nm. Reactions for kinetics were initiated by addition of 1-2 μL of **3e** or **3f** in acetonitrile per mL of the dioxane-water reaction mixture (1.0- or 3.0-mL total volume) which had been allowed to equilibrate to 37 °C. For product analyses, reaction mixtures were diluted with 1 volume of water and extracted with 1:1 hexane-ethyl acetate. After drying and evaporation of the organic solvents, the residue was dissolved in 2.5% ethyl acetate in hexane and analyzed by HPLC on the Perkin-Elmer HS-3 silica column as described above. The kinetics of these reactions generally exhibited an apparent lag phase during the first half-life; pseudo-first-order rate constants were determined from the later portion of the reaction where log $(A_\infty - A_t)$ vs time was linear. The cause of this apparent induction period is unclear. Neither the induction period, the final rate, nor the product distribution was affected by the presence of oxygen, as shown by a pair of parallel experiments in 3 M HCl in which one reaction mixture was degassed by purging with argon and a second was treated with pure oxygen prior to addition of adduct **3f**.

In an experiment to determine the effect of 2,2'-thiodiethanol [bis(2-hydroxyethyl) sulfide] on product distribution, 4 M solutions of 2,2'-thiodiethanol and bis(2-hydroxyethyl) ether in dioxane were prepared volumetrically and equal volumes of 6 M aqueous perchloric acid were mixed with appropriate mixtures of the above organic solutions such that the final concentration of 2,2'-thiodiethanol plus bis(2-hydroxyethyl) ether was held constant at 2 M. This was done to minimize medium effects that might result from the large amounts of thiodiethanol used. Because of the possibility that trace contamination of the thiodiethanol with free mercaptoethanol could influence product ratios, samples of reaction mixtures were neutralized, tested with Ellman's reagent,[19] and found to contain <0.05 mM free thiol. A reaction mixture containing 26 mM mercaptoethanol gave no benzo[*c*]phenanthrene product. Reverse-phase HPLC (Du Pont Zorbax phenyl column) of this mixture indicated the formation of the aromatic dehydration products as well as a broad peak that was slightly less polar than the initial adducts. This material (not seen in reaction mixtures containing 2,2'-thiodiethanol alone) had a UV spectrum similar to that of the initial adducts and was stable under the reaction conditions. Although this peak was not further characterized, we speculate that it corresponds to one or two isomeric bisthiol adducts formed by nucleophilic attack of the added mercaptoethanol.

The time course of adduct isomerization in the presence of 3 M hydrochloric acid was monitored by removal of 2-mL aliquots from a 10-mL reaction mixture and quenching with 5 mL of a solution containing 6 mmol of sodium hydroxide and 1 mmol each of sodium dihydrogen phosphate and disodium hydrogen phosphate. Products were extracted with three (ca. 1 mL) portions of ethyl acetate. After solvent evaporation, the residues were dissolved in 0.2 mL of methanol and portions were analyzed by HPLC on a Du Pont Zorbax phenyl column, 4.2 × 250 mm, eluted with 60% acetonitrile in water at 1.5 mL/min for 5 min, followed by a linear gradient to 100% acetonitrile in 15 min: rt 8.1 (**3e**), 8.6 (**3f**), 11.2 (benzo[*c*]phenanthrene), and 16.2 min (**3g** plus **3h**). The ratio of adducts **3e** and **3f** was determined at 318 nm, and the ratio of products was determined at 255 nm.

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(19) Ellman, G. L. *Arch. Biochem. Biophys.* **1959**, *82*, 70-77.

Biosynthesis of NCS Chrom A, the Chromophore of the Antitumor Antibiotic Neocarzinostatin

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Abstract: Biosynthetic studies on neocarzinostatin chromophore A (NCS Chrom A) were carried out on the basis of the incorporation of singly and doubly ^{13}C labeled acetate precursors as well as radiolabeled [*methyl*-^{3}H]methionine, [^{14}C]sodium bicarbonate, and [^{14}C]sodium acetate by cultures of *Streptomyces carzinostaticus* (ATCC #15944 F-42). The results suggest that the *N*-methyl of the fucosamine and the *O*-methyl of the naphthoic acid moieties are derived from methionine via *S*-adenosylmethionine and the cyclic carbonate carbonyl carbon from carbonate. The acetate incorporation results show that the C_{12} naphthoic acid ring is derived from a hexaketide. The intriguing C_{14} cyclic carbonate/bicyclo[7.3.0]dodecadienediyne ring system, on the other hand, appears to be derived from a minimum of eight head to tail coupled acetate units which is discussed in terms of the oleate-crepenynate biosynthetic pathway for polyacetylenes. The related C_{15} enediyne ring skeleton in the esperamicin/calicheamicin class of antitumor antibiotics may be similarly derived. These incorporation experiments provide independent support for the unprecedented structure of NCS Chrom A.

Neocarzinostatin (NCS) is a member of a family of macromolecular antitumor antibiotics obtained from culture filtrates of *Streptomyces*.[1] The drug causes DNA strand breakage in vivo and in vitro in a reaction greatly stimulated by a sulfhydryl compound.[2] All biological activity resides in a methanol-extractable nonprotein chromophore that is tightly and specifically bound to an apoprotein (M_r = 11 000).[3] In previous reports[4] we have elaborated on the structure of the major component NCS Chrom A and its relationship to two active minor components B and C. We proposed that NCS Chrom A consists of a cyclic

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(1) Ishida, N.; Miyazaki, K.; Kumagai, K. M.; Rikimaru, M. *J. Antibiot.* **1965**, *18*, 68-76.
(2) Goldberg, I. H. In *Mechanisms of DNA Damage and Repair*; Simic, M., Grossman, L., Upton, A. C., Eds.; Plenum Press: New York, 1986; Vol. 38, pp 231-244.
(3) (a) Napier, M. A.; Holmquist, B.; Strydom, D. J.; Goldberg, I. H. *Biochem. Biophys. Res. Commun.* **1979**, *89*, 635-642. (b) Kappen, L. S.; Napier, M. A.; Goldberg, I. H. *Proc. Natl. Acad. Sci. U.S.A* **1980**, *77*, 1970-1974. (c) Ohtsuki, K.; Ishida, N. *J. Antibiot.* **1980**, *33*, 744-750. (d) Suzuki, H.; Mura, K.; Kumada, Y.; Takeuchi, T.; Tanaka, N. *Biochem. Biophys. Res. Commun.* **1980**, *94*, 255-261.

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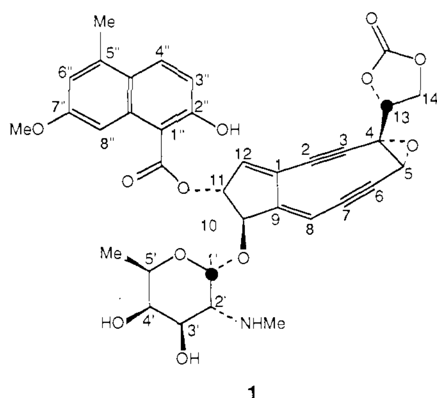
carbonate (1,3-dioxolan-2-one), 2-hydroxy-5-methoxy-7-methyl-1-naphthoate (recently revised to 2-hydroxy-7-methoxy-5-methyl-1-naphthoate),[5] and (methylamino)galactose (*N*-methylfucosamine)[6] moieties linked to an incompletely defined C_{12} substructural unit containing a highly strained epoxide ring which contributes toward its instability.[4c,7] This completed assignment of all oxygens in the molecule and ruled out the presence of an aliphatic aromatic diacyl peroxide as previously proposed by Edo et al.[8] The instability of NCS Chrom A has made its structural investigation quite challenging. The light-sensitive chromophore, when unprotected by the apoprotein, is extremely unstable in aqueous solution,[3b,9] especially upon concentration, and treatment with a variety of reagents results in decomposition. A heavy reliance on spectroscopic methods was indicated early in our structural work because of the limited quantities of NCS available. We found that the chromophore could be successfully stabilized in cold CD_3CO_2D or CD_3OD/CD_3CO_2D mixtures[4a,b] in sufficient concentration to obtain NMR data including preliminary ^{13}C NMR parameters[4c] and decided to explore the potential of biosynthetic incorporation of ^{13}C-enriched substrates. All critical carbons of the naphthoic acid, cyclic carbonate, and unknown C_{12} moieties were found to be acetate derived with good incorporation, which opened up the possibility of determining the complete carbon–carbon connectivity pattern of the unknown substructure from a ^{13}C homonuclear chemical shift correlation map (e.g. 2D-INADEQUATE).[10]

During this work Edo et al.[11a] reported on the complete structure determination of NCS Chrom A, **1**, based primarily on NMR investigation of a stable chlorohydrin derivative, incorporating



1

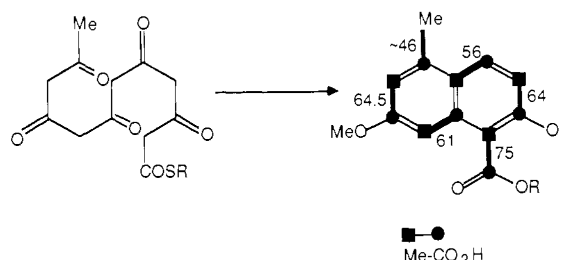
our previous findings and proposing for the C_{12} substructure the unprecedented bicyclo[7.3.0]dodecadienediyne ring system. Moreover, the absolute stereochemistry of the molecule as in **1** has very recently been determined.[11b] In this report we communicate our independent findings in support of the structure work as well as to provide an insight into the biosynthetic origin of the carbon skeletons of the naphthoic, cyclic carbonate, and epoxy bicyclo[7.3.0]dodecadienediyne ring systems and, by analogy, of the related C_{15} enediyne ring skeleton in the esperamicin/calicheamicin class of antibiotics.

Table I. ^{13}C NMR Assignments and $^1J_{cc}$ of [1 + 2-^{13}C]- and [1,2-$^{13}C_2$]Acetate Labeled NCS Chrom A

carbon	[1 + 2-^{13}C]acetate[a] δ, ppm[c]	$^1J_{cc}$, Hz[d]	[1,2-$^{13}C_2$]acetate[b] δ, ppm[c]	$^1J_{^{13}C-^1H}$, Hz	$^1J_{cc}$, Hz
C1''	108.7 s (2)		105.9 s (2)		obsc
C1''-CO_2	172.4 s (1)		173.3 s (1)		75
C2''	162.5 s (1)		165.8 s (1)		64
C3''	116.5 d (2)	62	116.9 d (2)	166	64
C4''	132.9 d (1)	62	134.5 d (1)	159	56
C4a''	124.4 s (2)		124.5 s (2)		~55
C5''	138.6 s (1)	60	138.6 s (1)		~46
C5''-Me	19.9 q (2)		~20.0 q (2)		obsc
C6''	118.2 d (2)	63	118.1 d (2)	160	64.5
C7''	161.2 s (1)		161.1 s (1)		64.5
C7''-OMe	55.8 q		55.7 q		
C8''	103.4 d (2)	69	104.8 d (2)	161.5	61
C8a''	135.4 s (1)		135.5 s (1)		61
C1	130.4 s (1)		130.2 s (1)		~72
C2	87.6 s (2)		87.7 s (2)		188
C3	97.9 s (1)		97.7 s (1)		188
C4	63.8 s (2)		63.9 s (2)		57
C5	55.2 d (1)		55.1 d (1)	198.5	s (103)[d]
C6	100.0 s (2)		99.8 s (2)		186
C7	90.8 s (1)		90.8 s (1)		186
C8	106.8 d (2)	91	106.6 d (2)	172	81
C9	161.1 s (1)		160.8 s (1)		81
C10	82.0 d (2)	48	81.3 d (2)	152	43
C11	82.8 d (1)	~45	83.0 d (1)	161	43
C12	140.0 d (2)	45	140.0 d (2)	179.5	72
C13	76.4 d (1)	32	75.8 d (1)	163	57 (32)[d]
C14	68.2 dd (2)	32	67.9 dd (2)	156, 160	s (32)[d]
CC=O	~160.0 s		155.9 s		
C1'	95.9 d		95.7 d		
C2'	59.5 d		59.3 d		
C2'-NMe	33.5 q		33.5 q		
C3'	68.6 d		68.1 d		
C4'	72.6 d		72.7 d		
C5'	69.2 d		69.1 d		
C5'-Me	16.5 q		16.4 q		

[a] At ~0 °C in CD_3OD/CD_3CO_2D (~9:1). [b] At ~5 °C in CD_3CO_2D. [c] Those carbons enriched by [1-^{13}C]- and [2-^{13}C]acetate are designated (1) and (2), respectively. Abbreviations: s = singlet, d = doublet, q = quartet, obsc = obscured. [d] $^1J_{cc}$ (hertz) values of satellites.

Scheme I. Postulated Biosynthetic Pathway of Naphthoic Acid Ring of NCS Chrom A from Labeling Pattern with [1-^{13}C]-, [2,^{13}C]- and [1,2-$^{13}C_2$]Acetates[a]



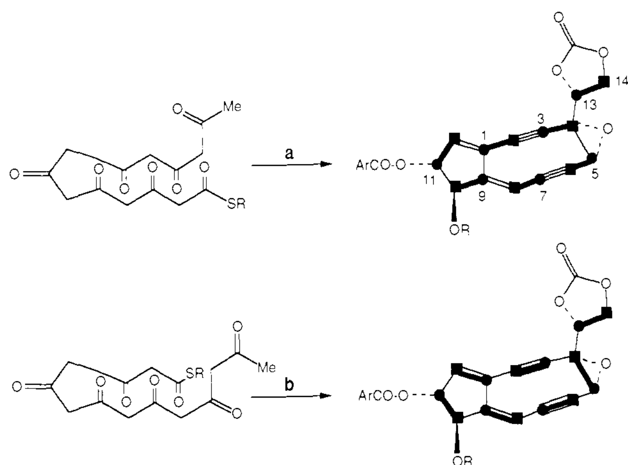
[a] $^1J_{cc}$ (hertz) are indicated for each acetate pair.

Results and Discussion

Incorporation of ^{13}C-Labeled Acetate. To produce biosynthetically ^{13}C-enriched material for determination of the structure and biosynthesis of NCS Chrom A, four incorporation experiments with singly and doubly ^{13}C-enriched acetate were carried out with shake cultures of *Streptomyces carzinostaticus* (ATCC #15944 F-42). High-purity NCS Chrom A uncontaminated with NCS chromophores B, C, and D (see the Experimental Section) was obtained through a multistep isolation procedure including reconstitution after HPLC purification of the labile, extracted chromophore with apo-NCS. Multiple incorporations of [^{13}C]-acetate within a single molecule of chromophore were observed in some cases under the feeding experiments employed. ^{13}C NMR assignments of Edo et al.[11] as well as our own were used, after

(4) (a) Albers-Schonberg, G.; Dewey, R. S.; Hensens, O. D.; Liesch, J. M.; Napier, M. A.; Goldberg, I. H. *Biochem. Biophys. Res. Commun.* **1980**, *95*, 1351–1356. (b) Napier, M. A.; Goldberg, I. H.; Hensens, O. D.; Dewey, R. S.; Liesch, J. M.; Albers-Schonberg, G. *Biochem. Biophys. Res. Commun.* **1981**, *100*, 1703–1712. (c) Hensens, O. D.; Dewey, R. S.; Liesch, J. M.; Napier, M. A.; Reamer, R. A.; Smith, J. L.; Albers-Schonberg, G.; Goldberg, I. H. *Biochem. Biophys. Res. Commun.* **1983**, *113*, 538–547.
(5) Shikuya, M.; Toyooka, K.; Kubota, S.; *Tetrahedron Lett.* **1984**, *25*, 1171–1174.
(6) Edo, K.; Akiyama, Y.; Saito, K.; Mizugaki, M. *J. Antibiot.* **1986**, *39*, 1615–1619.
(7) Lee, S. O.; Goldberg, I. H. *Mol. Pharmacol.* **1988**, *33*, 396–401.
(8) Edo, K.; Ito, M.; Ishida, N.; Koide, Y.; Ito, A.; Haga, M.; Takahashi, T.; Susuki, Y.; Kusano, G. *J. Antibiot.* **1982**, *35*, 106–110.
(9) Kappen, L. S.; Goldberg, I. H. *Biochemistry* **1980**, *19*, 4786–4790.
(10) Bax, A.; Freeman, R.; Frenkiel, T. A.; Levitt, M. H. *J. Magn. Reson.* **1981**, *43*, 478–483.
(11) (a) Edo, K.; Mizugaki, M.; Koide, Y.; Seto, H.; Furihata, K.; Otake, N.; Ishida, N. *Tetrahedron Lett.* **1985**, *26*, 331–334. (b) Myers, A. G.; Proteau, P. J.; Handel, T. M. *J. Am. Chem. Soc.* **1988**, *110*, 7212–7214.

Scheme II. Alternative but Apparent Biosynthetic Pathways of C_{14} Substructure of NCS Chrom A from Labeling Pattern with [1-^{13}C]- and [2-^{13}C]Acetates

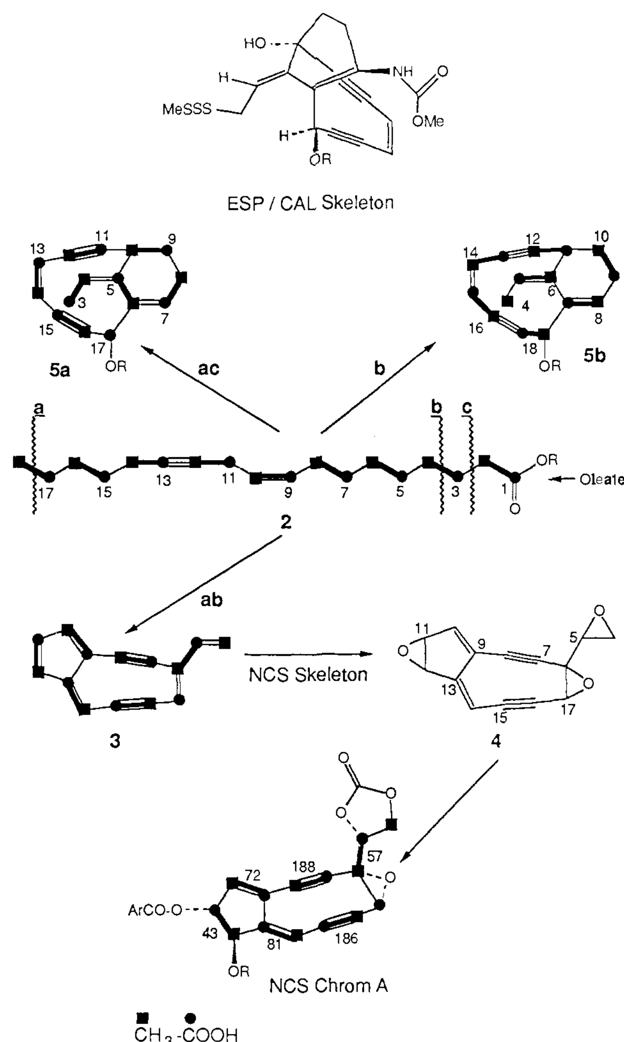


revision of some of the quaternary carbons on the basis of the modified structure for the naphthoic acid moiety.[5] Our assignments, based on chemical shift comparison and selective heteronuclear (^{1}H) decoupling experiments, are in agreement with those of Edo et al.[11] with the exception of C11 and C12, which need to be reversed.

The incorporation results indicated that six carbons of the C_{12} naphthoic acid and seven carbons of the C_{14} bicyclic diyne/cyclic carbonate ring systems are enriched by [1-^{13}C]- and [2-^{13}C]-acetate, respectively (see Table I), with no labeling observed in the methoxyl, *N*-methyl, carbonate, and sugar carbons. This suggests that both ring systems are derived from six and seven acetate units, respectively, as depicted in Schemes I and II. Incorporation experiments with [*methyl*-^{3}H]methionine and [^{14}C]sodium bicarbonate provide evidence that the *N*-methyl of the fucosamine and the *O*-methyl of the naphthoic acid moieties derive from methionine via *S*-adenosylmethionine (SAM) and the cyclic carbonate carbon is carbonate derived. The naphthoic acid moiety therefore appears to consist of a single polyketide chain of six intact acetate units which can fold in only one way (Scheme I). Thus, it can be envisaged that the C_{14} substructure is derived from a single heptaketide chain of seven head to tail coupled acetate units, but no distinction can be made between the two possible pathways a and b in Scheme II with the evidence obtained thus far.

Our original objective to establish the carbon–carbon connectivities of the C_{14} substructure of NCS Chrom A led us to experiments with doubly labeled [1,2-$^{13}C_2$]acetate and mixed-labeled [1 + 2-^{13}C]acetate which shed further light on the polyketide origin of the C_{14} ring system. In the case of the [1 + 2-^{13}C]acetate incorporation many one-bond couplings were evident for protonated carbons. Likewise for [1,2-$^{13}C_2$]acetate-enriched NCS Chrom A, protonated carbon doublets were clearly visible, whereas many of the quaternary carbons were weak, allowing us, nevertheless, to identify all acetate pairs (see Table I). Establishment of carbon–carbon connectivities between acetate pairs via double quantum coherence NMR[10] was not feasible however because of the low S/N. Some connectivities could be made by inspection, which shall be discussed below. The matching of $^{1}J_{cc}$ values confirmed the polyketide origin of the naphthoic acid ring as depicted in Scheme I, but contrary to our expectations, the carbon signals for the epoxide C5 and cyclic carbonate C14 carbons were observed as singlets and found to be inconsistent with the labeling patterns a and b for the C14 substructure in Scheme II. Loss of methyl and carboxylate carbons from either end of an octaketide chain appears to be implicated. This unexpected result is explicable, however, in light of previous studies on the biosynthesis of polyacetylenes. It has been shown that naturally occurring polyacetylenes, whether cyclized or not, derive from oleic acid via the now well-accepted oleate–crepenynate

Scheme III. Proposed Biosynthetic Pathway ab of C_{14} Substructure of NCS Chrom A from Labeling Pattern with [1,2-$^{13}C_2$]Acetate via the Oleate–Crepenynate Pathway (Ref 12)[a]



[a] It should not be inferred that structures **3** and **4** are viewed as intermediates (see the text). $^{1}J_{cc}$ (hertz) are indicated for each acetate pair. The pathway (ac or b) is also postulated for the related C_{15} enediyne ring skeleton of the esperamicin/calicheamicin class of antibiotics. The numbering scheme of both skeletons reflect the origin of the carbons from oleate or crepenynate.

pathway[12] and that shortening of the C_{18} fatty acid is common and can occur by loss of carbon from either end. Loss of carbon from the carboxylate end is more common,[13] but loss of the terminal methyl carbon of the C_{18} fatty acid has been demonstrated in the case of mycomycin[12c] as well as other polyacetylenes.[14] Cultures of *Resinicium bicolor* indicate that mycomycin is derived from C5 to C17 of crepenynate,[12c] a nonaketide, and although our results are consistent with the degradation of two carbons from an octaketide, the degradation of four carbons from a nonaketide would be consistent with these and prior studies of polyacetylene biosynthesis.

We therefore favor a linear C_{18} polyketide precursor of acetate units coupled in head to tail fashion for the C_{14} substructure of NCS Chrom A, which can be extended to the esperamicin/cal-

(12) (a) Bu'Lock, J. D. In *Comparative Phytochemistry*; Swain, T., Ed.; Academic Press: New York, 1966; Chapter 5. (b) Jones, E. R. H.; Piggin, C. M.; Thaller, V.; Turner, J. L. *J. Chem. Res., Synop.* **1977**, 68–69. (c) Ahmed, M.; Hearn, M.; Jones, E. R. H.; Thaller, V. *J. Chem. Res., Synop.* **1977**, 125.

(13) Bohlmann, F.; Burkhardt, T.; Zdero, C. *Naturally Occurring Acetylenes*; Academic Press: New York, 1973.

(14) Hodge, P.; Jones, E. R. H.; Lowe, G. *J. Chem. Soc. C* **1966**, 1216–1221.

icheamicin[15] class of antibiotics as well, as illustrated in Scheme III. For simplicity, the numbering in Scheme III for both skeletons reflect the origin of the carbons from oleate or crepenynate (**2**). This scheme would suggest that all oxygens attached at C4, C5, C10, C11, C13, and C14 are introduced by oxidation at a late stage of the biosynthetic pathway of a long deoxygenated precursor similar to that recently proposed for mevinolin by Vederas et al.[16] Whereas the present experiments preclude further definitive conclusions to be drawn regarding later steps of the biosynthetic pathway, the presence of an epoxide, as well as the juxtaposition of the remaining two pairs of oxygens in NCS Chrom, suggests epoxidation reactions as responsible for the introduction of the six oxygen functions either before, during, or after ring formation. This is illustrated for the latter case in Scheme III (**3** → **4**), where structures **3** and **4** should not be viewed as intermediates. The presence of epoxides in polyacetylenes is not uncommon[13] whereas the presence of a cyclic carbonate moiety has recently been demonstrated in a C_{16} triyne carboxylic acid isolated from *Actinomycetes*[17] and *Microbispora*[18] species. A linear chain oleate–crepenynate pathway may also be postulated for the esperamicins/calicheamicins as illustrated in pathways ac and b (Scheme III). Loss or retention of the terminal methyl group characterizes the two pathways. The scheme differs from that proposed by Schreiber and Kiessling[19] in that a dual polyketide pathway would have to be invoked to account for the postulated common precursor to both neocarzinostatin chromophore and the esperamicin/calicheamicin class of antibiotics. In particular, they hypothesize that C3 of **5a** (or C4 of **5b**) is derived from the C_1 pool whereas our work suggests its origin as C3 or C4 of crepenynate (**2**).

Structure Support. We wish to draw attention to those features of the acetate incorporation experiments which are consistent with the unprecedented structure **1** for NCS Chrom A. The structure of the naphthoic acid ring system has been rigorously established by synthesis[5] and supported by our incorporation results (see Table I), so our discussion will concentrate on the C_{14} substructure. The correlation of $^1J_{cc}$ values and their magnitude from the doubly labeled [1,2-^{13}C]acetate spectra implicated the presence of two single ($^1J_{cc}$ = 43 and 57 Hz), two trisubstituted olefinic ($^1J_{cc}$ = 72 and 81 Hz), and two acetylenic bonds ($^1J_{cc}$ = 186 and 188 Hz),[20] which settles the number of rings of the C_{14} moiety as four. The head to tail linkage of these acetate units as shown in Scheme III provides immediate corroboration of the novel skeleton. Assignment of the resonance at 63.9 ppm to the epoxide quaternary carbon and its appearance as a doublet ($^1J_{cc}$ = 57 Hz) with the same coupling as the cyclic carbonate methine establishes the link between these two rings. Moreover, the only carbon signals in the [1-^{13}C]acetate spectrum which showed a resolved geminal ^{13}C–^{13}C coupling of 14 Hz are attributable to the epoxide methine and one of the acetylenic carbons, which therefore extends the connectivity of C7 through C6–C5–C4–C13 to C14. Without prior knowledge of the assignments for the two acetylenic units, the data are also consistent with connectivity of C2–C3 to the linear sequence C5–C4–C13–C14 at C4 as C3 geminal coupling to C5 is also a possibility. Initially, it was surprising that resonances for C13 and C14 both showed satellites ($^1J_{cc}$ = 32 Hz) in the mixed [1 + 2-^{13}C]acetate labeled spectrum, which subsequently became clear from the doubly labeled result that C13 and C14 belong to a different pair of acetate units. The remaining sp^3–sp^3 single bond characterized by $^1J_{cc}$ = 43 Hz must carry the naphthoic acid and *N*-methylfucosamine moieties and was shown to be linked to one of the trisubstituted double bonds, both C11 and C12 having satellites characterized by $^1J_{cc}$ = 45 Hz in the mixed-[1 + 2-^{13}C]acetate spectrum. These data therefore establish the sequence C1–C12–C11–C10. The protonated carbons at C5 and C8 have satellites characterized by $^1J_{cc}$ = 103 and 91 Hz, corresponding to C5–C6 and C7–C8, respectively. These unusually high values as well as that for C8–C9 ($^1J_{cc}$ = 81 Hz) and the appreciably deshielded chemical shift of C9 at 160.9 ppm are consistent with a highly strained ring system previously implicated[4c] by the large ^{13}C–^{1}H coupling constant ($^1J_{CH}$ = 198.5 Hz) of the epoxide methine carbon.

Conclusion

In summary, the present work provides support for the unprecedented structure of NCS Chrom A and demonstrates that the C_{12} and C_{14} ring skeletons of the chromophore are formed by a polyketide pathway. The C_{12} naphthoic acid ring is derived from six intact acetate units, linked in head to tail fashion, whereas the novel C_{14} dienediyne ring skeleton incorporates six intact acetates and two terminal acetate units which undergo C–C bond cleavage. It is proposed that the C_{14} chain is derived from degradation of oleate via the now well-accepted oleate–crepenynate pathway[12] for polyacetylenes (Scheme III) rather than by de novo synthesis from acetate. This is contrary to the proposal by Schreiber and Kiessling,[19] who suggest a common nonlinear biosynthetic precursor to the C_{14} and C_{15} ring skeletons of NCS Chrom A and the esperamicin/calicheamicin class of antitumor antibiotics, respectively. The C_{15} enediyne ring skeleton of the latter antibiotics can similarly be derived via the oleate–crepenynate pathway (Scheme III).

Experimental Section

Materials. Sodium salts of 90% enriched [1-^{13}C]-, [2-^{13}C]- and [1,2-$^{13}C_2$]acetic acid were purchased from Merck Isotopes. Radiolabeled [*methyl*-^{3}H]methionine (1 Ci/mmol), [^{14}C]$NaHCO_3$ (40 mCi/mmol), [1-^{14}C]NaOAc (40 μCi/mmol) and [2-^{14}C]NaOAc (70 μCi/mmol) were obtained from New England Nuclear Corp.

Chromatography. High pressure liquid chromatography (HPLC) of the NCS chromophore was carried out as described previously[4c] on a Waters μBondapak C18 column using a gradient of 56–84% MeOH buffered with 10mM NH_4OAc pH 4. Under these conditions the retention times of the NCS chromophores were as follows: NCS Chrom A, 44 min; NCS Chrom B, 33 min; NCS Chrom C, 47 min; NCS Chrom D, 50 min. For semipreparative separations an Altex Ultrasphere ODS column (5 μm, 10 mm × 25 cm) was employed using 5 mL/min of 78% MeOH containing 10 mM NH_4OAc.

Column chromatography of NCS and apo-NCS was carried out at 4 °C in the dark with an automatic fraction collector taking 10 min fractions at 0.35 mL/min.

Gel filtration was carried out on a 15 mm × 60 cm column of Sephadex G-50 fine using 20 mM NH_4OAc pH 4 buffer.

Anion-exchange chromatography was carried out on a 15 mm × 20 cm column of DEAE Sephadex A-25, and the column was eluted with 30 mL of 25 mM NaCl, 20 mM NH_4OAc pH 4 followed by a 300 mL linear gradient from 25 to 125 mM NaCl in the same buffer. NCS eluted at 50 mM NaCl, Apo-NCS at 100 mM NaCl. NCS containing NCS Chrom A was partially resolved from NCS containing NCS Chrom D, the latter having a shorter retention time.

Cation-exchange chromatography was carried out on a 15 mm × 45 cm column of SP Sephadex C-25, and the column was eluted with 45 mL of 20 mM NaCl, 50 mM NaCOOH pH 3 followed by a 300 mL linear gradient from 20 to 100 mM NaCl in the same buffer. NCS eluted at

(15) (a) Golik, J.; Clardy, J.; Dubay, G.; Groenewold, G.; Kawaguchi, H.; Konishi, M.; Krishnan, B.; Ohkuma, H.; Saitoh, K.; Doyle, T. W. *J. Am. Chem. Soc.* **1987**, *109*, 3461–3462. (b) Golik, J.; Dubay, G.; Groenewold, G.; Kawaguchi, H.; Konishi, M.; Krishnan, B.; Ohkuma, H.; Saitoh, K.; Doyle, T. W. *J. Am. Chem. Soc.* **1987**, *109*, 3462–3464. (c) Lee, M. D.; Dunne, T. S.; Siegel, M. M.; Wang, C. C.; Morton, G. O.; Borders, D. B. *J. Am. Chem. Soc.* **1987**, *109*, 3464–3466. (d) Lee, M. D.; Dunne, T. S.; Wang, C. C.; Ellestad, G. A.; Siegel, M. M.; Morton, G. O.; McGahren, W. J.; Borders, D. B. *J. Am. Chem. Soc.* **1987** ,*109*, 3466–3468.
(16) Moore, R. N.; Bigan, G.; Chan, J. K.; Hogg, A. M.; Nakashima, T. T.; Vederas, J. C. *J. Am. Chem. Soc.* **1985**, *107*, 3694–3701.
(17) (a) Kempf, A. J.; Hensens, O. D.; Schwartz, R. E.; Sykes, R. S.; Wichmann, C. F.; Wilson, K. E.; Zink, D. L.; Zitano, L. *Abstracts of the New York Academy of Science 1st International Conference on Drug Research in Immunologic and Infectious Diseases*; *Antifungal Drugs: Synthesis, Preclinical and Clinical Evaluation*; Garden City, Long Island, New York, Oct 8, 1987; p 7. (b) Lewis, M. D.; Menes, R. *Tetrahedron Lett.* **1987**, *28*, 5129–5132.
(18) Wright, J. J.; Puar, M. S.; Pramanik, B.; Fishman, A. *J. Chem. Soc., Chem. Commun.* **1988**, 413–414. Patel, M.; Conover, M.; Horan, A.; Loebenberg, D.; Marquez, J.; Mierzwa, R.; Puar, M. S.; Waitz, J. A. *J. Antibiot.* **1988**, *41*, 794–797.
(19) Schreiber, S. L.; Kiessling, L. L. *J. Am. Chem. Soc.* **1988**, *110*, 631–633.
(20) (a) Wray, V. In *Progress in NMR Spectroscopy*; Elmsley, J. W.; Feeney, J., Sutcliffe, L. H., Eds.; Pergamon Press: Oxford, 1979; Vol. 13, pp 117–256. (b) Horak, R. M.; Steyn, P. S.; Vleggaar, R. *Magn. Reson. Chem.* **1985**, *23*, 955–1039. (c) Lambert, J.; Klessinger, M. *Magn. Reson. Chem.* **1987**, *25*, 456–461.

55 mM NaCl, apo-NCS at 65 mM NaCl. NCS containing different chromophores was partially resolved. The order of elution was NCS Chrom A, NCS Chrom D, NCS Chrom C, and NCS Chrom B.

NCS Production. *S. carzinostaticus* (ATCC #15944 F-42)[1] as maintained on YME agar slants at 27 °C. Twenty-four single-spore isolates were screened for antibiotic production. The best strains were used for NCS production. To ensure uniformity, cultures were started from spores from 2 week old slants which had been suspended in pH 7.2 phosphate-buffered saline and kept at −70 °C in 50% glycerin. Prior to inoculation approximately 90% of the glycerin was removed by centrifugation. Production cultures were grown according to the method of Ishida et al.[1] in baffled shake flasks. Typically 7.5 mg/L NCS was obtained.

Antibiotic activity was estimated by paper disk diffusion bioassay using *Bacillus subtilis* spores (ATCC 6051). For a more accurate determination of NCS Chrom A the HPLC integral was used.

90% of the NCS chromophore produced was NCS Chrom A. The remaining 10% was not NCS Chrom B as previously found in dated clinical ampoules of NCS[4b] but a new chromophore which we will call NCS chromophore D. NCS Chrom D has similar UV and fluorescent spectra as NCS Chrom A and is equally active in the bioassay used. Like NCS chromophores B and C, it is much less reactive than NCS Chrom A toward mercaptans.[4b] When the reaction of NCS Chrom A at 4 °C in 100 mM NaOAc-buffered MeOH pH 6.7 with 0.02 M 2-mercaptoethanol was monitored by loss of 440-nm fluorescence when excited at 340 nm, a $t_{1/2}$ of 1.3 min was measured. NCS Chrom D reacted with a $t_{1/2}$ of 6.2 min under the same conditions. When freshly prepared NCS was allowed to age for 6 months at −20 °C, NCS Chrom B was formed. Since neither NCS Chrom A nor NCS Chrom C have been shown to be converted to NCS Chrom B,[4b] perhaps NCS Chrom D is the precursor. Indeed, on storage of the apo-NCS Chrom D complex a product with the same HPLC retention time as NCS Chrom B is formed. It is worth noting that no incorporation of $[^{14}C]CO_2$ into NCS Chrom D was observed.

Biosynthetic Labeling of the NCS Chromophore. The best specific activities were obtained by a 20-h incubation of the bacterial mycelium from 12-h production cultures in nongrowth medium according to the method of Kudo et al.[21] to which labeled precursors were introduced. Typically the yield of NCS was a third of that obtained from the production medium.

[*methyl*-^{3}H]Methionine Incorporation. Mycelium from one flask of production medium was treated as above with a 1.9 mCi [*methyl*-^{3}H]-methionine (1 Ci/mmol). NCS Chrom A (0.4 mg equiv) (sp act. 70 mCi/mmol) was obtained. The naphthoic acid[5,22] portion of the NCS chromophore (obtained by hydrolysis) had approximately one-half of the specific activity associated with the intact chromophore. This shows that the *N*-methyl of the fucosamine and the *O*-methyl of the naphthoic acid probably derive from methionine via SAM. On the basis of the incorporation of two methyl groups, the specific incorporation was 3.5%.

[^{14}C]Acetate Incorporation. Mycelium was incubated as above with 25 mg of low specific activity [^{14}C]acetate (40 μCi/mmol [1-^{14}C]NaOAc or 70 μCi/mmol [2-^{14}C]NaOAc). Specific activities of NCS Chrom A indicated 10–15% specific incorporation based on 13 acetates per molecule. The naphthoic acid portion accounted for approximately one-half of the radioactivity incorporated.

[^{14}C]Bicarbonate Incorporation. Mycelium from one flask of production medium was treated as above with 0.1 mCi of [^{14}C]$NaHCO_3$ (40 mCi/mmol). NCS Chrom A with a specific activity of 0.13 mCi/mmol was obtained (0.3% specific incorporation).

[^{13}C]Acetate Incorporation. Mycelium was treated as above with 25 mg of 90% ^{13}C-labeled sodium acetate per flask of nongrowth medium (100 mL). Twenty-four flasks were grown at a time.

Flasks (5 × 24) treated with [1-^{13}C]NaOAc yielded, after purification, 43 mg equiv of NCS Chrom A.

Flasks (5 × 24) treated with [2-^{13}C]NaOAc yielded, after purification, 40 mg equiv of NCS Chrom A.

Flasks (5 × 24) treated with [1,2-$^{13}C_2$]NaOAc yielded, after purification, 45 mg equiv of NCS Chrom A.

Flasks (15 × 24) treated with a mixture of [1-^{13}C]NaOAc and [2-^{13}C]NaOAc (12.5 mg each per flask) yielded, after purification, 42 mg equiv of NCS Chrom A.

Purification of Biosynthetically ^{13}C-Labeled NCS Chromophore A. Culture filtrates were acidified with oxalic acid to pH 3.5 and filtered. $(NH_4)_2SO_4$ (650 g) was added per liter with stirring on ice to precipitate the proteins which were collected by centrifugation (16 000*g*, 30 min). The pellet was taken up in 20 mM NH_4OAc pH 4 and dialyzed against the same buffer for several hours at 4 °C. After lyophilization, the crude NCS was desalted on a gel filtration column. NCS-containing fractions were pooled and lyophilized. The NCS chromophore was extracted from the crude NCS by repeated extraction with 0.1 M HOAc in MeOH at 0 °C. The extracted NCS chromophore was purified by HPLC and recomplexed with addition of a 20% excess of purified apo-NCS,[3b] and excess MeOH was removed by rotary evaporation without heat. The purified apo-NCS Chrom A complex was dialyzed as before, lyophilized, and passed through a gel filtration column, and the NCS-containing fractions were lyophilized.

Purification of Apo-NCS. Methanol-extracted NCS was dissolved in water with dilute NH_4OH and adjusted to pH 4 with HOAc. The protein was purified by gel filtration chromatography followed by cation-exchange chromatography and anion-exchange chromatography. The purified protein was dialyzed, lyophilized, and passed through a gel filtration column as before, and the apo-NCS-containing fractions were lyophilized and extracted several times with 0.1 M HOAc in MeOH. The apo-NCS was dissolved in water with dilute NH_4OH and adjusted to pH 4 with HOAc.

^{13}C NMR Spectroscopy. The NMR experiments were performed on a Varian SC-300 spectrometer. ^{13}C-enriched NCS protein samples were extracted with 3–5 mL of CD_3CO_2D at ambient room temperature, centrifuged, and filtered, and the filtrate was concentrated to ~0.4 mL under reduced pressure in a warm-water bath to prevent the solution from freezing. All handling of the chromophore was carried out in the dark. Broad-band ^{1}H decoupled, gated, and SFORD ^{13}C NMR spectra were obtained on these solutions in 5-mm tubes at a probe temperature near 5 °C with TMS as reference. In order to perform the experiment at lower temperatures and to prevent the solution from freezing, the mixed [1 + 2-^{13}C]acetate enriched sample was worked up slightly differently. After concentration to ~0.4 mL, CD_3OD was added repeatedly after each concentration step whereby a final solvent mixture was obtained containing ca. 9:1 CD_3OD/CD_3CO_2D. Differences in chemical shift were noted and advantageously revealed the C5″-CH_3 resonance labeled by [2-^{13}C]acetate which was otherwise obscured by the CD_3CO_2D solvent peak near 20 ppm.

Losses are incurred in the extraction procedure of the labile chromophore especially on concentration for in situ ^{13}C NMR. In our experience the chromophore is unstable at concentrations above 3–5 mg/0.4 mL in CD_3CO_2D near 0 °C. For maximum S/N therefore, with the quantities of enriched NCS protein typically in the range 40–45 mg (theoretical yield of NCS Chrom A 2.4–2.7 mg), solutions in CD_3CO_2D were concentrated to a volume suitable for a 5-mm NMR tube whereas natural abundance spectra could only be obtained with significantly more material and a wider bore tube.[4c] Peak enhancements could therefore not be accurately determined.

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(21) Kudo, K.; Kikuchi, M.; Ishida, N. *Antimicrob. Agents Chemother.* **1972**, *1*, 289–295.

(22) Edo, K.; Katamine, S.; Kitami, F.; Ishida, N.; Koide, Y.; Kusano, G.; Nozoe, S. *J. Antibiot.* **1980**, *33*, 347–351.