the polar reagent $NaBH_4$ to the C-2 and C-6 positions of the biradical species **3b** (see Scheme 1) and suggests that at least for the $NaBH_4$ reaction, the participation of intermediate radical cations should not be ruled out.[27]

Whereas addition of thiolate to a conjugated diene or triene ring system is not unexpected, the extreme ease with which this occurs for NCS Chrom A under the mild conditions of pH and temperature and the fact that $NaBH_4$ can substitute for thiol,[8,28] strongly suggests that the ring skeleton is a highly strained and reactive system. NMR evidence in support of the ring strain in NCS Chrom A has recently been reported.[5,16] Especially the ease of nucleophilic attack at C-12 in the borohydride reaction at ambient room temperature in acidic methanol[5] or aqueous media[8,27] is, in our view, unprecedented.[29] By analogy, reduction of the acetylenic epoxide **7** to the allenic alcohol **8** (see Fig. 3) with the more reactive $LiAlH_4$ reagent occurs only after 6 hours under refluxing conditions in THF.[30] To facilitate the reaction with NCS Chrom A, a concerted mechanism proceeding *via* a borohydride adduct involving the C-2″ phenolic OH group, appeared at first attractive, analogous to the intermediacy of $LiAlH_4$ adduct **10** postulated for the rearrangement involving reduction of various acetylenic alcohol substrates **9**, where X can serve as a wide variety of leaving groups (see Fig. 3).[31] However, the observed β-face attack by the reagent from the less hindered side at C-12, *trans* to the naphthoic acid substituent at C-11, as discussed earlier, does not support such a proposal.

It is of interest to compare the reaction of NCS Chrom A with other nucleophiles. Reactions involving hydrochloric and perchloric acid in methanol lead exclusively to epoxide ring opening with formation of the chlorohydrin **12** and diol monomethyl ether **13** derivatives respectively.[6,32] The monomethyl ether **13** is inactive and the chlorohydrin **12** half as active as the epoxide in DNA strand scission activity in the presence of dithiothreitol at pH 8.[32] It can therefore be argued that with ring strain being comparable for both compounds but less than in the epoxide, the leaving group ability of the substituent at C-5 as well as the nucleophilicity of the nucleophile, is of paramount importance

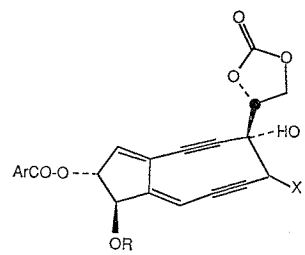
Fig. 3. Reduction of acetylenic substrates with lithium aluminum hydride (LAH).[30,31]



X denotes a wide variety of leaving groups, *e.g.*, halogen, hydroxy, alkoxy (epoxy), tetrahydropyranyloxy and trialkylammonium.[31]

in triggering the aromatization process. The propensity of thiols for addition to unsaturated systems, in this case involving nucleophilic attack at C-12, suggests as unlikely, the initiation of the aromatization process by epoxide ring opening involving a transient thiol adduct **14**.

It can be envisaged that in the reaction with DNA, the radical formed at C-2 or C-6 can abstract a hydrogen atom from the C-5′ of deoxyribose of mainly dT and dA residues[11] to form a carbon-centered radical at C-5′. Under aerobic conditions, the C-5′ radical can add dioxygen to form a peroxyl radical intermediate, leading to oxidation at C-5′ to the aldehyde,[9] or to the formation of covalent adducts of the DNA sugar.[33,34] The role of the second radical requires clarification especially in light of the fact that despite a postulated related biradical intermediate, the mechanism of action for the calichemicin/esperamicin[35] and NCS class of antitumor compounds appears to be different causing double and single DNA strand breaks, respectively. However, it has been found that at certain DNA sequences, such as AGC, NCS oxidizes C-1′ to form phospho-diester-linked 2-deoxyribonolactone,[36] generating an abasic site at the C residue and a direct strand break at the T residue, two nucleotides to the 3′ side on the complementary strand[37,38] *i.e.*, a double-stranded lesion suggesting H abstraction from two different sites.[27] The involvement of the second radical in the postulated intermediacy of a labile NCS-oxygen-DNA adduct of the type involving a peroxide link between C-2 or C-6 of **4a** and C-5′ of ribose seemed at first attractive but its susceptibility to thiol-induced decomposition[33,34,37,39] leading to a thiol adduct of the type **4a** (implicating hydrogenolysis of a vinyloxy or phenoxy C–O bond) is difficult to envisage.



12 X=Cl
13 X=OMe
14 X=SCH_2COOMe

Ar and R denote the naphthoate and N-methyl-fucosamine moieties as in **1** respectively.

Experimental

^1H and ^{13}C NMR spectra were recorded on a Varian SC-300 spectrometer in CD_3COOD at 25°C using TMS as internal standard.

$NaBH_4$ or $NaBD_4$ and methyl thioglycolate reaction products were isolated by methods previously

reported.[4] In each case, dialyzed, lyophilized NCS protein (~0.01 mM) was treated in 0.1 M acetic acid (or CD_3COOD) with excess $NaBH_4$ or $NaBD_4$ (~300 equivalents) and methyl thioglycolate (2~5 equivalents) respectively.

^{13}C NMR chemical shifts and $^1J_{CH}$ (Hz) were obtained for indene in CD_2Cl_2: 39.5 t (128), 121.3 d (159), 124.1 d (157), 125.0 d (159), 126.6 d (159), 132.4 d (165), 134.7 d (169), 144.2 s, 145.3 s.

Acknowledgments

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