Roles of chromophore and apo-protein in neocarzinostatin action
(reconstitution/isoelectric focusing/pre-neocarzinostatin/chromophore activity, release, and protection)

LIZZY S. KAPPEN, MARY A. NAPIER, AND IRVING H. GOLDBERG
Department of Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

Communicated by Arthur B. Pardee, January 21, 1980

ABSTRACT The methanol-extractable, nonprotein chromophore of the antitumor, protein antibiotic neocarzinostatin (NCS) has at least the full activity of the parent compound in inhibiting DNA synthesis and growth of HeLa cells and in causing DNA strand breaks in vivo and in vitro. In vitro DNA strand scission by the chromophore is markedly stimulated by 2-mercaptoethanol and is inhibited by guanidine hydrochloride and α-tocopherol. By high-pressure liquid chromatography, this activity has been localized to fractions eluting at >90% methanol and having fluorescence emission at 430 nm (excitation at 340 nm). The apo-protein of NCS is inactive by itself but complexes with the chromophore so as to regulate its availability during the in vivo reaction. In DNA strand scission the chromophore acts rapidly at both 0° and 37°C, whereas native and reconstituted NCS are inactive at 0°C and slowly active at 37°C. Complex formation with apo-NCS stabilizes the chromophore. Reconstitution of NCS (pI 3.3) from chromophore and apo-protein (pI 5.2) was shown by both activity studies and isoelectric focusing on polyacrylamide gels. "Pre-NCS," the biosynthetic precursor of NCS, is identical to apo-NCS in amino acid composition, spectral properties, isoelectric focusing on polyacrylamide gels, and ability to complex with isolated chromophore to form material with all the properties of native NCS.

Neocarzinostatin (NCS), an antitumor antibiotic, is a single-chain, acidic protein of molecular weight 10,700 with two disulfide bonds; its amino acid sequence is known (1). Considerable evidence has been presented to indicate that cellular DNA is the major target in the action of NCS and that the drug causes DNA strand breakage both in vivo and in vitro (reviewed in ref. 2). NCS introduces single-strand breaks almost exclusively at thymidylate and adenylate residues in DNA in vitro (3–5) in a reaction greatly stimulated by a sulfhydryl compound (3, 6–9) and dependent on oxygen (9–11). Evidence for the existence of an active, labile form of NCS that causes single-strand breaks in linear and supercoiled DNA has been presented (9, 12). With the recent demonstration that NCS contains non-protein, chromophoric material that has UV-visible absorption above 300 nm and fluorescence emission at 420 nm and 400 nm and that can be separated from the protein (13), the question arises as to its possible role in NCS action. Indirect evidence for such a role comes from our finding that NCS protein from which the chromophore(s) has been removed or destroyed by various procedures has lost virtually all of its biological activity (12, 14).

In this paper we offer direct evidence that the isolated chromophore(s) is responsible for both the in vivo and in vitro effects of NCS. Furthermore, we show that holo-NCS, with the properties of native NCS, can be reconstituted from the isolated chromophore and apo-protein. Finally, factors that were earlier shown to influence NCS activity can now be assigned as to acting via the chromophore or the apo-protein.

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MATERIALS AND METHODS

NCS (clinical form, Kayaku Antibiotics) was kindly provided by W. T. Bradner (Bristol Laboratories, Syracuse, NY) and was stored frozen in 0.015 M sodium acetate buffer at pH 5.0. A preparation of "pre-NCS" and a purified sample of macromomycin, a chromophore-lacking protein antibiotic, were generously provided by T. S. A. Samy (Sidney Farber Cancer Institute). Pre-NCS is an inactive, biosynthetic precursor of NCS produced in the culture filtrate of Streptomyces carzinostaticus variant F-41 (15) and has the same amino acid composition as NCS, although deamination of the asparagine in position 83 has been reported (16). The UV-visible absorption and fluorescence spectra of the "pre-NCS" were found to be identical to those reported for apo-NCS (13, 14). Comparison of the biological properties of NCS and macromomycin has been reported (12, 17). The assays for NCS-induced scission of [methyl-3H]thymidine-labeled linear λ DNA and supercoiled pMB9 DNA have been described (9, 17). DNA synthesis and drug-induced DNA strand scission in HeLa cells were measured as reported (17). All manipulations involving NCS and its extraction products were carried out in the dark.

Preparation of NCS Chromophore and Apo-Protein. Nonprotein chromophore was extracted from lyophilized NCS with dry, redistilled methanol at 0°C. The protein residue was washed twice with methanol and the first wash was added to the original methanol extract. The final volumes of the methanol-extracted chromophore and of the protein dissolved in H2O were half the volume of the original NCS solution. Both solutions were kept at −70°C. The chromophore in methanol contained less than 1% contaminating protein as estimated by Lowry determination (18) and amino acid analysis. Each fraction had the UV-visible absorption and fluorescence characteristics previously reported (13).

Reconstitution of NCS. Equal volumes of the chromophore in methanol and the NCS protein (methanol extracted or pre-NCS) in H2O were mixed at 0°C and the methanol was removed under reduced pressure. The aqueous solution was lyophilized and the residue was dissolved in half the volume of H2O present in the original solution of NCS. Reconstitution was monitored by isoelectric focusing on polyacrylamide gels using a pH gradient of 2.5 to 4.0 (14). The protein bands were stained with Coomassie blue prior to photography.

Chromatography of Chromophore. For high-pressure liquid chromatography (HPLC) we used a model A2C/GPC-204 chromatograph from Waters Associates, a µBondapak C18 column (7.4 mm X 30 cm), a 254-nm absorbance detector, and a Schoeffel model SP 970 fluorescence detector (340 nm excitation; 418 nm emission cutoff filter). The 100% methanol-soluble fraction of NCS was injected in a small volume and

Abbreviations: NCS, neocarzinostatin. HPLC, high-pressure liquid chromatography.

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Table 1. In vitro DNA strand scission by NCS and derivatives

<table>
<thead>
<tr>
<th>Addition</th>
<th>µg/ml</th>
<th>% conversion, form I DNA to form II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromophore</td>
<td>0.05</td>
<td>68</td>
</tr>
<tr>
<td>Chromophore</td>
<td>0.25</td>
<td>100</td>
</tr>
<tr>
<td>Reconstituted NCS</td>
<td>0.05</td>
<td>61</td>
</tr>
<tr>
<td>Apo-NCS</td>
<td>0.5</td>
<td>1.7</td>
</tr>
<tr>
<td>Native NCS</td>
<td>0.05</td>
<td>68</td>
</tr>
</tbody>
</table>

Incubations (250 µl) contained 0.62 µg of supercoiled (88% form I) pMB9 DNA (1.8 x 10^4 cpm), 50 mM Tris at pH 8.0, 10 mM 2-mercaptoethanol, and the drug. Because addition of chromophore in methanol gave a final concentration of 2% methanol in the reaction, the same amount of methanol was added to the drug-free controls and the other drug-containing samples. After incubation (37°C, 20 min) the reaction mixtures were analyzed on 5-30% alkaline sucrose gradients (17). The nicked form (form II) of DNA was quantitated.

RESULTS

Activity of Isolated Chromophore. The isolated chromophore was as active as native NCS and reconstituted NCS in inducing single-strand breaks in supercoiled pMB9 DNA (Table 1) and in inhibiting DNA synthesis (Table 2). When HeLa cells (1 x 10^5/plate) were exposed to drug for 45 min and the number of cells was determined after 5 days of incubation, the concentrations (expressed in µg equivalent of native NCS per ml) for 50% inhibition of cell growth were: chromophore, 0.02 µg/ml; native NCS, 0.02 µg/ml; apo-NCS, >10 µg/ml. Identical results were found when drug-induced DNA strand scission in HeLa cells was followed (data not shown). When crystalline bovine serum albumin was substituted for apo-NCS in the reconstitution, no activity was noted. Apo-NCS has little residual activity. Similar results were obtained with apo-NCS prepared by methanol treatment or Amberlite XAD-7 chromatography (12-14) and with pre-NCS.

Requirement for 2-Mercaptoethanol and Effect of Other Compounds. The requirement for 2-mercaptoethanol in the production of acid-soluble radioactivity from [3H]thymidine-labeled λ DNA by the chromophore is shown in Fig. 1. As true for native NCS (9, 12), about 10 mM 2-mercaptoethanol was required for optimal reaction. Furthermore, in common with the parent reaction there was no effect of 10 mM EDTA, but 5 mM Mg^{2+}, 200 mM NaCl, 50 mM guanidine hydrochloride, and 0.5 mM α-tocopherol all caused significant inhibition (Table 3). Unlike the reaction with native NCS, however, isopropanol at <0.5 M had little effect; above this concentration it inhibited the reaction progressively.

Effect of Incubation Temperature on DNA Scission. In contrast to native or reconstituted NCS, the chromophore was essentially as active at 0°C as at 37°C (Fig. 2). At 0 and 37°C the reaction with the chromophore was over within 2.5 and 1 min, respectively. At 37°C the reaction due to native or reconstituted NCS was considerably slower than that due to chromophore, and the extent of the reaction (at 30 min) with chromophore was consistently 50-100% greater than that with native NCS. Chromophore complexed with apo-protein as native or reconstituted NCS was inactive in DNA scission at 0°C, and this effect could be duplicated by addition of apo-protein just before the reaction with the chromophore was started (Fig. 3). No such inhibition was found with the addition of either of two other proteins, bovine serum albumin or macromycycin; in fact, some stimulation of the reaction was found.

Table 2. Effect of NCS and derivatives on DNA synthesis

<table>
<thead>
<tr>
<th>Addition</th>
<th>µg/ml</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromophore</td>
<td>0.6</td>
<td>61</td>
</tr>
<tr>
<td>Apo-NCS</td>
<td>0.6</td>
<td>2</td>
</tr>
<tr>
<td>Reconstituted NCS</td>
<td>0.6</td>
<td>65</td>
</tr>
<tr>
<td>Native NCS</td>
<td>0.6</td>
<td>62</td>
</tr>
</tbody>
</table>

HeLa cells (9 x 10^5/ml) were preincubated with the drugs for 40 min at 37°C before addition of [3H]thymidine. The radioactivity incorporated into DNA in 40 min was measured (17). In the absence of drug, 1.8 x 10^4 cpm was incorporated into DNA. All tubes contained 1% methanol which did not significantly affect the incorporation of radioactivity in the controls.

Table 3. Effect of different compounds on chromophore-induced breakage of DNA

<table>
<thead>
<tr>
<th>Addition</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA* (10 mM)</td>
<td>0</td>
</tr>
<tr>
<td>NaCl (200 mM)</td>
<td>57</td>
</tr>
<tr>
<td>MgCl₂ (5 mM)</td>
<td>53</td>
</tr>
<tr>
<td>α-Tocopherol (0.5 mM)</td>
<td>96</td>
</tr>
<tr>
<td>Guanidine hydrochloride (50 mM)</td>
<td>47</td>
</tr>
</tbody>
</table>

Reaction mixtures (100 µl) contained the components given in Fig. 1 and 10 mM 2-mercaptoethanol. The reaction was started by the addition of chromophore equivalent to 96 µg of NCS per ml. Trichloroacetic acid-soluble radioactivity was determined after 30-min incubation at 37°C. Chromophore alone solubilized 27.4% of the radioactivity.

* Inhibition found with EDTA was equal to that found with an equivalent concentration (40 mM) of NaCl.
Protection from Loss of Chromophore Activity by Apo-NCS. There was a marked loss of activity of the chromophore when it was preincubated in aqueous solution in the absence of apo-NCS at either 0 or 37°C for 5 min before the reaction was started by the addition of 2-mercaptoethanol (Fig. 4). The loss of activity was greater at 37°C than at 0°C. Native NCS lost no activity during preincubation at either temperature. The activity loss of the chromophore at 0°C was instantaneous and may be due in part to its poor aqueous solubility in the absence of apo-NCS. The activity loss at both preincubation temperatures could be avoided to a considerable extent by the addition of apo-NCS to the preincubation. This protective effect was maximal at 50–75 μg/ml, and, in the experiment in which preincubation was at 0°C, was equivalent to 84% of the activity of native NCS in the absence of added apo-NCS. At higher apo-NCS levels, the competition between the protein and the DNA for interaction with the chromophore became dominant. Neither bovine serum albumin nor macromomycin at similar concentrations provided any protection. Pre-NCS was as effective as apo-NCS in protecting the chromophore.

Reconstitution of NCS. The preceding experiments (Tables 1 and 2; Figs. 2–4) provide evidence for the functional reconstitution of NCS from isolated chromophore and apo-protein.

Reconstitution occurred both under dilute conditions (in which the two components were added to an incubation mixture) and under concentrated conditions (in which a solution containing both components was lyophilized). The regeneration of NCS could also be detected by isoelectric focusing on polyacrylamide gels (Fig. 5). Apo-NCS prepared by removal of the chromophore by methanol or by Amberlite XAD-7 chromatography has a pI of about 3.2 (12, 14), as does the biosynthetic precursor of NCS, pre-NCS (7, 15); the pI of NCS is about 3.3. Material with pI 3.3 was formed by colyophilization of apo-protein with chromophore. Increasing amounts of chromophore converted

![Graph](image-url)

**FIG. 2.** Comparison of the DNA scission activities of chromophore (●), NCS (△), and reconstituted NCS (○) at 0°C (A) and 37°C (B). Reaction mixtures (800 μl) similar to those in Fig. 1 containing 10 mM 2-mercaptoethanol but no drug were equilibrated at the incubation temperatures before start of the reaction by addition of 96 μg of drug per ml. All reactions contained 8% methanol. At times indicated, 100 μl of each mixture was withdrawn for the determination of trichloroacetic acid-soluble radioactivity.

![Graph](image-url)

**FIG. 3.** Effect of addition of apo-NCS on DNA scission by chromophore at 0°C. Incubations (100 μl) similar to those in Fig. 2 contained various levels of apo-NCS. The reaction was started by addition of chromophore (in 8 μl of methanol) equivalent to 96 μg of NCS per ml. After 10 min, trichloroacetic acid-soluble radioactivity was measured; 100% activity with chromophore alone represents 17% of trichloroacetic acid-solubilized radioactivity.

![Graph](image-url)

**FIG. 4.** Effect of preincubation of chromophore in the presence or absence of apo-NCS on its DNA scission activity. Chromophore (in 8 μl of methanol containing the equivalent of 9.6 μg of NCS) was preincubated for 5 min at 0°C (△) or 37°C (○) in a total volume of 90 μl containing 11 mM Tris (pH 8.0) and 0.35 μg of λ DNA. DNA scission reaction was initiated by the addition of 1 μmol (10 μl) of 2-mercaptoethanol. Where there was no preincubation (○), chromophore was added last. After 45 min at 37°C, trichloroacetic acid-soluble radioactivity was determined. Native NCS made 18.5% of the DNA acid-soluble with or without preincubation.
more apo-NCS to NCS. Two very faint bands (pl about 3.4 and 3.5) present in native NCS, but not in apo-NCS or the chromophore, appeared in the reconstituted sample. Whether these represent multiple chromophore-containing forms of NCS remains to be determined. There is good correspondence between the relative activities of native and reconstituted NCS in DNA scission at 37°C (Fig. 2) and their actual content of material with pl 3.3 (Fig. 5). Heating of the reconstituted NCS at 60°C for 1 hr or exposure to long-wave UV (Mineralight Lamp, model UVSL-58, 366 nm) for 1 hr at 15 cm distance reconverted it to the apo-NCS form with pl 3.2. Similar treatment of the isolated chromophore, but not of the apo-protein, prevented reconstitution. Pre-NCS was also converted by chromatophore into material with the pl of NCS. Reconstituted NCS had a circular dichroism spectrum (unpublished data) typical of native NCS and different from that of apo-NCS (14) or chromophore alone.

**DISCUSSION**

The nonprotein chromophoric material extracted from NCS by 100% methanol has at least the full activity of the parent compound in inducing DNA strand breakage in vitro and in vivo and in inhibiting DNA synthesis and growth in HeLa cells. By HPLC we have identified the active material as that eluting with >90% methanol and possessing the 420-nm fluorescence emission of native NCS. It seems likely that the other chromophoric materials obtained by HPLC represent inactive degradation products. This interpretation is compatible with earlier findings that different experimental conditions can lead to an increase of the 490-nm fluorescent material at the expense of the 420-nm material (ref. 13; unpublished data). How the active component participates in the oxygen-dependent damage to DNA is not yet known, but it appears not to involve metals as revealed by experiments with chelators (Table 3; refs. 7 and 9) and by analysis of native NCS for transition metals such as iron, molybdenum, zinc, and copper (unpublished data). Presumably, the chromophore participates directly in reduction-oxidation reactions and, perhaps, through its conversion to a free radical form causes direct damage to the deoxyribose backbone of the DNA (discussed in ref. 2). Such a mechanism is supported.

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**Table 4. In vitro DNA strand scission by HPLC fractions**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Relative concentration*</th>
<th>% conversion, form I to form II DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock chromophore</td>
<td>1.00</td>
<td>69.3</td>
</tr>
<tr>
<td>HPLC fractions:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>2.9</td>
<td>2.7</td>
</tr>
<tr>
<td>II</td>
<td>0.43</td>
<td>68.0</td>
</tr>
<tr>
<td>III</td>
<td>6.2</td>
<td>62.7</td>
</tr>
<tr>
<td>IV</td>
<td>4.2</td>
<td>38.7</td>
</tr>
</tbody>
</table>

* Reaction conditions were identical to those in Table 1.

† Concentration was 0.1 μg equivalent of native NCS per ml.

**HPLC of Methanol-Extracted Chromophore.** HPLC of the methanol-soluble chromophore showed the separation of a heterogeneous mixture of UV-absorbing and fluorescent components (Fig. 6). The major 254-nm-absorbing components eluted with 90–100% methanol, after 45 min. The two major fluorescent components eluted at 18 min (54% methanol; a minor 254-nm-absorbing component) and 49 min (97% methanol; the major 254-nm-absorbing component) with emission at 490 nm (excitation, 390 nm) and 420 nm (excitation, 340 nm), respectively. The absorption profile consistently showed two overlapping peaks between 48 and 49 min which are not resolved in the fluorescence profile except at very low concentrations.

In preliminary studies we have tested the activity of the major absorbing and fluorescing components. Fractions (0.8 min) of the two fluorescent components, I (17.1–17.9 min) and II (47.9–48.7 min), and fractions III (51.5–52.3 min) and IV (53.5–54.3) eluting after peak II were assayed for in vitro pMb9 DNA scission activity (Table 4). Fraction I was inactive at 3 times the equivalent chromophore mixture concentration, whereas fraction II gave DNA strand scission at least equivalent to that given by the chromophore mixture. This fraction was also active in inhibition of DNA synthesis and growth and in DNA strand scission in HeLa cells. Fractions III and IV showed activity only at high relative concentrations. These results and data not reported here indicate that the active components with fluorescence emission at 420 nm elute late from the column, after 35 min, at high methanol concentrations. The components with 490-nm fluorescence emission are inactive.

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**FIG. 5.** Isoelectric focusing of reconstituted NCS on polyacrylamide gel. The gel (5%; pH 2.5–4.2) was prefocused at 10 W for 45 min. After application of the sample (24 μg of protein), electrophoresis was done at 25 W (70 mA) for 3 hr at 2°C in the dark. Lanes: 1, native NCS; 2, apo-NCS; 3, apo-NCS + chromophore (24 μg of NCS equivalent); 4, apo-NCS + chromophore (60 μg of NCS equivalent); 5, pre-NCS + chromophore (24 μg of NCS equivalent); 6, pre-NCS + chromophore (60 μg of NCS equivalent); 7, pre-NCS; 8, native NCS. Chromophore alone (60 μg of NCS equivalent) showed no bands.

**FIG. 6.** HPLC of NCS chromophore: 50 μl of NCS (1.3 mg equivalent per ml in methanol) was injected and elution was at 2.0 ml/min with a gradient of 35–100% mobile phase solvent B in solvent A (---). --- Fluorescence emission (excitation at 340 nm, with 418-nm cutoff filter); --- absorbance at 254 nm. Recovery of material absorbing at 254 nm was about 90%.
by the observed inhibition of the reaction with the chromophore (Table 3) and with NCS (9) by the potent peroxyl radical scavenger, \( \alpha \)-tocopherol.

Although it has been speculated that the requirement for a sulfhydryl compound might be due to reduction of NCS protein disulfides to generate an active form of the drug (9, 11, 19), the data in Fig. 1 clearly show that mercaptan functions in the absence of the protein, presumably by reducing the chromophoric material. Similarly, the inhibition of the NCS reaction by high ionic strength and \( \text{Mg}^{2+} \) (9) is also found with the chromophore alone and is probably related to the interaction between the activated chromophore and the DNA. Because guanidine hydrochloride treatment of NCS results in changes in its fluorescence properties (13), its inhibition of the activity of the chromophore may be a direct effect.

What then is the role of the protein in NCS? We have shown that the chromophore is markedly stabilized by interaction with the apo-protein. The apo-protein protects the chromophore not only against heat-induced instability but also against that found at 0°C in aqueous solution. The latter action may reflect the ability of the protein to provide the hydrophobic environment necessary to maintain the chromophore in solution until its activated form can react with the DNA. Furthermore, the availability of the chromophore for interaction with its target DNA is determined by its ability to dissociate from the protein. Thus, at 0°C chromophore associated with NCS protein is inactive in DNA strand scission but becomes available for scission at 37°C. Free chromophore, however, is fully active at 0°C. Another DNA strand scission agent, the glycopeptide bleomycin, is also fully active at 0°C (20), but its action is believed to involve free radical generation via \( \text{Fe}^{2+} \) oxidation (20). These data indicating that the nonprotein chromophore possesses full biological activity and that it is active after release from its complex with the apo-protein of NCS may explain previously unrecognized findings that NCS covalently bound to agarose or Sepharose is cytotoxic (21, *).

The difference between the kinetics and extent of DNA breakage at 37°C due to chromophore and those due to native or reconstituted NCS resembles earlier results obtained with native NCS reactions stimulated by isopropanol (or other organic solvents and denaturants) (9, 12). The latter effect (the \( V_{\text{max}} \) but not the \( K_m \), was increased) was attributed to the solvent-induced unfolding of the protein to generate an active form of the drug (9, 12). Such unfolding of the protein would lead to the release of the activated chromophore for its interaction with DNA. Because the chromophore is fully active in its free state, isopropanol results in no stimulation of this reaction. The decreased extent of DNA breakage found with holo-NCS compared with free chromophore (or when isopropanol is included in the reaction with the former (12)) is presumably due to the fact that the former reaction is much slower than the latter and the opportunity for inactivation of the drug is greater (12). The ability of apo-NCS to complex the chromophore also explains the inhibitory effect of the apo-protein on the DNA strand scission reaction by chromophore (Figs. 2 and 3) and native NCS (12). Added apo-protein would be expected to compete with the DNA for the chromophore. In fact, such competition between DNA and apo-protein has been observed (ref. 12; unpublished data).

The finding that a preparation of pre-NCS, like the protein stripped of chromophore by solvent or chromatography on Amberlite XAD-7, can be reconstituted into a material with the properties of native NCS supports our thesis that pre-NCS is the apo-protein of NCS (2). Not just any protein will substitute for NCS apo-protein in its interaction with the chromophore from NCS. Neither bovine serum albumin nor macromomycin was effective. This reaction appears to be specific for the NCS apo-protein and is similar to the requirement found earlier in the inhibition of NCS-induced DNA strand scission (12). Finally, based on our finding that the chromophore is eluted from CM-cellulose by a pH gradient after NCS, we proposed that the pl of NCS results from combination of the more negatively charged apo-NCS with a positively charged chromophore (14).

The results on reconstitution as analyzed by isoelectric focusing are consistent with this interpretation. Furthermore, because reconstitution at suboptimal ratios of chromophore to apo-protein fails to generate bands between pl 3.2 and pl 3.3, in the latter material presumably the chromophore-to-protein ratio is 1.

**Note Added in Proof.** We have recently also succeeded in extracting the nonprotein chromophore of the antitumor protein antibiotic amomycin and have found it to have the cytotoxic properties (17) of the parent material.

We thank Jeanne Thivierge for her excellent technical assistance. Dr. D. J. Strydom for performing the amino acid analyses, and Dr. B. Vallee for making available HPLC and spectrophotometric instrumentation. We thank Drs. B. Holmquist and D. J. Strydom for helpful discussions. This work was supported by U.S. Public Health Service Research Grant GM 12573 from the National Institutes of Health. L.S.K. was partially supported by Grant RR 05831 from the Biomedical Research Support Grant Program, National Institutes of Health and M.A.N. by a fellowship from the Interdisciplinary Programs in Health Program at the Harvard School of Public Health.
