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Neocarzinostatin Chromophore

Assignment of Spectral Properties and Structural Requirements for Binding to DNA

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SUMMARY

The site responsible for the mercaptan (or borohydride)-stimulated DNA scission activity of neocarzinostatin chromophore (NCS-Chrom) is located in the central C_{12}-subunit of the molecule. This has been determined by studies of the characteristic spectral properties of the chromophore and its reduction products and of the spectral changes induced by their interaction with its apoprotein (apo-NCS) and DNA. The UV-visible absorption, fluorescence, CD, and MCD spectral properties of the major nonprotein chromophoric component of neocarzinostatin (NCS-Chrom *A*) are assigned to its component substructures, the 2-hydroxy-5-methoxy-7-methyl-1-naphthoate, the five-membered cyclic carbonate ring (1,3-dioxolan-2-one), the 2,6-dideoxy-2-methylaminogalactose, and the incompletely defined C_{12}-subunit which links the other three residues. Although the major source of its UV-visible absorption is the naphthoic acid residue (HNA-NCS), a significant absorption from ~260 to 330 nm is due to the presence of the highly unsaturated C_{12}-subunit. The presence of the C_{12}-subunit and, to a lesser extent, the cyclic carbonate reduces the intensity of the fluorescence emission of the fluorophore of NCS-Chrom *A*, the HNA-NCS subunit. The CD activity of NCS-Chrom *A* is also due to the presence of the C_{12}-subunit. The MCD activity of NCS-Chrom *A*, however, is completely accounted for by the naphthoic acid residue. A role for the C_{12}-subunit in the binding of NCS-Chrom to DNA or apo-NCS is indicated by the resultant absorption hypochromicity in the region assigned to the C_{12}-moiety. Limited modification of the C_{12}-subunit (by mercaptan or borohydride) inactivates the chromophore for DNA strand scission, although both products still bind DNA. The naphthoic acid residue alone is not sufficient for binding to DNA. Therefore, the intercalation of the naphthoate residue between DNA base pairs requires the binding of NCS-Chrom to DNA probably via electrostatic interaction between the positively charged 2-methylamino group of the galactose residue and the negatively charged oxygens of the phosphate in the DNA backbone. The C_{12}-unit is viewed as forming a short-lived reduction-activated species that, in the presence of oxygen, causes single-strand breaks in DNA. The cyclic carbonate residue is not required for *in vitro* DNA strand scission activity but affects its stability with respect to hydrolysis and reactivity with mercaptan. If DNA is absent the activated species decompose to inactive products, including a mercaptan (or hydrogen) addition product of the C_{12}-subunit.

INTRODUCTION

The antitumor antibiotic NCS[2] is a 1:1 complex of a and *B*, nonprotein chromophoric components extracted from clinical NCS; NCS-Chrom *C*, methanolysis product of *A*; apo-NCS, protein component of NCS; NCS-Chrom *D*, pH 8 hydrolysis product of NCS-Chrom *A*; HNA-NCS, 2-hydroxy-5-methoxy-7-methyl naphthoic acid isolated from NCS-Chrom; Me-HNA-NCS, methyl ester of HNA-NCS; NCSAS-1 and NCSAS-2, isolated methyl mercaptan and methyl thioglycolate reaction products of NCS-Chrom *A*, respectively; NCSAH, isolated sodium borohydride reaction product of NCS-Chrom *A*; HPLC, high-performance liquid chromatography; MCD, magnetic circular dichroism; CD, circular dichroism; RFI, relative fluorescence intensities; pH_m, pH meter readings of solutions containing high methanol concentrations.

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[2] The abbreviations used are: NCS, neocarzinostatin; NCS-Chrom *A*

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highly unsaturated nonprotein chromophoric compound and an acidic protein (M_r 10,700) (1, 2). The isolated chromophoric material, NCS-Chrom, possesses all of the biological activity of NCS (3, 4) and is very labile (5, 6). The chromophore-free protein is inactive by itself, but binds the chromophore tightly ($K_d \sim 10^{-10}$) and specifically (6); it both stabilizes the chromophore and controls its release for interaction with DNA (3–6). Extraction of lyophilized NCS with acidic methanol removes essentially 100% of the bioactivity into the methanol, with 90% of the *in vitro* activity located in a single component, NCS-Chrom *A*, and the remaining 10% in a second component, NCS-Chrom *B*, of equal *in vitro* DNA strand scission activity but only 5% of the *in vivo* activity of NCS-Chrom *A* (2). NCS-Chrom *A* consists of three subcomponents, 2-hydroxy-5-methoxy-7-methyl-1-naphthoate, 2,6-dideoxy-2-methylaminogalactose, and a five-membered cyclic carbonate ring (1,3-dioxolan-2-one), all of which are linked to a 12-carbon (C_{12})-containing subunit of incompletely defined structure (7, 8). NCS-Chrom *B* is formally equivalent to a hydrolysis/decarboxylation product of NCS-Chrom *A* and possesses a diol-residue of the cyclic carbonate subunit (8). A third biologically active derivative of NCS-Chrom *A* has been isolated, NCS-Chrom *C*, which is a methanolysis product of the cyclic carbonate of NCS-Chrom *A* with *in vitro* activity equal to that of *A* and 80% of the *in vivo* activity of NCS-Chrom *A* for Hela cell growth inhibition (2, 8).

Because of the lability of the C_{12}-subunit, it has not been possible to isolate it directly. Also, the high degree of unsaturation of the subunit has made completion of the structure assignment by NMR analysis difficult. Studies to complete the structure of NCS-Chrom are continuing. It is possible, however, to obtain substantial information on the active site of NCS-Chrom by studying its spectral properties and those of its derivatives which either do not possess this unit or possess it in a modified form. These results are used to interpret the spectral changes occurring on interaction of NCS-Chrom *A* with DNA and apo-NCS and permit assignment of functions to the different chemical components of NCS-Chrom.

MATERIALS AND METHODS

NCS (clinical ampules from Kayaku Antibiotics) was stored frozen in 0.015 M sodium acetate (pH 5). All chemicals were of reagent grade. ^{3}H-NaBH$_4$ (250 mCi/mmole) was purchased from New England Nuclear Corporation (Boston, Mass.). Absorption spectra were recorded on a Cary 219 spectrophotometer, and uncorrected fluorescence spectra were obtained on a Perkin-Elmer 512 spectrofluorometer. MCD spectra were measured in a Cary Model 61 CD instrument equipped with a Varian Model V4145 superconducting solenoid at a field strength of 40 kG. CD spectra for corresponding MCD spectra were measured under identical experimental conditions in the absence of a magnetic field.

NCS-Chrom *A* was prepared by extraction of lyophilized NCS with 0.1 M acetic acid in methanol and purified by HPLC. All studies were done using HPLC-purified NCS-Chrom *A* and its derivatives. HPLC was performed as described (2). The elution time of various chromophore derivatives is listed in Table 1. The absorption and fluorescence measurements for the pH titrations were made in a water-jacketed cuvette holder at 4° in a 1 × 1 cm cuvette fitted with a spinning bar and pH electrode. Additions of NaOH or HCl were made to a starting solution in 0.003 M ammonium formate (or 0.0014 M ammonium acetate), 0.01 M sodium citrate, and 0.01 M Tris in 10% methanol.

TABLE 1

Elution time of NCS-Chrom A and derivatives from a reverse-phase μC_{18} HPLC column

See Materials and Methods for chromatrography conditions

Compound	Elution time
	min
NCS-Chrom *A*	63
NCS-Chrom *B*	58.5
NCS-Chrom *C*	67
HNA-NCS[a]	8.5
Me-HNA-NCS	47
NCSAS-1[b]	47.5
NCSAS-2	46.5
NCSAH[c]	42

[a] The elution time of HNA-NCS is pH-dependent, eluting much more slowly as the pH of the elution buffer is reduced.

[b] The equivalent products for NCS-Chrom *B* and *C* elute at 41 and 51 min, respectively.

[c] The equivalent product for NCS-Chrom *B* elutes at 41 min. Two products which elute at 42.5 and 46 min are observed for NCS-Chrom *C*.

RESULTS

Preparation, Isolation, and Spectral Characterization of Derivatives of NCS-Chrom A

2-Hydroxy-5-methoxy-7-methyl-1-naphthoic acid and methyl ester. HNA-NCS is found by HPLC and mass spectral analysis[3] as a minor fraction in various NCS-Chrom preparations. Substantial yields of HNA-NCS are obtained by treatment of NCS-Chrom *A* with 1 N sodium hydroxide in 90% methanol for 2 hr at 85°, followed by acidification with acetic acid and separation of the products by HPLC. Mass spectral analysis after trimethylsilylation reveals a molecular ion with m/e 376, corresponding to the di-trimethylsilyl derivative of a compound of the elemental composition $C_{13}H_{12}O_4$,[3] identical with the 2-hydroxy-5-methoxy-7-methyl-1-naphthoic acid reported earlier (7, 9).

Me-HNA-NCS was isolated from an NCS-Chrom preparation obtained by methanol extraction of lyophilized NCS containing sodium acetate. NCS-Chrom *A* undergoes methanolysis to NCS-Chrom *C* on storage at −20° (2, 8). This conversion is accompanied by the formation of small quantities of Me-HNA-NCS. Mass spectral analysis after trimethylsilylation reveals a molecular ion with m/e 318, corresponding to the mono-trimethylsilyl derivative of a compound with elemental composition $C_{14}H_{14}O_4$.[3]

HNA-NCS exhibits characteristic absorption and fluorescence spectral properties similar to but not identical with those of NCS-Chrom *A* (see ref. 2 and Fig. 3a). Furthermore, for HNA-NCS and its methyl ester, these properties show a characteristic pH dependence reflect-

[3] J. Liesch, personal communication.

ing the state of ionization of the carboxyl and hydroxyl substituents. At $pH_m \sim 6$ (Fig. 1a), absorption maxima occur in the UV and visible and, in contrast to NCS-Chrom *A*, a minimum is present near 275 nm. The fluorescence excitation and emission maxima of HNA-NCS, at 335 and 435 nm, respectively, are similar to those for NCS-Chrom *A* (2), but the relative intensity is substantially greater (Table 2). At pH 2, there is a red shift and hyperchromicity of the visible absorption resulting in a spectrum similar to that of Me-HNA-NCS [Fig. 1a and reported by Edo *et al.* (9)]. There is absorption hypochromicity of HNA-NCS at pH 14 with respect to pH 4 from 300 to 345 nm. The fluorescence emission maxima at pH 2, 8, and 14 for HNA-NCS (435, 405, and 420 nm) (Table 2) correspond to similar maxima for 2-hydroxy-1-naphthoic acid (435, 407, and 423 nm) (10). The characteristic changes in absorption which occur as the pH is raised above 2 indicate that the carboxyl group of the HNA-NCS is protonated (—COOH) at this pH. A similar shift from 296 to 302 nm occurs for salicylic acid upon protonation of the carboxyl group ($pK_a = 3$) or its conversion to the methyl ester (11). Ionization of the hydroxyl group of 2-hydroxy-1-naphthoic acid (pK_a 11.9) results in a shift of the 334- and 296-nm bands to 352 and 322 nm, respectively (10). The pH-dependent absorption changes of HNA-NCS are completely reversible by adjustment of the pH, and a $pK_a \sim 11$ (calculated from the pH titration of the absorbance at 250, 290, or 315 nm) is estimated for the ionization of the hydroxyl group of HNA-NCS. Corresponding shifts are observed in the fluorescence excitation maxima as the ionization states change. The fluorescence emission maxima and intensity are more sensitive than the absorbance to the electronic state and are used to determine the pK of the ionization (Fig. 2; Table 2). The intensity of the fluorescence emission of HNA-NCS decreases sharply as the pH is raised from pH 2 to 4, and further to pH 8. Near pH 8 the emission maximum shifts from 435 to 405 nm (Fig. 2; Table 2). Lowering to pH < 2 also results in a sharp reduction in the fluorescence intensity, but with no shift in the excitation or emission maxima. In strong alkali (Fig. 2; Table 2), the fluorescence intensity increases with respect to pH 8 and the emission maximum shifts to 420 nm. The changes in the excitation maxima correspond to the pH-dependent absorption changes. The fluorescence changes are completely reversible by lowering the pH. By analogy with the pH-dependent fluorescence changes of 2-hydroxy-1-naphthoic acid (10) and consistent with the absorption changes observed from pH 2 to 4 noted above, the fluorescence changes observed as the pH is increased most likely reflect the stepwise ionization of the hydroxynaphthoic acid cation (protonated carboxyl group) to the neutral molecule (pH 2), followed by dissociation of the carboxyl group to the singly charged anion (pH 2–4), and finally ionization of the phenolic hydroxyl group ($pK_a = 11$) (Scheme 1). 2-Hydroxy-1-naphthoic acid has fluorescence emission maxima at 435, 407, and 423 nm at pH < 1, pH 6, and pH 14, respectively (10).

HNA-NCS possesses strong MCD bands with minima at 245, 280 (sh), and 310 nm and maxima at 260 and 345 nm at $pH \sim 6$ in methanol (Fig. 1b). The visible bands

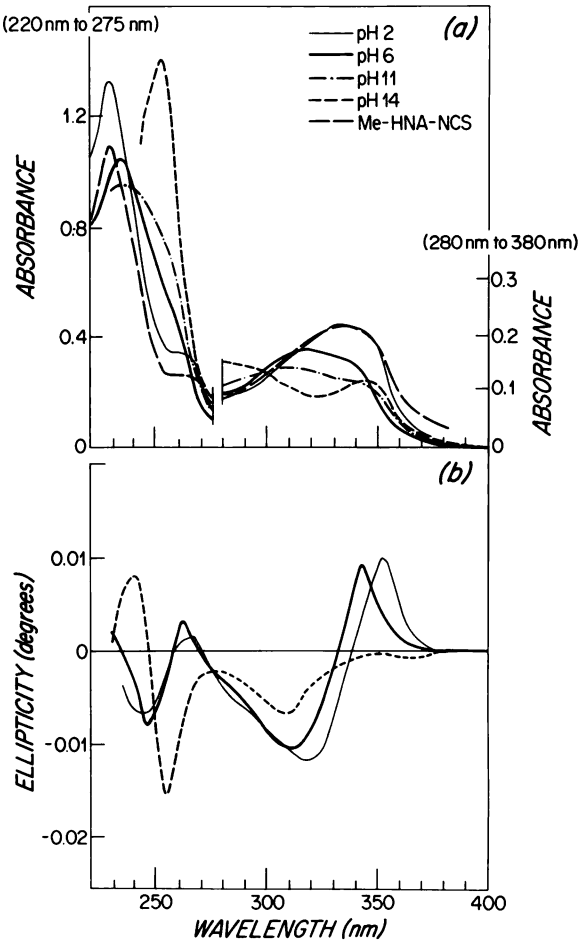


FIG. 1. *pH dependence of the absorption and MCD of the 2-hydroxy-5-methoxy-7-methyl-1-naphthoic acid and the absorption spectrum of its methyl ester*

a. HNA-NCS in 100% methanol, $pH_m \sim 6$ (—); addition of NaOH to 1 N (– – –); followed by addition of 10 N HCl to pH 2 (——). Spectrum of HNA-NCS at pH 11 (–·–). Absorption spectrum of Me-HNA-NCS in methanol (— —). Absorption spectra of HNA-NCS in 10% and 100% methanol and at 4° and 25° are similar.

b. The MCD spectrum of HNA-NCS. Spectra were obtained on a single sample of HNA-NCS ($A_{340} = 0.16$) in 100% methanol, $pH_m \sim 6$ (—); then NaOH added to 1 N (– – –), followed by addition of 10 N HCl to pH 2 (——).

are similar in shape but are red-shifted with respect to the corresponding bands of NCS-Chrom *A* (Fig. 3b and ref. 2). The MCD spectrum is sensitive to the substituents on the naphthalene, since 1-naphthoic acid, 2-naphthol, and 2-hydroxy-3-naphthoic acid exhibit very different MCD spectra (data not shown). In 1 N sodium hydroxide, the MCD spectrum changes dramatically. The 345-nm band is absent; maxima occur at 240 and 275 nm, and minima are at 255 and 310 nm. At pH 2 the MCD spectrum is similar in shape to the spectrum at pH 6, but the presence of the uncharged carboxyl group is reflected in the red shift of the visible bands. This spectrum is closer to that of NCS-Chrom *A*, NCSAS, and NCSAH (each an ester of HNA-NCS) (see below) than that for the ionized carboxylate species at pH ≥ 4.

Methylmercaptan (NCSAS-1) and methylthioglycolate (NCSAS-2) products of NCS-Chrom A. While the *in*

TABLE 2

Fluorescence spectral characterization of derivatives of NCS-Chrom A

Compound	Solvent	pH_m[a]	Fluorescence maxima		RFI[b]/A_{340}
			Excitation	Emission	
NCS-Chrom *A*	0.01 M Ammonium acetate, 65% methanol	5	250(sh), 270, 340	430	7
	1 N Sodium hydroxide, 10% methanol	14	250, 300, 340	420	4
NCS-Chrom *B*	0.01 M Ammonium acetate, 65% methanol	5	240, 270, 340	430	12
NCS-Chrom *C*	0.01 M Ammonium acetate, 65% methanol	5	240, 270, 340	430	15
HNA-NCS[c]	0.1 M HCl, 10% methanol	2	240, 260, 335	435	194
	1 M Ammonium acetate, 10% methanol	4	260, 335	435	99
	1 M Tris, 10% methanol	8	250–260, 330	405	46
	0.1 M Sodium hydroxide, 10% methanol	12	250, 300, 345	420	53
	1 M Sodium hydroxide, 10% methanol	14	250, 300, 345	420	65
Me-HNA-NCS[c]	0.1 M HCl, 10% methanol	2	240, 265, 335	435–440	120
	1 M Ammonium acetate, 10% methanol	4	250, 265, 335	435	132
	1 M Tris, 10% methanol	8	240–260, 330	430	85
	0.1 M Sodium hydroxide, 10% methanol	12	250, 300, 340	420	8
	1 N Sodium hydroxide, 10% methanol	14	250, 300, 340	420	8
NCSAS-1[c]	0.02 M Sodium citrate, 10% methanol	4	250(sh), 270, 335	440	130
	0.02 M Tris, pH 8, 10% methanol	8	240, 270, 335	440	90
NCSAS-2	0.02 M Sodium citrate, 10% methanol	4	250(sh), 270, 335	440	121
	0.02 M Tris, 10% methanol	8	240, 270, 335	440	72
NCSAH	0.02 M Sodium citrate, 10% methanol	4	250, 270, 340	440	120
	0.02 M Tris, 10% methanol	8	240, 270, 340	440	75

[a] Measured on standard pH meter with no correction for methanol concentration.
[b] RFI, relative fluorescence emission intensity measured with excitation at the long wavelength maximum. See Materials and Methods.
[c] See Fig. 2.

vitro DNA scission activity of methanol-extracted NCS-Chrom is highly dependent on the presence of a mercaptan (ref. 3 and reviewed in ref. 12), the chromophore is rapidly inactivated by the mercaptan in the absence of DNA (5). An intensely blue fluorescent (Table 3) product forms upon addition of the mercaptan to the C_{12}-subunit (7, 8). This is associated with absorption hypochromicity between 270 and 330 nm and increased absorption from 330 to 400 nm (Fig. 3a). A study of the kinetics of the fluorescence increase of NCS-Chrom *A*, *B*, and *C* in 0.02 M methylmercaptan/0.09 M ammonium acetate (pH 6.5) in 70% methanol shows a mean lifetime, τ, of $A < C < B$, the same relative order as of the rate of hydrolytic

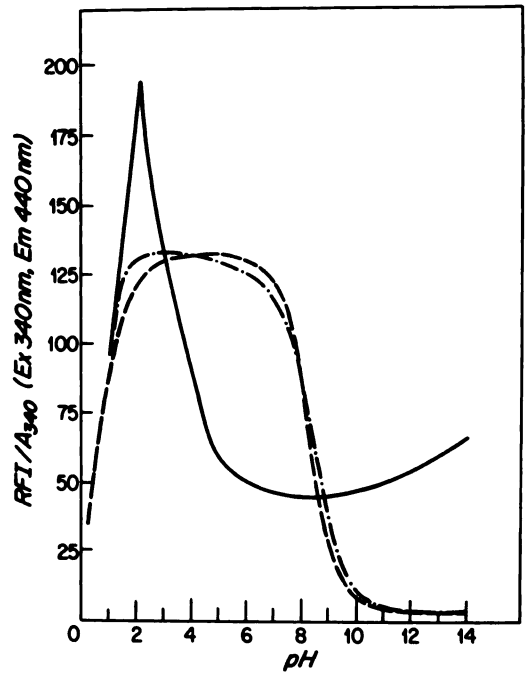


FIG. 2. *pH titration of the relative fluorescence intensity per absorbance at 340 nm of HNA-NCS (——), Me-HNA-NCS (– – –), and NCSAS-1 (-·-·)*

The absorbance at 340 nm for NCS-Chrom *A*, HNA-NCS, Me-HNA-NCS, and NCSAS-1 are 0.03, 0.012, 0.082 and 0.016, respectively, in 10% methanol. The fluorescence intensities were measured at 0° with excitation and emission wavelengths of 340 and 430 nm, respectively. See Materials and Methods.

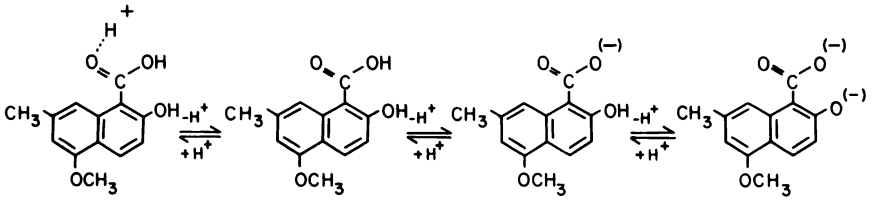
TABLE 3

Relative fluorescence intensity of NCS-Chrom A treated with methylmercaptan at pH 5 and 6.5

Fluorescence spectral data were obtained from recorded, uncorrected excitation and emission spectra excitation maxima at 340 nm and emission maxima at 440 nm, at the indicated times. All samples contained NCS-Chrom *A* (A_{340} = 0.12, A_{330} = 0.15), purified by HPLC as described under Materials and Methods, in 0.02 M ammonium formate (pH 4)/70% methanol. Equivalent volumes of methanol were added for sodium acetate and mercaptan additions.

Treatment condition	pH_m[a]	RFI_{440}/A_{330}	
		1.75 hr	5.5 hr
None	5	7	10
0.1 M Sodium acetate[b]	6.5	10[c]	24[c]
0.033 M CH_3SH, 0.1 M sodium acetate[d]	6.5	113	123
0.033 M CH_3SH[e]	5	54	104

[a] As measured with standard pH meter with no correction for the high methanol concentration.
[b] Addition of sodium acetate in methanol.
[c] Fluorescence increase is measured at the shoulder of the 490-nm fluorescence generated at this pH.
[d] Addition of sodium acetate in methanol, followed by addition of methylmercaptan in methanol.
[e] Addition of methylmercaptan in methanol.



SCHEME 1

decomposition at pH 8 (Table 4). The reaction of NCS-Chrom *A* with mercaptan is pH-dependent, occurring slowly at low pH and more rapidly near pH 7 (Table 3). The high methanol concentration was chosen to reduce the competitive spontaneous hydrolytic loss of active chromophore (6) [i.e., formation of NCS-Chrom *D* with 490-nm fluorescence (2)]. Treatment of HNA-NCS with methyl mercaptan under similar conditions causes no absorption or fluorescence changes, indicating that the changes observed above are due to the reaction at a residue other than the naphthoic acid. Furthermore, since NCS-Chrom *B* and *C* are also activated for DNA strand scission by mercaptan (2), the intact cyclic carbonate subunit is not required for this activity.

The CD activity of the NCS-Chrom *A* is substantially reduced (about 10% residual ellipticity at 260 nm) by the reaction with mercaptan (Fig. 3c). The presence of a negative CD band at 238 nm observed for native NCS treated with mercaptan (ref. 13 and confirmed in this laboratory) is not seen for the protein-free chromophore treated with mercaptan, although the residual CD activity may be related. There is virtually no change in the 355-nm MCD band of NCS-Chrom *A* with mercaptan treatment (Fig. 3b), further supporting the absence of change in the chromophoric unit responsible for this activity (i.e., the naphthoate moiety) by the reaction of mercaptan with NCS-Chrom *A*.

Reverse-phase HPLC of the products of mercaptan-treated NCS-Chrom *A* separates a major UV-absorbing and intensely blue fluorescent peak which elutes at 47.5 min (Table 1), several minor intensely blue fluorescent peaks, and no peak eluting as active NCS-Chrom *A*. The major product (NCSAS-1) is an addition product of the mercaptan to the C_{12}-subunit of NCS-Chrom *A*, with no apparent structural change in the naphthoic acid and galactose residues as shown by ^{1}H-NMR and mass spectral analyses (7, 8).[4] Maxima occur in the absorption spectrum of NCSAS-1 at 265, 275 (sh), 330, and 350 (sh) nm. The CD spectrum of NCSAS-1 is reduced in magnitude and is qualitatively different from that of NCS-Chrom *A*. The intense blue fluorescence (Table 2) and absorption spectral properties (Fig. 4a) are consistent with those of an ester of the naphthoic acid. The pH titration profile of NCSAS-1 is virtually identical with that of Me-HNA-NCS (Fig. 2), $pK_a \sim 8.5$, indicating that the presence of the sugar and modified C_{12} residues does not affect the naphthoate ionization characteristics. The stability of this derivative is reflected in the reversibility of the pH titrations.

In contrast to the high pH required for methyl mercaptan and 2-mercaptoethanol to react with NCS-Chrom *A*, methylthioglycolate reacts very quickly with NCS-Chrom *A* at pH ≤ 4, facilitating the preparation of more concentrated samples without the competing formation of NCS-Chrom *D*. The major product, NCSAS-2, elutes at 46.5 min (Table 1) and is structurally similar to NCSAS-1.[4] The absorption, fluorescence, and CD spectral properties of NCSAS-2 (Fig. 4; Table 2) are very similar to those of NCSAS-1. The CD spectrum exhibits negative extrema at 275 and 285 nm, and positive extrema at 310, 320, and ~ 340 nm (sh). The MCD spectrum (Fig. 4b) also shows the characteristic HNA-NCS pattern with extrema and intensities similar to those of the reaction mixture of NCS-Chrom *A* and methylmercaptan.

Sodium borohydride reduction product. The requirement for mercaptan in the *in vitro* DNA strand scission activity of NCS-Chrom *A* can be replaced by the presence of $NaBH_4$[5] as was reported for native NCS (12). Pretreatment of NCS-Chrom *A* with $NaBH_4$ in the absence of DNA, as with mercaptan, results in its inactivation.[5] Furthermore, changes in the absorption and fluorescence spectral properties resulting from borohydride treatment are similar to those observed for mercaptan treatment. It was of interest, therefore, to compare the products of the reaction mixtures by HPLC, to characterize the major product from the borohydride reaction, and to compare their interaction with DNA.

HPLC analysis of the reaction products NCS-Chrom *A* with $NaBH_4$ shows an intensely fluorescent major peak eluting at 42 min (NCSAH), HNA-NCS at 8.5 min, and a peak at 13 min with very low fluorescence intensity and other minor peaks. Similar products are formed with NCS-Chrom *B* and *C*, except that with NCS-Chrom *C* two major products are formed (Table 1). Reduction with [^{3}H]$NaBH_4$ results in the radiolabel's eluting with the major product of NCS-Chrom *A*, *B*, and *C* (two peaks). The ^{1}H-NMR and mass spectra of NCSAH are similar to those of NCSAS.[4]

Reaction with $NaBH_4$ instead of mercaptan appears to affect only the CD activity of the molecule (Table 2; Fig. 5). The absorption (264, 272, 285, 320, 330, and 350 (sh) nm), fluorescence excitation (250, 270, and 340 nm), and emission (440 nm) maxima, intensity, and MCD extrema [315 (neg) and 360 (pos) nm] are very similar to those of NCSAS-1 and -2. The ultraviolet CD spectrum is different from that of NCSAS-1 and -2 with a positive maxima at 275, but the maxima in the visible, 310 and 350 (sh) nm, are similar to those of the mercaptan products (Fig. 5).

[4] O. Hensens, J. Liesch, *et al.*, manuscript in preparation.
[5] L. S. Kappen and I. H. Goldberg, unpublished observation.

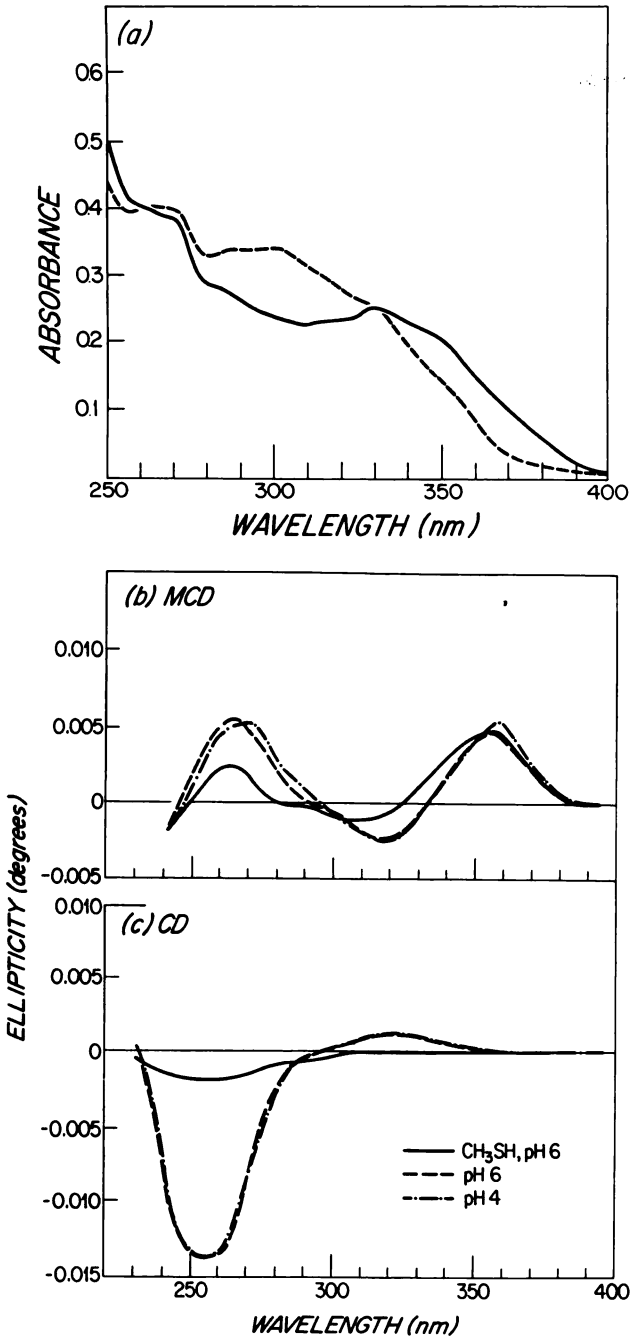


FIG. 3. *Effect of methylmercaptan treatment on the absorption, MCD, and CD spectra of NCS-Chrom A*

a, Absorption spectrum of NCS-Chrom *A* (- - -), treated with 0.02 M methylmercaptan, 4.5 hr, at 4°, in 0.1 M sodium acetate/0.02 M ammonium formate/70% methanol, pH 6 (——); MCD (b) and CD (c) spectra were obtained on a single sample of NCS-Chrom *A* (A_{340} = 0.12) in 0.02 M ammonium formate, pH 4 (-·-) in 85% methanol; addition of 0.1 M ammonium formate, pH 6 (- - -), followed by addition of methylmercaptan to 0.01 M at 4° for 1.5 hr (——). Spectra were obtained at ambient temperature.

pH Titration of Absorption and Fluorescence Spectral Properties of NCS-Chrom A

Although HNA-NCS is clearly the predominant source of the absorption, fluorescence, and MCD properties of NCS-Chrom *A*, there still remain spectral properties unaccounted for by the naphthoate or its ester. Also, although HNA-NCS and Me-HNA-NCS possess functional groups that reversibly ionize without deterioration of the original spectral properties, NCS-Chrom *A* possesses labile spectral properties (as well as labile biological activity). In aqueous 10% methanol, at pH > 4, the absorption, fluorescence, CD, and MCD spectra of NCS-Chrom *A* change immediately (Fig. 6 and ref. 2). This rapid deterioration of spectral properties at pH 7 is inhibited in 90% methanol, in agreement with experiments on the stability of the biological activity of NCS-Chrom in solutions of high methanol concentrations (5). Titration of the absorption spectrum (Fig. 6a and b) from pH 3.6 to 8 reveals an absorption decrease at 250 and 300 nm (and an increase at 380 nm) which resembles the pH inactivation profile of isolated chromophore previously reported (5). The appearance of the 380-nm absorption correlates with the appearance of 490-nm fluorescence (with excitation maximum at 380 nm) and is due to the formation of the apparent hydrolysis product of NCS-Chrom *A* (NCS-Chrom *D*, described in ref. 2) and is irreversibly destroyed at pH > 11. The presence of the naphthoate residue in the degradation products is indicated by the maxima at 250, 280 (sh), and 350 nm observed at pH ≥ 12. If the sample is brought to pH 14 directly, no NCS-Chrom *D* is formed. Irreversible degradation of the active chromophore occurs as the pH is raised above 4, since attempts to reverse the changes by returning to pH ≤ 4 immediately after an increase of ≤ 1 pH unit were unsuccessful. However, the changes from pH 8 to 12 attributable to the naphthoate are completely reversed by lowering the pH.

Binding of Derivatives of NCS-Chrom A to Calf Thymus DNA

The binding of methanol-extracted NCS-Chrom and its 2-mercaptoethanol reaction product(s) (6) and of HPLC-purified NCS-Chrom *A* (2) to DNA and evidence for the intercalation of the naphthoic acid residue of NCS-Chrom into DNA (14) have been reported. With

TABLE 4

Relative reaction rates of NCS-Chrom A, B, and C with methylmercaptan and with aqueous pH 8 buffer

Chromophore	τ		
	Mercaptan[a]		pH 8, aqueous[b]
	RT	0°	0°
		min	
NCS-Chrom *A*	2.5	14	0.6
NCS-Chrom *B*	10	68	2.2
NCS-Chrom *C*	7	30	1.2

[a] Chromophore samples *A*, *B*, and *C* (A_{340} = 0.05, 0.05, and 0.04) in 0.02 M methylmercaptan/0.09 M ammonium acetate in 70% methanol, pH_m = 6.5. Fluorescence emission monitored at 440 nm (excitation at 340 nm).

[b] Chromophore samples *A*, *B*, and *C* (A_{340} = 0.008, 0.007, and 0.015) in 0.08 M Tris (pH 8) in 13% methanol. Fluorescence emission monitored at 490 nm (excitation at 380 nm).

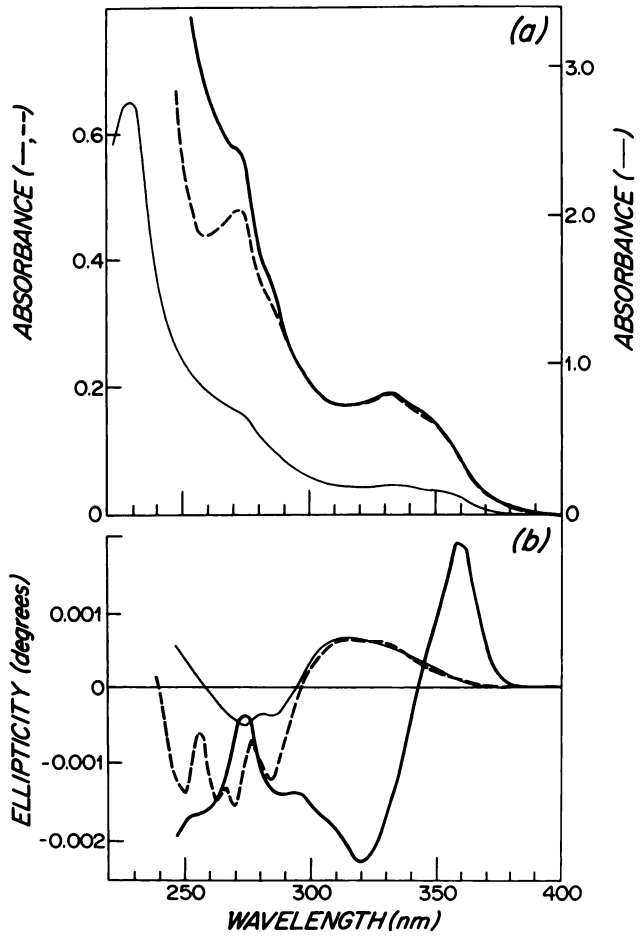


FIG. 4. *Absorption, MCD, and CD spectra of purified mercaptan addition products of NCS-Chrom A with CH_3SH (NCSAS-1) or CH_3OCOCH_2SH (NCSAS-2)*

a, Absorption spectrum of NCSAS-1 (- - -) and NCSAS-2 (——) in 0.01 M acetic acid in methanol. b, CD spectra of NCSAS-1 (- - -) and NCSAS-2 (——) and the MCD spectrum of NCSAS-2 (—) in 0.01 M acetic acid in methanol (see a). Samples were purified by HPLC.

the relatively low pK of the naphthoic acid, it is unlikely that the negatively charged carboxylate would be able to bind to DNA at pH ≥ 4. However, the methyl ester of HNA is uncharged at pH < 8, so it is possible to study its interaction with DNA at pH ~ 4. The DNA binding studies (Fig. 7b) (by quenching of the naphthoate blue fluorescence) show that very little interaction occurs even at very high ratios of DNA to chromophore, indicating the requirement of more than the uncharged naphthoic acid for binding to DNA.

Although the purified major mercaptan (NCSAS-1 and -2) and borohydride (NCSAH) products of NCS-Chrom *A* possess no DNA strand scission activity at up to 100-fold concentrations of that of NCS-Chrom *A*,[6] the products do, however, interact with DNA (Fig. 7a and b). The interaction results in a reduction of the fluorescence emission intensity and a shift in the fluorescence emission maximum from 440 to 420 nm at high DNA to chromophore ratios. The binding curves (Fig. 7b) for each of the three derivatives is similar to that for NCS-Chrom and the 2-mercaptoethanol reaction product(s) (6). Therefore, active chromophore is not required for binding to DNA, but components other than the ester of the naphthoic acid alone are required.

[6] M. A. Napier and I. H. Goldberg, unpublished observation.

DISCUSSION

The absorption, fluorescence, and MCD spectral properties of biologically active NCS-Chrom *A* and the changes in these properties resulting from various chemical treatments are consistent with the presence of HNA-NCS. However, the fluorescence intensity of NCS-Chrom *A* is reduced relative to that of HNA-NCS. Furthermore, NCS-Chrom *A* possesses absorption transitions and Cotton effects which cannot be assigned to the naphthoate moiety and therefore must be due to the presence of the remainder of the molecule.

The HNA-NCS moiety is the source of the blue fluo-

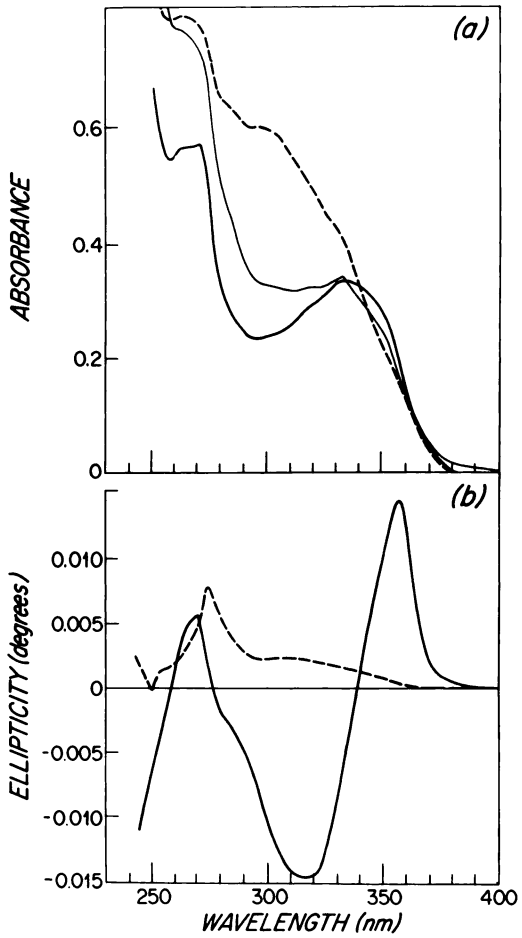


FIG. 5. *Absorption spectra of the reaction mixture of NCS-Chrom A with $NaBH_4$ and the absorption, CD, and MCD spectra of the major product isolated by HPLC (NCSAH)*

a. The absorption spectra of NCS-Chrom *A*, 0.01 M ammonium acetate, pH_m = 4, in 70% methanol, before (- - -) and after (——) treatment with freshly prepared aqueous $NaBH_4$ (0.015 M, final concentration, pH_m = 8.5) at 4° and of NCSAH (__) in 0.01 M acetic acid in methanol.

b. The MCD (——) and CD (- - -) spectra of NCSAH (see Fig. 4a) in 0.01 M acetic acid in methanol.

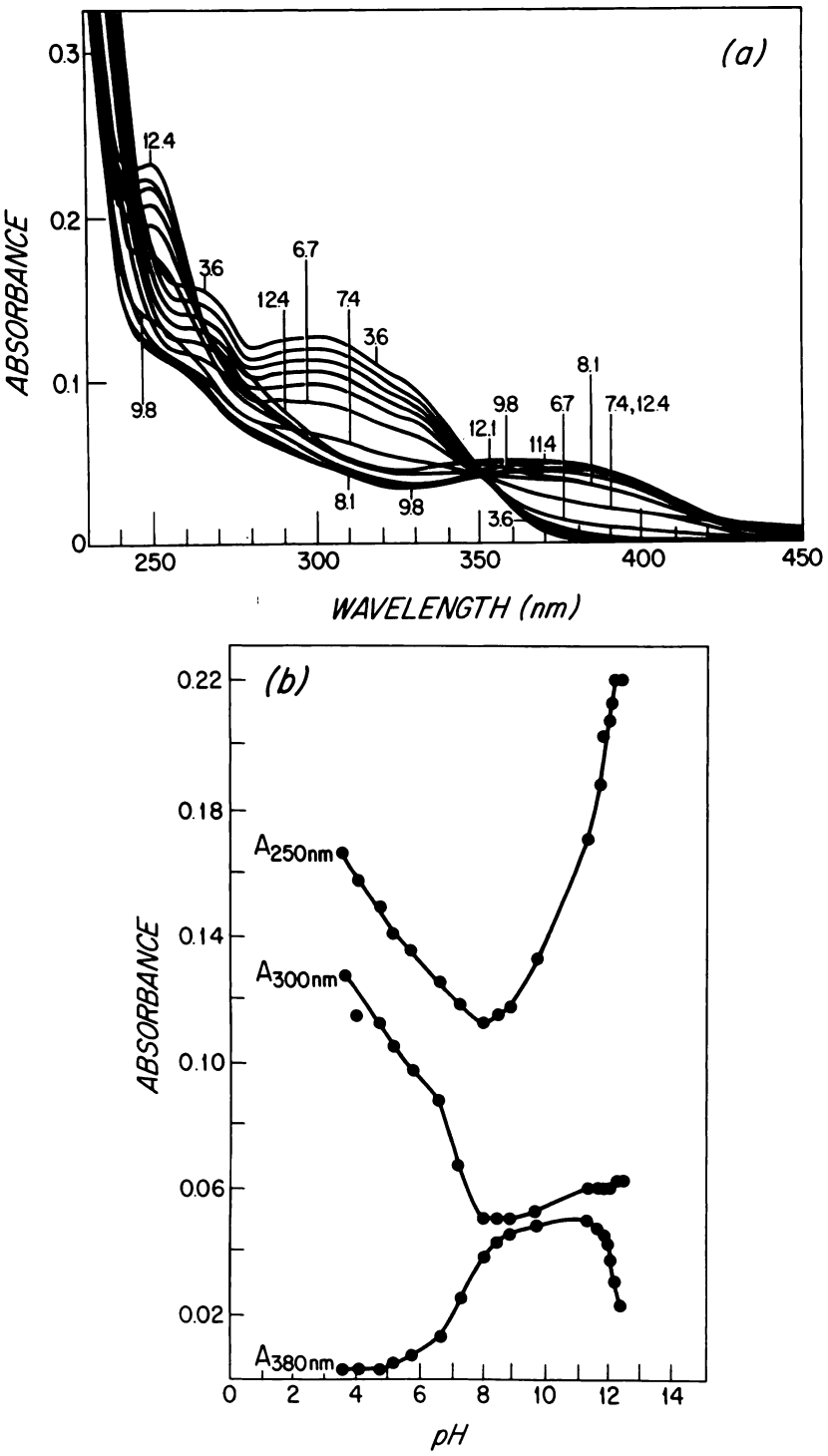


FIG. 6. *Stepwise pH titration of the absorption spectrum of NCS-Chrom A*

a. Spectra were obtained at 4° on a single sample of HPLC-purified NCS-Chrom in 0.003 M ammonium acetate, 0.01 M Tris, 0.01 M sodium acetate, and 10% methanol from pH 3.6 to pH 12.4 with addition of 1 N or 10 N NaOH (final dilution 5%). The pH value for each curve is indicated. The lines between pH 3.6 and 6.7 at 300 nm represent, successively, pH values of 4.1, 4.8, 5.2, and 5.9.

b. pH titration curves of NCS-Chrom *A* absorption (at the indicated wavelengths) from low to high pH (data taken from spectra in a).

rescence, the long wavelength MCD band, and of major features of the absorption spectrum. The isolated hydroxynaphthoic acid (HNA-NCS) prepared from NCS-Chrom *A* exhibits spectral properties, a phenolic ionization ($pK_a \sim 11$) of the naphthoyl hydroxyl proton, and carboxyl and carbonyl ionizations similar to those of 2-hydroxynaphthoic acid (10) and salicylic acid (11). The extinction coefficient of NCS-Chrom *A* at 340 nm of

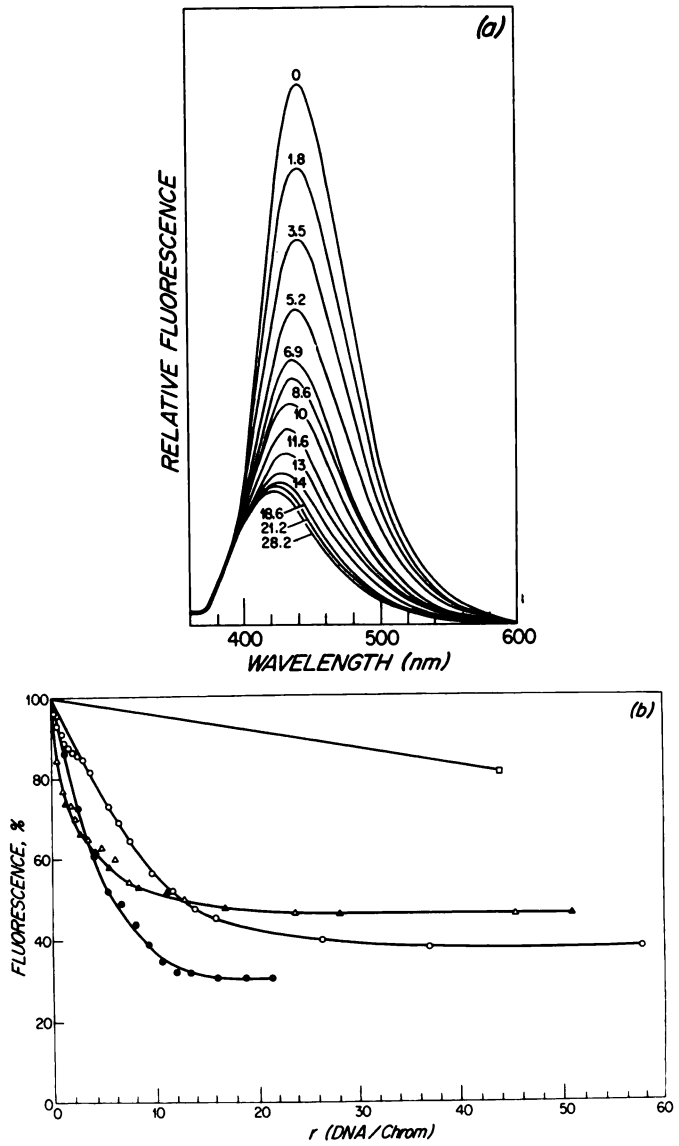


FIG. 7. *Binding of derivatives of NCS-Chrom A to DNA*

a. Fluorescence emission spectra (excitation at 340 nm) of NCSAS-1, alone (A_{340} = 0.013, 0.02 M sodium citrate (pH 4)/10% methanol at 25°) and with successive additions of concentrated calf thymus DNA. DNA concentrations in micromolarity are indicated for each spectrum.

b. Fluorescence at 435 nm was measured after addition of DNA to the chromophore solution at 25° of Me-HNA-NCS (□), NCSAS-1 (●), NCSAS-2 (△, ▲, separate experiments), and NCSAH (○) as indicated. The total chromophore concentrations, calculated assuming a molar extinction coefficient equal to that of isolated NCS-Chrom *A* of 9500 at 340 nm (14) for each derivative, are 1.26, 1.35, 0.526, and 2.21 μM, respectively. No shift in the fluorescence maximum is observed for Me-HNA-NCS even at a 400-fold excess of DNA. For the remaining three derivatives, at ratio of DNA to Chrom ~ 5, the emission maximum begins to shift to a lower wavelength. At DNA to Chrom ratios of approximately 27, 45, and 60 for NCSAS-1, NCSAS-2, and NCSAH, respectively, the emission maximum has shifted from 440 to 420 nm, and the excitation maximum shifts from 340 to 360 nm.

~ 9,500 (14) is consistent with values near 10,000 reported for the long wavelength absorption maxima of various naphthoic acids (15).

Although the fluorescence excitation and emission spectra of NCS-Chrom *A* arise from the hydroxynaphthoate group, the fluorescence intensity of HNA-NCS is substantially quenched in the parent NCS-Chrom *A* molecule (Table 2). The source of the quenching is removed by treatment with mercaptan or borohydride (Tables 2 and 3), resulting in increased intensity and a pH dependence of the blue fluorescence (Fig. 2) equal to that of Me-HNA-NCS. These results are consistent with the presence of a naphthoate ester, not the free acid, and with the modification but not the removal of the unsaturated C_{12}-substructure. The HNA residue is not modified by these treatments, and is not the direct source of the asymmetry since these treatments result in the irreversible loss of the CD activity of NCS-Chrom *A* with no change in the MCD activity. Thus, the Cotton effect observed for NCS-Chrom *A* (and *B* and *C*) is either directly due to the asymmetry of the C_{12}-subunit or its interaction with the asymmetrical sugar residue (still present in the mercaptan and borohydride products).

The absorption spectrum of the unsaturated C_{12}-sub-

unit covalently bound to HNA-NCS, the sugar residue, and the cyclic carbonate is represented by the absorption difference spectra between active NCS-Chrom *A* and its mercaptan treatment product (Fig. 8a) (and HNA-NCS at pH 2 or the borohydride product, data not shown), exhibiting maximal hypochromicity in the 290- to 300-nm region. This difference spectrum probably reflects the electronic interaction of the C_{12}-substructure with the remainder of the chromophore molecule which results in a modification of the spectral properties of both the naphthoate and C_{12}-subunits. The loss of absorption near 300 nm resulting from mercaptan (Fig. 8a) treatment of NCS-Chrom *A* and the absence of any apparent change in the HNA unit as evidenced by the MCD, fluorescence, absorption, ^{1}H-NMR, and mass spectral properties, together with the ^{1}H-NMR and mass spectral evidence for the mercaptan addition to the C_{12}-unit (7, 8),[4] suggest that these reactions modify the C_{12}-chromophoric unit and/or the manner in which it interacts with the naphthoate group.

Addition of apo-NCS (2) or DNA (2, 14) to NCS-Chrom *A* results in absorption changes very similar to those produced by the above chemical treatments (alkali, mercaptan, and $NaBH_4$), with hypochromicity centered near 310 nm and hyperchromicity near 365 nm, with a crossover near 345 nm (Fig. 8b and c). However, these additions result in quenching of the fluorescence with a shift of the emission from 440 to 420 nm and stabilization of the labile chromophore (5, 6) and are reversible by removal of the macromolecule. The red shift in absorption on binding of NCS-Chrom *A* to apo-NCS is also reflected in the long wavelength MCD band of *A* (at 355 nm) which occurs at 365 nm in native NCS. The similarity of the difference spectra generated when NCS-Chrom *A* is treated with mercaptan and the difference spectra produced on complex formation with apo-NCS or DNA suggests that in the latter instances similar modifications in the electronic structure of the C_{12}-unit of NCS-Chrom *A* or in its interaction with HNA-NCS take place, resulting in hypochromicity near 300 nm but without destruction of the chemical entity required for bioactivity. Electric dichroism data (14) indicate that the naphthoate moiety may be the DNA-intercalating unit and that the electronic transition responsible for the absorption at 310 nm is oriented either randomly or perpendicular to the plane of the nucleotide molecules. Whatever the nature of the interaction of the NCS-Chrom *A* with DNA, it results in a modification of the electronic structure of NCS-Chrom *A* that is responsible for the 310-nm absorption, presumably the C_{12}-subunit.

Based on the instability of the C_{12}-substructure, the relative stability of the naphthoate and galactose units to the cofactors required in the DNA scission reaction, and the absence of a requirement for the cyclic carbonate subunit, it seems likely that the C_{12}-unit is the chemically reactive group (but whose reactivity is modified by the cyclic carbonate unit) that forms a short-lived mercaptan-activated species which in the presence of oxygen causes selective oxidation of the 5′-carbon of deoxyribose in DNA, resulting in the formation of nucleoside 5′-aldehyde at the 5′ end of a strand-break (16) or of a covalent NCS chromophore-DNA adduct on this same

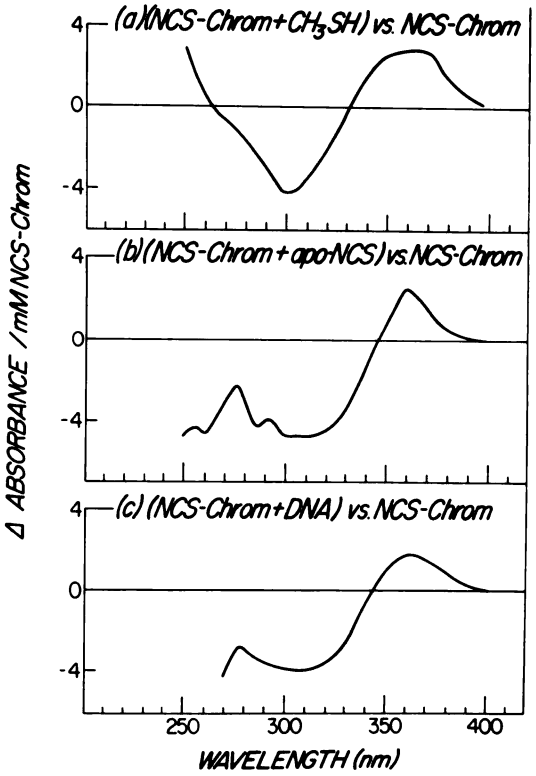


FIG. 8. *Absorption difference spectra of products from various treatments of NCS-Chrom A versus NCS-Chrom A at pH 4*

a, NCS-Chrom *A* treated with 0.02 M methylmercaptan, pH 6 (see Fig. 3a). b, NCS-Chrom *A* bound to apo-NCS at 1:1 molar ratio (absorption of apo-NCS subtracted from absorption of product) (2). c, NCS-Chrom *A* bound to calf thymus DNA, molar ratio of NCS-Chrom *A* to nucleotide = 0.148 (absorption of DNA subtracted from spectrum of product) (2). Absorption was calculated per millimolar NCS-Chrom *A*. Control, NCS-Chrom *A* absorption at pH 4.

carbon (17, 18). In the absence of DNA the mercaptan-activated NCS-Chrom decomposes to inactive products, including a mercaptan addition product. In addition to intercalation of the naphthoate moiety between DNA base pairs, it seems likely that the complex is stabilized by electrostatic interaction between the positively charged 2-methylamino group on the chromophore sugar residue and the negatively charged oxygen atom of the phosphate in the DNA backbone. The interaction of the sugar residue and/or the C_{12}-subunit is required before intercalation, since the methyl ester of HNA-NCS does not bind to DNA.

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