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Role of Epoxide in Neocarzinostatin Chromophore Stability and Action

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SUMMARY

To determine the role of the epoxide moiety of the nonprotein chromophore of the antitumor antibiotic neocarzinostatin in its ability to damage DNA, the diol monomethyl ether derivative was prepared, and its stability and biological properties were studied. This analogue was found to be more stable than the epoxide (about 9-fold), but to be much less active in nicking supercoiled DNA and in forming covalent adducts with poly(dA-dT). However it is able to bind noncovalently to DNA and to the neocarzinostatin apoprotein. Another analogue, the chlorohydrin derivative, is about half as active as the epoxide in the DNA scission reaction and appears to produce the same covalent adducts with poly(dA-dT) as does the epoxide, suggesting that both compounds undergo similar types of activation by thiol. These results indicate that the epoxide moiety of the neocarzinostatin chromophore is an important part of the highly unsaturated, strained bicyclo[7.3.0]dodecadiendiyne in the thiol-dependent, DNA-damaging reaction. It may be involved in the activation of the drug to its active species and/or may be the site of new bond formation in its reaction with DNA.

NCS, an antitumor antibiotic (holoprotein) produced from the culture filtrate of *Streptomyces carzinostaticus*, consists of two components: an acidic apoprotein (NCS-A; molecular weight 11,093, pI 3.26) (1) and a noncovalently bound labile nonprotein chromophore (NCS-C_e; molecular weight 659) (2). NCS-C_e (3–6) is primarily responsible for the biological activity of NCS, while NCS-A plays an important role as a carrier and stabilizer of NCS-C_e (7, 8). NCS-A is inactive by itself in terms of both binding and damaging DNA, but it binds NCS-C_e tightly and specifically (K_d 1×10^{-10} M) (9). Not only does NCS-A stabilize NCS-C_e from degradation, but it also controls the release of NCS-C_e to allow interaction with DNA (7, 9).

NCS-C_e has a relatively strong affinity for DNA (K_d 10^{-6} M) (9–12), especially in the regions rich in dT and dA residues in the minor groove (13), and produces single-strand breaks mainly by abstracting a hydrogen from the C-5′ of deoxyribose on dT and dA (14) to produce a carbon-centered radical at C-5′, which adds dioxygen to form a peroxyl radical intermediate, leading to oxidation of C-5′ to the aldehyde (14–18) or to the formation of covalent adducts on the DNA sugar (19–22). The formation of nucleoside 5′-aldehyde at the 5′-end of a strand break requires oxygen and is in competition with the formation of stable DNA-drug adducts, whose production is optimal under anaerobic conditions (21).

NCS-C_e consists of three main structural subunits: a substituted naphthoic acid, an aminosugar (D-fucosamine), and a bicyclo[7.3.0]dodecadiendiyne epoxide (Fig. 1A).

We have proposed that the naphthoic acid moiety intercalates into DNA in the minor groove, and the positively charged aminosugar moiety interacts with the negatively charged sugar phosphate backbone of DNA (23). These two anchors may serve to juxtapose the bicyclo[7.3.0]dodecadiendiyne epoxide moiety with the deoxyribose of primarily thymidylate residues in DNA (24). The cyclic carbonate (1,3-dioxolan-2-one) moiety appears to take part in the passage of the NCS-C_e through cellular and nuclear membranes, judging from the activities (25, 26) of the two derivatives of the cyclic carbonate, the vicinal diol and the acyclic hydroxy carbonate. The two derivatives possess *in vitro* DNA scission activity equal to the NCS-C_e with the cyclic carbonate but have about 5% and 80%, respectively, of the *in vivo* activity of the NCS-C_e.

Two analogues of the epoxide (NCS-C_e, 1), chlorohydrin (NCS-C_c, 2) (5, 27) and diol monomethyl ether (NCS-C_d, 3), were therefore prepared as the first step to help identify the potential active site of the NCS-C_e (Fig. 1A). Our results, showing that the diol monomethyl ether is inactive in DNA scission in contrast to the epoxide and chlorohydrin, although

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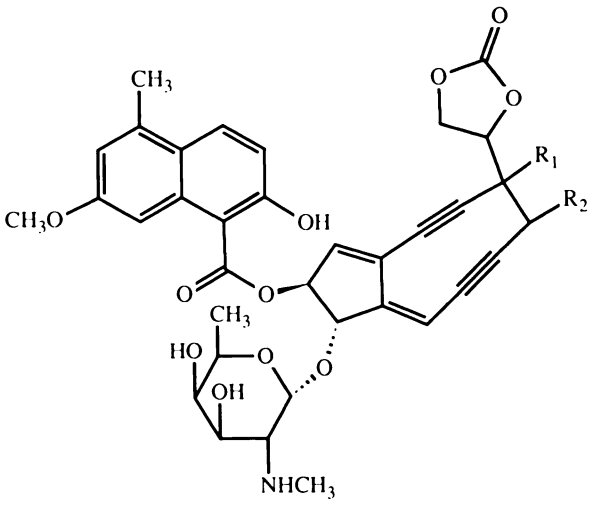
ABBREVIATIONS: NCS, neocarzinostatin holoprotein; NCS-C, neocarzinostatin chromophore (C_e, epoxide; C_d, diol monomethyl ether; C_c, chlorohydrin); NCS-A, neocarzinostatin apoprotein; FPLC, fast protein liquid chromatography; HPLC, high performance liquid chromatography; DTPA, diethylenetriaminepentaacetic acid; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; FAB-MS, fast atom bombardment mass spectrometry.



1 $R_1, R_2 = O$

2 $R_1 = OH$ $R_2 = Cl$

3 $R_1 = OH$ $R_2 = OCH_3$

A

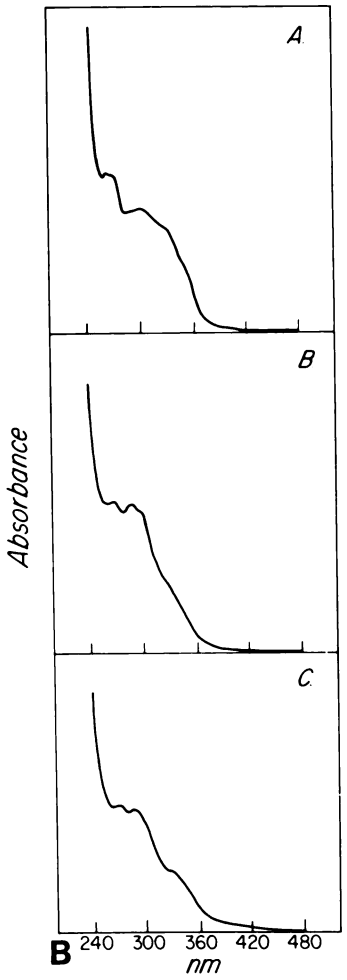


Fig. 1. Derivatives of NCS-C: NCS-C_e (1*A*), NCS-C_c (2*B*), and NCS-C_d (3*C*). Structures 1–3 (A) and UV absorption spectra *A–C* (B) of NCS-C are shown. The spectra were taken in 20 mM sodium citrate/citric acid, pH 4.0, containing 5% (v/v) methanol at 5° (0.2 absorbance unit full scale). See Materials and Methods for details.

it complexes with DNA, strongly suggest that the epoxide functionality is important in the activity of the NCS-C_e toward DNA, probably in a cooperative manner with the highly unsaturated strained bicyclo[7.3.0]dodecadiendiyne moiety. This conclusion differs from a report (27) in which the epoxide moiety was judged to be relatively unimportant since both NCS-C_e and NCS-C_c were found to be biologically active.

Materials and Methods

All procedures involving NCS-C were carried out in the dark, and NCS-C was normally stored at −70° in brown vials.

Neocarzinostatin chromophore (NCS-C). NCS [λ_{max}(nm) 274, 287.5, 341; λ_{min}(nm) 255] was either obtained from clinical ampules (Kayaku Antibiotics, Tokyo, Japan) or produced by fermentation of *Streptomyces carzinostaticus* employing a procedure modified from the one previously described (28, 29). NCS from fermentation was purified by successive chromatography on a Sephadex G-50 column using 20 mM NH_4OAc/HOAc (pH 4.8), FPLC on a Mono-Q HR 5/5 anion exchange column (Pharmacia Fine Chemicals) (30), with a stepwise gradient using 20 mM NH_4OAc/HOAc, pH 4.8, and 20 mM NH_4OAc/HOAc, pH 4.8, containing 1.0 M NaCl, and a Sephadex G-25 desalting column using 20 mM NH_4OAc/HOAc, pH 4.0.

NCS-C_e (1, Fig. 1A; ϵ_{341} 8,800 $M^{-1}cm^{-1}$)[1] was extracted overnight in methanol from NCS containing 20 mM sodium citrate/citric acid, pH 4.0, after dialysis against distilled water, buffering with 20 mM sodium citrate/citric acid, pH 4.0, and then lyophilization, as described previously (10). Its concentration was determined by UV absorption spectroscopy (ϵ_{341} 10,800 $M^{-1}cm^{-1}$) (10) after reconstitution with an excess of highly purified NCS-A [λ_{max}(nm) 278.5; λ_{min}(nm) 248]. Purity was checked by HPLC on an analytical Microsorb C_{18} column with an 80-min convex gradient elution [50–80% (v/v) methanol/water containing 10 mM NH_4OAc/HOAc, pH 4.0] at 1 ml/min: UV [20 mM sodium citrate/citric acid, pH 4.0, in 5% (v/v) methanol/water) (Fig. 1B), λ_{max}(nm) 261, 270, 286, 299, 313, 327, 347.

Synthetic derivatives of NCS-C. A stirred solution of NCS-C_e in methanol was treated with HCl (2.0 M in methanol) at a final concentration of 0.3 M at 0° for 24 hr. The excess HCl was removed under nitrogen in an ice bath to provide NCS-C_c (2, Fig. 1A) in 47.9% yield.

Perchloric acid ($HClO_4$; 2.0 M in methanol) was added dropwise to a stirred solution of NCS-C_e in methanol at 0° to give a final concentration of 0.6 M. The reaction was allowed to proceed in an ice bath for 24 h, and then the excess of $HClO_4$ was precipitated by adding KCl (4.0 M in water), and the mixture was centrifuged at 8,000 rpm for 30 min at 5° to afford NCS-C_d (3, Figure 1-1) in 45.7% yield. Alternatively, NCS was dialyzed against distilled water at 5°, lyophilized in the presence of 20 mM sodium citrate/citric acid, pH 4.5, and then treated with prechilled 0.6 M $HClO_4$ in methanol at 0° overnight. The mixture was centrifuged at 10,000 rpm for 30 min to remove the NCS-A, prechilled saturated aqueous KCl was added, and the supernatant was subjected to subsequent purification.

The crude derivatives were purified by HPLC using an analytical Microsorb C_{18} column with a 40-min isocratic elution [65%(v/v) methanol/water containing 10 mM NH_4OAc/HOAc, pH 4.0) followed by an 80-min convex gradient elution [65–75% (v/v) methanol/water containing the same buffer) at 1 ml/min. It was monitored by both UV absorbance at 254 nm and fluorescence (excitation 340 nm, emission

[1] S. H. Lee and I. H. Goldberg, unpublished data.

420 nm). The purified derivatives were concentrated by lyophilization after removal of most methanol under nitrogen: NCS-C_d, UV [20 mM sodium citrate/citric acid, pH 4.0, in 5% (v/v) methanol/water] (Fig. 1B); λ_{max}(nm) 267, 285, 298, 326, 347. FAB-MS (m/z): MH^+ 692, 462, 419, 241, 215, 160. NCS-C_c, UV [20 mM sodium citrate/citric acid, pH 4.0, in 5% (v/v) methanol/water] (Fig. 1B); λ_{max}(nm) 269, 287, 297, 328, 347. FAB-MS (m/z): MH^+ 696.

The representative retention times for NCS-C, when subjected to an 80-min convex gradient elution [65–75% (v/v) methanol/water containing 10 mM NH_4OAc/HOAc, pH 4.0), are as follows: 85.5 min for NCS-C_e, 76.8 min for NCS-C_c, and 73.3 min for NCS-C_d.

Neocarzinostatin apoprotein (NCS-A). NCS-A was further purified by Rohm & Haas Amberlite XAD-7 column chromatography (7) followed by FPLC, using the same solvent system as above. Its concentration was determined by UV absorption spectroscopy (ϵ_{278} 14,400 $M^{-1}cm^{-1}$) (10) in 20 mM sodium citrate/citric acid, pH 4.5, at 15°.

DNA. Sonicated, nuclease S1-treated and phenol extracted calf thymus DNA was prepared as previously described (9).

Relaxation of supercoiled DNA. Various amounts of methanolic NCS-C solutions were added to aqueous solutions containing ϕX 174 RF DNA (Bethesda Research Laboratories, Gaithersburg, MD) in ice, to give final concentrations of 0, 0.005, 0.1, 1, or 15 μM NCS-C, 100 μM ϕX 174 RF DNA, 3 mM DTPA, 5 mM DTT, and 3 mM NaOAc/HOAc, pH 5.0, in 50 μl total volume. The reactions were initiated by adding 0.5 M Tris-HCl, pH 8.0, to a final concentration of 30 mM; then, the mixtures were incubated at room temperature for 30 min. Subsequently, 20 μl of a gel-loading solution containing 80% (v/v) formamide, 0.1% (w/v) xylene cyanol, 0.1% (w/v) bromphenol blue, and 1 mM EDTA, pH 8.0 was added, and samples were loaded onto 1% (w/v) agarose horizontal slab gel containing 1 μg/ml ethidium bromide, 40 mM Tris-HCl, pH 7.9, and 5 mM NaOAc/1 mM EDTA. Electrophoresis was run in the same buffer at 110 V.

Formation of NCS-C-DNA adducts. The anaerobic reactions were carried out as previously described (21) using poly(dA-dT) as DNA and DTT as sulfhydryl cofactor. A typical reaction mixture contained 30 μM NCS-C (NCS-C_e, NCS-C_c, or NCS-C_d), 500 μM poly(dA-dT), 3 mM EDTA, pH 4.0, 1 mM DTT, and 60 mM Tris-HCl, pH 8.0, in 400 μl. The reaction mixtures containing the adducts were purified by HPLC on a μBondapak C_{18} analytical column, after being digested successively with deoxyribonuclease II (type V from bovine spleen; Sigma), endonuclease S1 (*Aspergillus oryzae*; Sigma or Miles), and acid phosphatase (type III from potato; Sigma). A 20-min isocratic elution with water containing 10 mM NH_4OAc/HOAc, pH 4.5, was followed by a 120-min convex gradient elution [0–70% (v/v) methanol/water containing the same buffer] at 1 ml/min. It was monitored both by UV absorbance at 254 nm at 0.08 absorbance unit full scale and by fluorescence (excitation 340 nm, emission 420 nm) at 0.1 μamp sensitivity range.

Binding study. Spectroscopic measurements were performed on a Perkin-Elmer model 552A UV-VIS Spectrophotometer at 5°. Small volumes of either NCS-A to give a final concentration of 10 μM or calf thymus DNA to give a final concentration of 100 μM were added to the sample cuvette containing 5 μM NCS-C in 20 mM NH_4OAc/HOAc, pH 5.0, in either 10 or 5% (v/v) methanol/water, and to the reference cuvette.

Stability of NCS-C. The fluorescence changes at 495 nm (excitation at 390 nm) were taken by adding 100 μl of 15 μM NCS-C in methanol to the cuvette containing 400 μl of all the other components such as 20 mM NH_4OAc/HOAc, pH 4.0, or 20 mM Tris-HCl, pH 8.0, or various amounts of DNA in 20 mM Tris-HCl, pH 8.0, at 25°.

Results

Relative activity of NCS-C. ϕX 174 RF DNA, a supercoiled DNA (form I), was treated with various amounts of NCS-C (C_e, C_c, and C_d), in the presence of DTT to compare the activity of the analogues toward DNA. At 15 μM, both NCS-C_e and NCS-C_c degraded DNA completely, whereas NCS-C_d simply relaxed form I to give single-nicked circular duplex DNA (form II). The quantitative difference in relative activity between NCS-C_e and NCS-C_c becomes evident at 0.1 μM and 0.005 μM. At 0.1 μM, NCS-C_e converted most of form I to form II, whereas more of form I remained at the same dose level of NCS-C_c. At 0.005 μM, NCS-C_e converted approximately half of form I to form II, whereas NCS-C_c resulted in far less conversion to form II. The respective ratios of form I and form II between NCS-C_e and NCS-C_c indicate that NCS-C_e is at least twice as active as NCS-C_c. This result is supported by a similar experiment based on their antitumor activity (27) which showed that NCS-C_e was twice as active as NCS-C_c. However, NCS-C_d had much less activity (<1%) compared with NCS-C_e in that it converted form I to form II completely only at a drug level of 15 μM, whereas NCS-C_e provided a comparable result at 0.1 μM. In other words, virtually no form II was produced by NCS-C_d at 0.1 μM, a level which resulted in almost complete conversion of form I to form II by NCS-C_e; NCS-C_d provided comparable cleavage at a dose level of 15 μM. Therefore, NCS-C_e is approximately 150-fold more active in producing single-strand nicks in DNA than NCS-C_d.

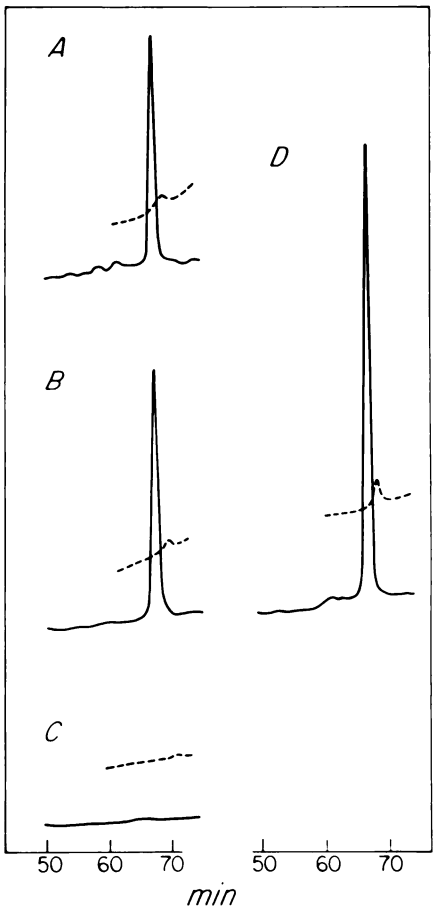


Fig. 2. HPLC profile of major adduct of NCS-C to poly(dA-dT). A 20-min isocratic elution with water containing 10 mM NH_4OAc/HOAc, pH 4.5, was followed by a 120-min convex gradient elution with 0–70% (v/v) methanol/water containing the same buffer at 1 ml/min [UV absorbance (– – –) at 254 nm (0.08 absorbance unit full scale) or fluorescence (—) at 340 nm (excitation) and 420 nm (emission) (0.1-μamp sensitivity range)]: A, NCS-C_e; B, NCS-C_c; C, NCS-C_d; and D, A and B co-chromatographed. Stable DNA-drug adducts were prepared as described under Materials and Methods.

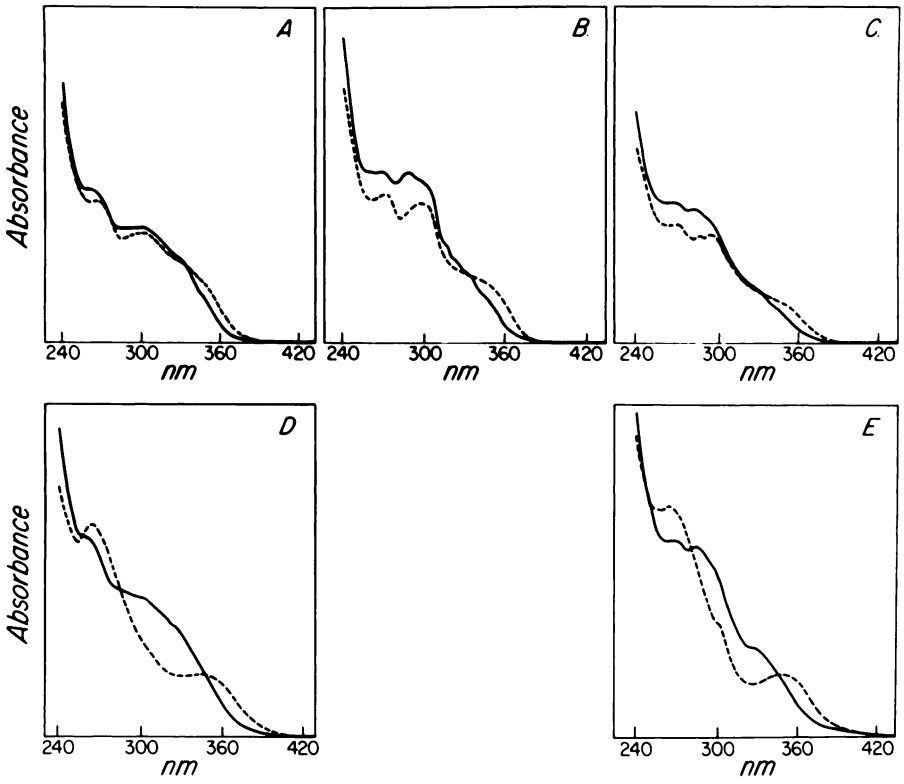


Fig. 3. Effects of NCS-A and DNA binding on the UV absorption spectra of NCS-C. Spectral changes upon binding are shown for free (—) and NCS-A-bound (– – –) NCS-C in 20 mM sodium citrate/citric acid, pH 4.0, containing 10% (v/v) methanol (A, NCS-C_e; B, NCS-C_c; and C, NCS-C_d) and for free (—) and calf thymus DNA-bound (– – –) NCS-C in 20 mM NH_4OAc/HOAc, pH 5.0, containing 5% (v/v) methanol (D, NCS-C_e; and E, NCS-C_d). The spectra of 5 μM NCS-C before and after addition of concentrated solutions of either NCS-A to give a final concentration of 10 μM or DNA to give a final concentration of 100 μM were taken at 5° (0.2 absorbance unit full scale). An equal amount of either NCS-A or DNA was added to the reference cuvette for bound spectra.

Anaerobic production of adducts in the presence of DTT. Treatment of poly(dA-dT) with NCS-C in the presence of DTT under anaerobic conditions resulted in the formation of two stable adducts, one as a major one. They were isolated as NCS-C adducts of trinucleotides, either d(TpApT) or d(ApTpA),[2] after the DNA was digested with enzymes. Although both NCS-C_e and NCS-C_c afforded positive results, NCS-C_d did not give any adduct, suggesting that NCS-C_d is biologically inactive. The major adducts from NCS-C_e and NCS-C_c eluted together when co-chromatographed on HPLC, indicating that both of these compounds resulted in apparently the same product (Fig. 2).

Binding study. The binding of the NCS-C derivatives, NCS-C_e, NCS-C_c, and NCS-C_d, was monitored by changes in UV spectra upon the addition of either apoprotein (Figure 3, A–C) or calf thymus DNA (Fig. 3, D and E) in an appropriate aqueous buffer containing methanol to determine whether the synthetic derivatives, NCS-C_c and NCS-C_d, retain binding affinity of NCS-C_e toward the apoprotein and DNA. However, the modifications of the epoxide moiety to give either chlorohydrin or diol monomethyl ether did not seem to alter the binding affinity of NCS-C in spite of the difference in their biological activity.

Stability. Spontaneous degradation of NCS-C to a highly fluorescent degradation product (9) was measured by monitoring the increase in the intensity of fluorescence at 495 nm (excitation at 390 nm) at pH 8.0 in the absence or presence of DNA (Fig. 4, Table 1). The first order rate constants were calculated from the Guggenheim plots (31) to be 2.8×10^{-2} sec^{-1} (NCS-C_e), 8.9×10^{-3} sec^{-1} (NCS-C_c), and 3.4×10^{-3} sec^{-1} (NCS-C_d) at pH 8.0 (Fig. 4A, Table 1). It is easily recognizable that NCS-C_e at pH 8.0 in the presence of 0.75 mM DNA is almost as stable as at pH 4.0, indicating the ability of DNA to protect the otherwise unstable NCS-C_e under these conditions. The stabilities of the three forms of NCS-C vary in the order: NCS-C_e < NCS-C_c < NCS-C_d.

Discussion

NCS-C_d is a relatively stable (about 9-fold), biologically inactive analogue of NCS-C_e. It produces neither single-strand breaks nor adducts of DNA at drug concentrations comparable to those of the active form. However, NCS-C_c possesses the same activity profile as NCS-C_e, although it is not as potent.

Thiol activates DNA-bound NCS-C_e to a presumed free radical species that abstracts a hydrogen atom from C-5′ of the deoxyribose of mainly dT residues to form a carbon-centered radical at C-5′ (14). Under aerobic conditions dioxygen adds to the radical at C-5′ to form a peroxy radical derivative that eventuates in a strand break with mainly nucleoside 5′-aldehyde at the 5′-end [10–15% of the breaks result from scission at C(5′)-C(4′) to generate 3′-formyl phosphate-ended DNA, an energy-rich intermediate in strand breakage (32)]. In the absence of dioxygen the bound drug itself reacts with the radical to C-5′ to form a stable NCS-C-DNA adduct. It is reasonable to assume that NCS-C_e and NCS-C_c give rise to the same free radical species upon activation by a thiol, resulting in an opening of the epoxide or a nucleophilic substitution of the chloride, respectively, leading to the same adducts, as shown by the presence of a single peak on HPLC when their major adducts were co-chromatographed. The absence of either leaving group on NCS-C_d naturally rules out the possibility of this mechanism and hence it lacks significant biological activity as a result. Recently, a proposal has been made involving such a mechanism for the epoxide in drug activation (33).

Since the overall structural differences among the analogues

[2] S. H. Lee and I. H. Goldberg, unpublished data.

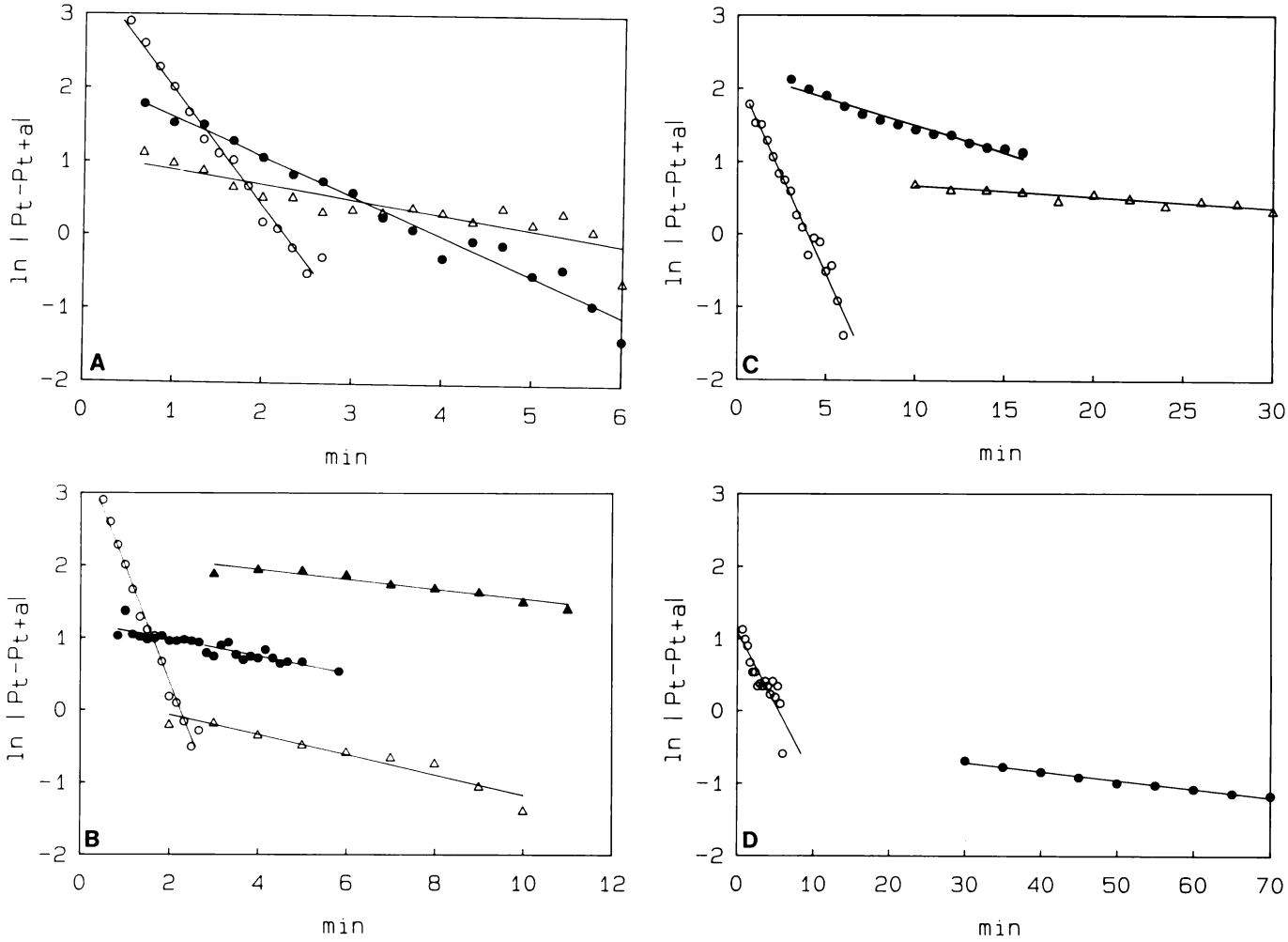


Fig. 4. Effect of pH and DNA on the stability of NCS-C at 25°. Spontaneous degradation of NCS-C was measured by monitoring the generation of fluorescence at 495 nm. A. Decomposition of NCS-C at pH 8.0: ○, NCS-C_e; ●, NCS-C_c; and △, NCS-C_d. B. Stabilization of NCS-C_e compared at pH 8.0 in the presence of 0 mM (○), 0.75 mM (●), or 1.5 mM (▲) DNA, and at pH 4.0 in the absence of DNA (△). C. Stabilization of NCS-C_c compared at pH 8.0 in the presence of 0 mM (○), 0.2 mM (●), and 1.5 mM (△) DNA. D. Stabilization of NCS-C_d compared at pH 8.0 in the presence of 0 mM (○) and 1.5 mM (●) DNA. $P_t - P_{t+a}$ is the change in fluorescence measured between time t and $t + a$ where a indicates constant time intervals.

TABLE 1

First order rate constants for NCS-C derivatives at 25°[a]

NCS-C derivative	pH	DNA	k,sec^{-1}
		mM	
NCS-C_e	8.0	0.0	2.81×10^{-2}
	8.0	0.1	1.10×10^{-2}
	8.0	0.75	1.72×10^{-3}
	8.0	1.5	1.35×10^{-3}
	4.0	0.0	1.97×10^{-3}
NCS-C_c	8.0	0.0	8.92×10^{-3}
	8.0	0.2	1.41×10^{-3}
	8.0	0.75	2.83×10^{-4}
	8.0	1.5	2.67×10^{-4}
NCS-C_d	8.0	0.0	3.35×10^{-3}
	8.0	1.5	2.06×10^{-4}

[a] A first order rate constant was obtained from the slope of a plot of $\ln |P_t - P_{t+a}|$ versus t.

of NCS-C as a whole are negligible in terms of recognition by either the apoprotein or DNA, both NCS-C_d and NCS-C_c possess high binding affinity toward them. This result supports our proposal that the substituted naphthoic acid and the positively charged aminosugar (D-fucosamine) moieties anchor the NCS-C to DNA so as to place the bicyclo[7.3.0]dodecadiendiyne epoxide moiety near the deoxyribose of mainly dT residues, allowing the chemical damage to take place.

Because of its relative stability and its very low DNA cleaving ability, NCS-C_d should prove to be useful in efforts to obtain stable complexes with oligodeoxyribonucleotides for X-ray crystallographic and DNA footprinting studies.

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