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NEOCARZINOSTATIN: CHEMICAL CHARACTERIZATION AND PARTIAL STRUCTURE OF THE NON-PROTEIN CHROMOPHORE

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Summary

The molecular formula $C_{35}H_{35}NO_{12}$ (mol.wt. 661) is proposed for the biologically active chromophoric component of neocarzinostatin. The partial structure 2 is proposed based on ^1H NMR and mass spectral data and consists, in part, of a 2,6-dideoxy-2-methylamino-galactose moiety and a naphthoic acid derivative. Special treatments required to obtain spectral data of the labile chromophore are described.

Neocarzinostatin (NCS[‡]), an acidic antitumor antibiotic (1), causes DNA strand breakage *in vivo* and *in vitro* in a reaction greatly stimulated by a sulfhydryl compound (reviewed in ref. 2). Napier et al. (3) have shown that NCS consists of two components, a protein (10,700 daltons), previously sequenced (1) and thought to be the active drug, and a very labile nonprotein chromophore extractable with methanol. Kappen et al. (4) have shown that the isolated chromophore possesses the full biological activity of NCS. The apoprotein protects the chromophore from inactivation and controls its release (4-7). Recently others have also reported the isolation of a chromophore of variable activity from NCS (8,9). In this communication we wish to report the results of our initial investigation of the structure of the chromophore group.

Abbreviations: NCS, neocarzinostatin; HPLC, high pressure liquid chromatography; TMS, SiC_3H_8; BSTFA, bis-trimethylsilyl-trifluoroacetamide; EDAX, energy dispersive X-ray fluorescence; NMR, nuclear magnetic resonance; ppm, parts per million; IR, infrared.

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MATERIALS AND METHODS

The starting material for these investigations consisted of clinical ampules of NCS, each containing approximately 1.3 mg of the drug in 2 ml 0.015 M sodium acetate, pH 5.0. The chromophore amounts to about 6% of the weight of NCS, or 78 µg per ampule. Lyophilized ampule contents were extracted with methanol at 0°C in the dark, the protein separated by centrifugation, and the supernatant subjected to HPLC on a reverse phase column in 0.01 M ammonium acetate, pH 4.0, in 50-80% methanol. Significant losses were incurred under these conditions due to the intrinsic high reactivity of the compound free of its apoprotein. On an analytical scale, higher yields of a more homogeneous, active product were obtained by extraction with glacial acetic acid in which the chromophore remained sufficiently stable for analysis Lyophilized ampule contents were first dialyzed against distilled water, lyophilized and redissolved in D_2O (H_2O for mass spectral studies) and again lyophilized. This material, approximately 10 mg, was extracted at room temperature with about 1 ml of glacial CD_3COOD (CH_3COOH for mass spectral and HPLC analysis, and bioassay) and, after concentration under reduced pressure, was directly analyzed by ^1H NMR at 300 MHz. The chromatographic analysis showed the presence of a major and varying amounts (10-20%) of a minor component, both shown to possess *in vitro* DNA strand scission activity (manuscript in preparation). By comparison with analyses on a homogeneous sample obtained from HPLC, the minor component did not manifest itself clearly in ^1H NMR or mass spectra. We, therefore, chose to work with the material without further separation. HPLC analyses of methanol extracts (0°C) of NCS showed several additional minor peaks (4,7). Extraneous signals were also observed in ^1H NMR spectra in CD_3OD. However, a comparison of these spectra with those obtained on acetic acid solutions proved valuable in the unambiguous assignment of the observed signals to a total of 29 non-exchangeable protons (Table I).

The NCS chromophore requires derivatization in order to yield mass spectra. Samples of the above described NCS extracts were evaporated to dryness at 0°C and the residue treated with BSTFA or $^2H_{18}$-BSTFA in dimethylformamide or pyridine. These reaction mixtures were analyzed from the direct probe inlet system of the mass spectrometer or via the gas-chromatographic inlet system using a short 1% Dexsil 300 on 100/120 Supelcoport column at about 200°C. Highly reproducible partial spectra, representing about one-third of the molecule, were obtained by either method. Substantial pyrolyzed residue remained in the sample tube. Analogous results were obtained by field-desorption mass spectrometry. The low volatility of the compound was attributed either to a higher than expected molecular weight, e.g. of a disulfide dimer, or to polymerization of the highly reactive compound prior to derivatization. Solutions of the chromophore in methanol or the residue after evaporation of glacial acetic acid solutions were, therefore, briefly exposed to 10 mM methylmercaptan in methanol at 0°C, re-evaporated and trimethylsilylated. By this method highly reproducible mass spectra of the methylmercaptan addition product of the NCS chromophore were obtained.

RESULTS AND DISCUSSION

Mass spectra of TMS derivatives obtained without prior mercaptan treatment show molecular ions of the di-TMS derivative of a compound of the elemental composition $C_{13}H_{12}O_4$ (M^+376.1528, calc. 376.1526; ^2H analog: M^+ 394). The compound appears to be identical with 2-hydroxy-5-methoxy-7-methyl-1-naphthoic acid which has recently been obtained as the methyl ester

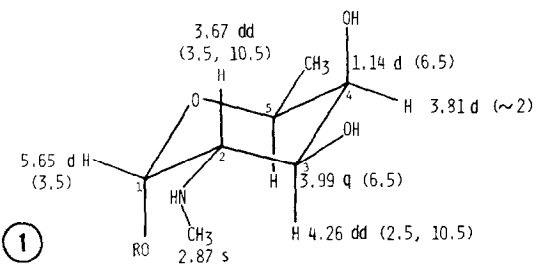
by Edo et al. (10) by treatment of NCS chromophore with sodium methoxide in methanol. Trimethylsilylation after treatment with methylmercaptan resulted in abundant ions of m/e 951.3883 and 997.3743, both containing four TMS groups (^{2}H analogs: m/e 987 and 1033). The analogous reaction with $HSCH_2COOCH_3$ resulted in ions at m/e 951 and 1055. The exact masses correspond to the compositions (excluding TMS groups) $C_{35}H_{37}NO_{12}$ (calc. 951.3897) and $C_{36}H_{39}NO_{12}S$ (calc. 997.3774). The mass spectrometric loss of thioaldehydes, at least in the case of enolic (11) and aromatic thioethers (12) and thioglycollic acids and esters (13) has been reported. The abundant high mass fragment at m/e 646.2695 ($C_{22}H_{24}NO_8 \cdot TMS_3$; calc. 646.2688) results from the loss of the naphthoic acid moiety as $C_{13}H_{13}O_4 \cdot TMS_1$ from the m/e 951 ion. Analysis of the corresponding mercaptan experiment in CD_3OD indicated that a reduction did not take place. Thus addition of methyl mercaptan has occurred and is corroborated by EDAX analysis which showed no sulfur in the native chromophore. This is also supported by molecular weight determinations by ultracentrifugation, both before and after treatment with methylmercaptan, which in each case gave values close to 650 daltons. These results, therefore, indicate that NCS chromophore has the elemental composition $C_{35}H_{35}NO_{12}$, mol. wt. 661. The tetra-TMS derivative indicates a minimum of four exchangeable hydrogens, and we assume that none of these has been newly formed in the mercaptan addition reaction. Further analysis of the mass spectra indicates that at least one more active hydrogen functionality is not available for trimethylsilylation, probably for steric reasons.

Twenty-nine of the remaining thirty protons are non-exchangeable and clearly observed. Their mutual relationships as defined by ^{1}H NMR analysis are listed in Table I. Signals attributable to the naphthoic acid derivative are indicated by a single asterisk. A second group of seven signals, identified by double asterisks, are assigned to a 2,6-dideoxy-2-methylamino-galactose shown in partial structure 1 (Fig. 1). This structure is compatible with all observed coupling constants, including the near-zero coupling between H4 and

TABLE 1. ^{1}H NMR Data on NCS Chromophore in CD_3CO_2D (25°C) and CD_3OD (~ 0°C, spiked with CD_3CO_2D)[a]

δ (CD_3CO_2D)		δ (CD_3OD)	
**1.14 d	(3H, J=6.5)	1.25 d	(3H, J=6.5)
*2.49 s	(3H)	2.60 s	(3H)
**2.87 s	(3H)	2.90 s	(3H)
**3.67 dd	(1H, J=3.5, 10.5)	~3.52 m	(obsc)
*3.73 s	(3H)	3.82 s	(3H)
**3.81 d	(1H, J≈2)	3.75 d	(1H, J≈2)
3.98 s	(1H)	4.16 s	(1H)
**3.99 q	(1H, J≈6.5)	4.03 q	(1H, J=6.5)
**4.26 dd	(1H, J=2.5, 10.5)	4.00 m	(1H)
4.42 dd	(1H, J=5, 9.5)	4.54 dd	(1H, J=5, 9)
4.62 dd	(1H, J=8, 9.5)	4.71 t	(1H, J≈8.5)
4.78 dd	(1H, J=5, 8)	4.87 dd	(1H, J=5, 8.5)
5.00 br.s	(1H)	5.06 br.s	(1H)
**5.65 d	(1H, J=3.5)	5.71 d	(1H, J≈3)
5.68 br.s	(1H)	5.88 br.s	(1H)
6.18 br.s	(1H)	6.23 br.s	(1H)
6.69 br.s	(1H)	6.81 br.s	(1H)
*6.82 br.s	(1H)	6.90 br.s	(1H)
*6.94 d	(1H, J=9)	7.03 d	(1H, J=9.5)
*7.81 br.s	(1H)	7.50 br.s	(1H)
*8.01 d	(1H, J=9)	8.09 d	(1H, J=9.5)

[a]Chemical shifts (δ) are given in ppm downfield of internal tetramethylsilane. Coupling constants in brackets are in Hz. Abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, obsc = obscured, br. = broad. Resonances marked by a single asterisk are those assigned to the naphthoic acid derivative. Those with a double asterisk are assigned to the aminohexose moiety.



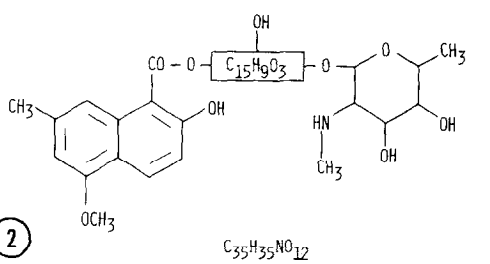


Figure 1. ^{1}H NMR assignments of the 2,6-dideoxy-2-methylamino-galactose substructure of the NCS chromophore. The chemical shift (δ, ppm) and multiplicity of each resonance with coupling constants (Hz) in parentheses are indicated next to the appropriate proton (see Table I). Ring carbon atoms are numbered 1-5.

Figure 2. Proposed partial structure of the NCS chromophore, elemental composition $C_{35}H_{35}NO_{12}$.

H5 (14). Abundant fragments in the mass spectra of the tetra-TMS derivative at m/e 320.1708 (^{2}H analog: m/e 338) and m/e 304.1755 (^{2}H analog: m/e 322) expected for partial strucutre 1, correspond to the compositions $C_7H_{14}NO_4 \cdot TMS_2$ (calc. 320.1713) and $C_7H_{14}NO_3 \cdot TMS_2$ (calc. 304.1764), respectively. Ions of m/e 175.0825 ($C_4H_7O_3 \cdot TMS_1$; calc. 175.0790) and 145.0904 ($C_3H_7NO \cdot TMS_1$; calc. 145.0923) are further breakdown products of m/e 320. With respect to the total active hydrogen count of NCS chromophore, it may be added that appropriate acetylation leads to triacetyl fragments of m/e 302.1234 (calc. 302.1240 for $C_{13}H_{20}NO_7$) and 286.1286 (calc. 286.1291 for $C_{13}H_{20}NO_6$) corresponding to the di-TMS fragments of m/e 320 and 304.

The mass spectral and ^{1}H NMR data describe the NCS chromophore in considerable detail and lead to the working hypothesis which is shown in partial structure 2 (Fig. 2). Treatment of NCS chromophore with ammonia, after exposure to mercaptan causes ammonolysis within the C_{15}-substructure, indicating a reactive carbonyl function. This carbonyl group may be the origin of the reported (8) IR absorption of NCS chromophore at about 1780 cm^{-1}. Furthermore, ammonolysis removes the naphthoic acid as the amide. These reactions do not affect the characteristic mass spectral fragments of the aminohexose moiety. The C_{15}-substructure is also responsible for the uptake of mercaptan and is associated with the so far unassigned ^{1}H NMR absorption of eight protons (Table I). Details of the above mentioned reactions and further interpretations will be the subject of a forthcoming paper.

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