in 25 ml. of benzene was refluxed for 10 min. on the steam bath. The bicarbonate-washed benzene solution on evaporation gave, after crystallization from aqueous methanol, 325 mg. (85%) of dehydrotremetone (VII), m.p. 83–85°. This, crystallized four times from the same solvent, gave material, m.p. 84.0–85.5° (lit.[12a] m.p. 87.5–88.5°); λ_{max}^{EtOH} 253, 280.5, 292.5 mμ (ε 39,100, 19,150, 14,900) [lit.[12a] λ_{max}^{EtOH} 252, 280, 292 mμ (ε 39,000, 19,000, 15,000)]. Mixed with a sample of VII isolated from *Aplopappus heterophyllus*,[11] m.p. 80–82° cor.; the mixture melted unchanged. The infrared spectra of the two materials were identical throughout, the chief bands being at 5.98, 7.00, 7.41, 7.74, 8.68, and 11.14 μ, all in carbon tetrachloride.

A mixture of this synthetic VII with a sample, m.p. 83.5–85.5° cor., from *Eupatorium urticaefolium*,[12a] melted at 83.5–85.0° cor. The infrared spectra of this natural dehydrotremetone[12a] in carbon tetrachloride was identical with the two curves obtained previously.

The oxime of dehydrotremetone, from aqueous methanol, melted at 131.5–133.5° (lit.[12a] m.p. 131–132°). A mixture of our oxime with the oxime of VII derived from *E. urticaefolium*[12a] melted at 132–133°.

The Dehydrotremetone–Maleic Anhydride Adduct.—A solution of 500 mg. of VII and 500 mg. of maleic anhydride in 25 ml. of benzene was refluxed for 10 hr. On cooling, 150 mg. of the maleic anhydride adduct, m.p. 200–207° dec., was obtained and a second crop by concentration of the mother liquor weighed 180 mg., m.p. 195–205° dec. Extensive recrystallization from aqueous acetone gave material melting at 205–210° dec., not improved by further recrystallization.

Anal. Calcd. for $C_{17}H_{14}O_5$: C, 68.45; H, 4.73. Found: C, 68.72; H, 4.23.

5-Acetyl-2-isopropylbenzofuran.—The hydrogenation of 200 mg. of dehydrotremetone (VII) in 8 ml. of ethyl acetate with 50 mg. of 5% platinum–charcoal at room temperature and a pressure slightly above atmospheric consumed 1 mole of hydrogen in 2.5 hr. and gave, after sublimation at 60–70° (6 mm.), 180 mg. of colorless needles, m.p. 46–48°. Resublimation gave material for analysis; m.p. 47.5–48.5°; λ^{CCl_4} 5.93 μ (no band at 11.14 μ); λ_{max}^{EtOH} 236.5 mμ (ε 21,100); λ_{infl}^{EtOH} 255 mμ (ε 5720).

Anal. Calcd. for $C_{13}H_{14}O_2$: C, 77.20; H, 6.98. Found: C, 77.58; H, 7.04.

Acknowledgment.—This investigation was supported by a Public Health Service grant CY-4817 from the National Cancer Institute, Public Health Service. We also are greatly indebted to Dr. L. H. Zalkow and to Dr. W. A. Bonner who so kindly provided materials isolated from plant sources.

The Structure and Biosynthesis of Nidulin[1,2]

WILLIAM F. BEACH[3] AND JOHN H. RICHARDS[4]

Contribution No. 2949 from the Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena, California

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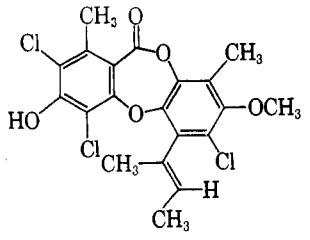
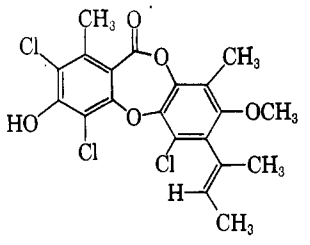
The structure of nidulin is confirmed by additional degradative evidence to be I. The biogenesis of this fungal metabolite is discussed and evidence is presented that it is derived from acetate and isoleucine.

In 1945, Kurung reported[5] that *Aspergillus ustus* produced a substance which was active *in vitro* in inhibiting the growth of *Mycobacterium tuberculosis* and *M. ranae*. Hogeboom and Craig[6] reported the isolation of two crystalline compounds from the same species of *Aspergillus*. Since these early reports much work on the metabolites of *A. ustus* (more properly now *A. nidulans*) has appeared,[7–12] and various partial structural proposals have been suggested.[8,10] Recently,[11,12] two complete structures for nidulin (I and II) have been advanced. Experiments described in the present paper revise the previous results which led to structure II and confirm structure I for nidulin.

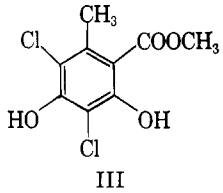


I



II

The structure of ring A of nidulin was established by the isolation of methyl 4,6-dichloro-*o*-sellinate (III)



III

as a degradation product of methyl nidulinate.[9] The presence of the depsidone nucleus was also deduced at an early date.[8] The nature of the five carbons attached to ring B was elucidated both by n.m.r. techniques[13] and by chemical degradations.[11]

The remaining question about the structure of nidulin concerns the distribution of groups about ring B. The assignment of structure I to nidulin[11] rested on the isolation of a dihydroxybenzoquinone derivative, $C_{11}H_{12}O_4$, obtained from nidulin by action of hydriodic acid on nidulin, followed by aerial oxidation of the resulting product in alkaline methanol. This substance was formulated as the 2,5-dihydroxybenzoquinone derivative, because its ultraviolet spectrum in neutral ethanol bears a close resemblance to that of 3-methyl-2,5-dihydroxybenzoquinone. The relative orientation of the two alkyl groups, the chlorine atom, and the oxygen substituent known to be disposed about ring B of

(1) Supported in part by a grant from the U. S. Public Health Service.

(2) Presented at the 140th National Meeting of the American Chemical Society, Chicago, Ill., September, 1961.

(3) National Institute of Health Predoctoral Fellow.

(4) Alfred P. Sloan Fellow.

(5) J. Kurung, *Science*, **102**, 11 (1945).

(6) G. H. Hogeboom and L. C. Craig, *J. Biol. Chem.* **162**, 363 (1946).

(7) W. E. Doering, R. J. Dubos, D. S. Noyce, and R. Dreyfus, *J. Am. Chem. Soc.*, **68**, 725 (1946).

(8) F. M. Dean, J. C. Roberts, A. Robertson, and K. B. Raper, *Nature*, **172**, 344 (1953).

(9) F. M. Dean, J. C. Roberts, and A. Robertson, *J. Chem. Soc.*, 1432 (1954).

(10) F. M. Dean, A. D. T. Erni, and A. Robertson, *ibid.*, 3545 (1956).

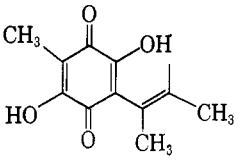
(11) F. M. Dean, D. S. Deorha, A. D. T. Erni, D. W. Hughes, and J. C. Roberts, *ibid.*, 4829 (1960).

(12) W. F. Beach and J. H. Richards, *J. Org. Chem.*, **26**, 1339 (1961).

(13) Ref. 12, p. 3011.

nidulin was thus established. The similarity of the ultraviolet spectra of 2,5-dihydroxybenzoquinone and the product of nidulin degradation in neutral ethanol, is, however, an inadequate basis for structural assignment. For example, the only known 2,6-dihydroxybenzoquinone derivative is 3,5-dimethyl-2,6-dihydroxybenzoquinone[14] whose physical properties are similar to those of the nidulin degradation product and which gives a bright blue solution when dissolved in alkali, a reported property of the nidulin degradation product, but not a characteristic behavior of 2,5-dihydroxybenzoquinone. Moreover, the ultraviolet spectrum of 3,5-dimethyl-2,6-dihydroxybenzoquinone in neutral ethanol is very similar to that of the nidulin degradation product (Fig. 1). Therefore, the previous evidence[11] is insufficient for establishing the structure of the degradation product as a 2,5-dihydroxybenzoquinone derivative or of nidulin as I.

It is, however, easily shown that the dihydroxybenzoquinone from degradation of nidulin does indeed have structure IV. The second ionization constants of 2,5-



IV

and 2,6-dihydroxybenzoquinone will differ markedly. In the former case, the two negative charges are conjugated essentially independently, each with one of the two carbonyl groups, whereas the two negative charges in the dianion of the 2,6-dihydroxybenzoquinone must share conjugation with the same carbonyl function. Therefore a 2,5-dihydroxybenzoquinone will be expected to have a significantly more acidic pK_a (2) than will a 2,6-derivative. The pK_a's of the appropriate substances were determined by observing the ultraviolet spectra of solutions at different pH's obtained by appropriate phosphate buffers. The value of pK_a (2) for 2,5-dihydroxybenzoquinone of 5.2 agrees well with that obtained by Schwarzenbach and Suter[15] from oxidation–reduction potential measurements and is in close accord with the value of 4.8 found for the nidulin degradation product. On the other hand the pK_a (2) for 3,5-dimethyl-2,6-dihydroxybenzoquinone of 8.8 is significantly different, proving unambiguously a 2,5-orientation of the hydroxyl groups in the nidulin degradation product.

This formulation is clearly incompatible with structure II proposed by ourselves[12] for nidulin. This proposal was based on an infrared argument which was found to be invalid when a crucial intermediate, which once analyzed for three chlorine atoms per molecule, was reanalyzed and found to contain only two chlorines per molecule. The derivative in question was prepared by refluxing dihydronidulin in glacial acetic acid for eight or nine hours in the presence of hydrobromic acid and red phosphorus. Under conditions strong enough to cleave the methyl ether, hydrolysis of the lactone and decarboxylation of the resulting acid also occur, as would be expected. Under these conditions neither diploicin (V) nor gangaleoidin (VI) loses chlorine while

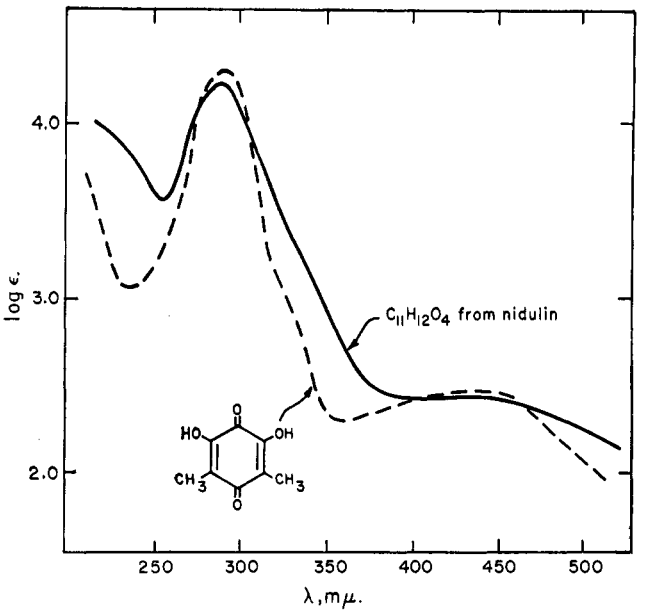
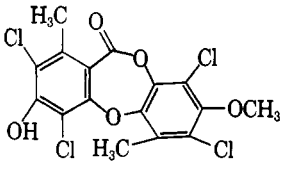
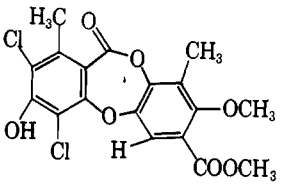


Fig. 1.—Comparison of the ultraviolet spectrum of synthetic 3,5-dimethyl-2,6-dihydroxybenzoquinone with that published for the new compound $C_{11}H_{12}O_4$ obtained from nidulin.



V

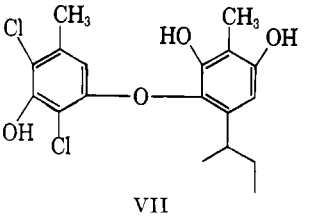


VI

dihydronidulin gives rise to a dechlorinated product, $C_{18}H_{20}O_4Cl_2$, dechlorodemethyldecarbodihydronidulin. Although the ability of hydrogen halide acids to cleave carbon–chlorine bonds has been casually mentioned,[11] this seems to be the first example of a dechlorination under such mild conditions.

In this particular case, the kinetic course of the reaction could be followed qualitatively. The hydrolysis of the lactone and decarboxylation of the resulting acid are quite complete within twenty minutes. Demethylation is essentially complete within one hour. Dechlorination proceeds at a considerably slower rate, with a half life of about two hours.

The structural assignment of dechlorodemethyldecarbodihydronidulin as VII, is based on two considera-



VII

tions. First the loss of chlorine from ring A would be most unusual as this ring has exactly the same substitution pattern as diploicin and gangaleoidin, neither of which lose chlorine under identical conditions. A second, independent argument, can be made on the basis of infrared spectral evidence. Thus, demethyldecarbodihydronidulin, a second product of the reac-

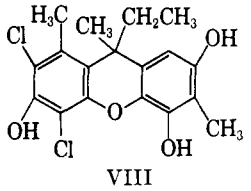
(14) H. Brunmayr, *Monatsch.*, **21**, 1 (1900).

(15) G. Schwarzenbach and H. Suter, *Helv. Chim. Acta*, **24**, 617 (1941).

tion, has two bands in the hydroxyl region; a band at 3534 cm.$^{-1}$, which is roughly twice as intense as a band at 3570 cm.$^{-1}$. The former absorption is attributed to two hydroxyl functions, which are hydrogen bonded to neighboring chlorine,[16] and the latter absorption, to a phenolic hydroxyl group which is bonded to oxygen.[17] Dechlorodemethyldecarbodihydronidulin has three hydroxyl absorptions of about equal intensity. These infrared absorptions suggest that one of the hydroxyl groups is hydrogen bonded to a chlorine (3533 cm.$^{-1}$), one to an oxygen (3566 cm.$^{-1}$), and one is free (3616 cm.$^{-1}$). Thus the net change in the formation of the dechloro derivative is the loss of a hydrogen bonding acceptor adjacent to one of the ring hydroxyls. Loss of chlorine from ring A would not lead to this observed result, and it is, therefore, concluded that a chlorine atom is lost from ring B which supports structure VII for dechlorodemethyldecarbodihydronidulin.

A final point in the chemistry of nidulin is the formation of a xanthene, postulated[11] to account for the isolation of ethylmethylmalonic acid on oxidation of an amorphous substance obtained by heating nidulin or *O*-methylisonidulin with hydriodic acid in glacial acetic acid solution. This xanthene could be formed by an acid-catalyzed internal Friedel–Crafts reaction effected by attack of the protonated olefinic side chain of ring B on the vacant site of ring A. We have isolated this xanthene, formed by refluxing nidulin in a mixture of glacial acetic acid, hydrobromic acid, and red phosphorus. The hydrolysis of the lactone and decarboxylation of the resulting acid was accomplished within twenty minutes. Demethylation also occurred easily and was complete within one hour. The loss of chlorine and xanthene formation proceeded at about equal rates and were about one-half and one-third complete, respectively, after three hours.

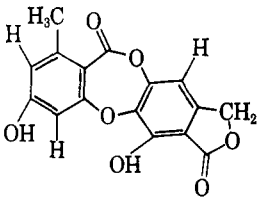
The structure VIII is assigned to the xanthene product, $C_{18}H_{18}O_4Cl_2$, thus obtained, primarily on the basis



VIII

of its n.m.r. spectrum (at 60 Mc.) which includes a triplet at 35 c.p.s. with a splitting of 7 c.p.s. from the methyl protons of the 9-ethyl group, a singlet at 112 c.p.s. from the protons of the 9-methyl group and singlets at 137 and 160 c.p.s. from the protons of the 1-and 6-arylmethyl groups. The relative intensities are in accord with these assignments.

All the foregoing observations are accounted for by the structure of nidulin I first proposed by Dean, *et. al.*[11] An alternative with the chlorine and methoxyl groups interchanged on ring B is possible. The former structure is favored because of the positive bleaching powder test (for resorcinol nuclei) that nornidulin has been observed to give.[9] Precedent also favors structure I. Of all known depsidones, only one, variolaric acid (IX), has a group other than hydroxyl or methyl at position 4′. It should be noted that even in variolaric acid, the oxygen function of ring B is in the 6′-position and *meta* to the lactone bridge.[18]

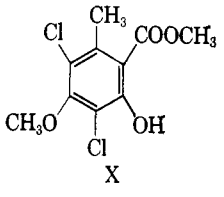


IX

Nidulin is a particularly attractive depsidone for biosynthetic studies because of its origin from a simple fungus rather than from a lichen, and we have undertaken a preliminary study of the biosynthesis of nidulin from labeled acetate and isoleucine.

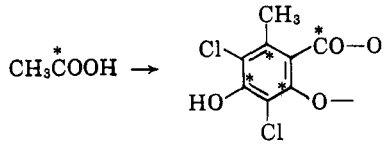
Birch,[19a] in particular, has examined the role of acetate in the biosynthesis of many natural substances, and the depsidones fit well into this theoretical framework. In particular, ring A of nidulin is expected to be derived from four molecules of acetate or, in light of recent developments,[19b] from one molecule of acetate and three molecules of malonate. The origin of ring B is open to speculation.

Sodium acetate-1-C^{14} was incorporated into nidulin produced by *A. nidulans* to the extent of 1.6%. Nidulin was degraded to decarbonidulin and to methyl 4,6-dichloroeverinate (X). The decarbonidulin possessed



X

86 ± 3% of the original activity present in nidulin, and the methyl 4,6-dichloroeverinate had 49 ± 3% of the total activity. Thus the carboxyl carbon attached to ring A had about one-fourth of the total ring A activity (*i.e.*, found 14 ± 3%, expected, 0.25 × 49 = 12%), and the total activity was divided essentially equally between the two rings.



The 2-butenyl side chain of nidulin is an unusual feature, not previously encountered as such in nature. *Aspergillus flavus* produces an acidic nitrogen-containing metabolite, aspergillic acid[20] (XI) which is readily recognized as an oxidized form of the diketopiperazine XII, of isoleucine (XIII), and leucine (XIV). Another metabolite, hydroxyaspergillic acid (XV) is produced simultaneously,[21] and loss of water from the side chain of this compound would produce the 2-but-2-enyl side

(16) A. W. Baker, *J. Am. Chem. Soc.*, **80**, 3598 (1958); A. W. Baker and W. A. Kalding, *ibid.*, **81**, 5904 (1959).

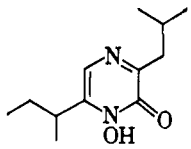
(17) A. W. Baker and A. T. Shulgin, *ibid.*, **80**, 5358 (1958).

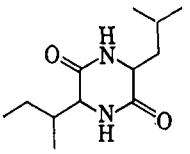
(18) NOTE ADDED IN PROOF.—Recent work has been adduced additional evidence for structure VI for the nidulin degradation product [B. W. Bycroft and J. C. Roberts, *J. Org. Chem.*, **28**, 1429 (1963)]. Moreover, a recent X-ray determination of the structure of nidulin confirms I (Mrs. Joyce McMillan and R. E. Dickerson, private communication).

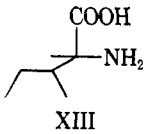
(19) (a) A. J. Birch, review on progress in "The Chemistry of Organic Natural Products," Springer-Verlag, Berlin, 1957, p. 186; (b) J. D. Bu'Lock and H. M. Smalley, *Proc. Chem. Soc.*, 209 (1961).

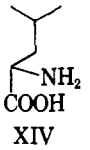
(20) G. T. Newbold, W. Sharp, and F. S. Spring, *J. Chem. Soc.*, 2679 (1951).

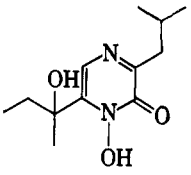
(21) J. D. Dutcher, *J. Biol. Chem.*, **232**, 785 (1958).


XI


XII


XIII


XIV

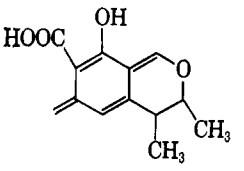

XV

chain characteristic of nidulin. These considerations led us to investigate the incorporation of isoleucine into ring B of nidulin. Recently hydroxyaspergillic acid has been shown[22] to be derived from leucine and isoleucine in the manner just discussed.

Uniformly labeled isoleucine was incorporated into nidulin to the extent of 4%. The isolation of ring A of the nidulin so produced as methyl 4,6-dichloroeverinate, having a specific activity of 13% of that of the original metabolite, demonstrated that 87% of the nidulin molecule's radioactivity is concentrated in the B ring. If isoleucine's role in the biogenesis of nidulin were solely to act as the substrate for the 2-but-2-enyl side chain, one would anticipate no activity in ring A in this experiment. However, isoleucine can be degraded to acetate *in vivo*. For example, studies[23] of isoleucine metabolism by enzymatic extracts of pig heart and rat liver have indicated that acetyl coenzyme A is one of the metabolic end products. If one assumes that all the carbon atoms of the skeleton of nidulin arise from either acetate or isoleucine, that all carbons from the same initial source are equally labeled in nidulin and that only one isoleucine is incorporated into the ring B skeleton, it may be calculated from the observed incorporations that those carbons which come directly from isoleucine are approximately nine times more radioactive than those which have come from isoleucine by way of acetate or other intermediates.

Alternately, if one assumes that the 13% activity found in ring A is equally divided between the eight carbon atoms of ring A, so that each has about 1.6% of the total activity, and that the six carbon atoms of ring B, which are not directly derived from isoleucine, each have a similar 1.6% of the total activity, there is then $100 - (13 + 6 \times 1.6) = 77.4\%$ of the total activity contained in the remaining five carbon atoms. This value strongly supports the selective incorporation of isoleucine into ring B.

It has been suggested[12] that the side chain of nidulin is related to skeletal features present in citrinin[24a] (XVI) and calphyllolide[24b] (XVII) and that the 2-butenyl systems in these metabolites might arise from acetate plus a one-carbon fragment. The results of the experiment with isoleucine suggest a different biogenesis for ring B of nidulin, in which a five-carbon fragment derived from isoleucine, three acetate (or malonate) units, and an additional one-carbon unit are combined as shown in Chart I. The side chain can be oxidized after formation of the ring, or the isoleucine might be incorporated as tiglic acid (*cis*-2-carboxy-2-butene). Tiglyl coenzyme A has been identified as an intermediate in the metabolism of isoleucine.[22]


XVI

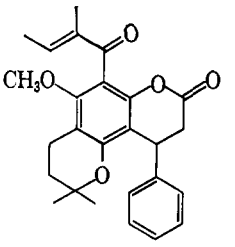
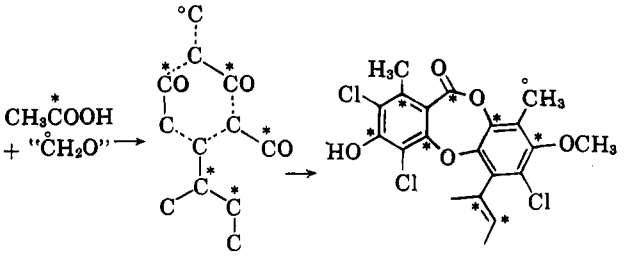

XVIII

CHART I



Rings B and A (orsellinic acid derived from acetate and malonate) can be joined, and the diphenyl ether bond formed by a one-electron oxidative coupling process.[25] The introduction of positive chlorine and formation of the *O*-methyl ether complete the biosynthesis of nidulin. The chlorination of ring B with concerted decarboxylation is well precedented.

It is of interest at this point to test whether this biogenetic scheme is in agreement with the data on acetate incorporation. The biosynthesis of isoleucine has been established in *Escherichia coli*.[26] If one assumes the same origin for isoleucine in *A. nidulans*, one will expect to find the observed equal distribution of activity between the two rings of nidulin. The acetate-C^{14} result is thus entirely compatible with the proposed biosynthetic pathway to nidulin (Chart I).

Experimental

Melting points are uncorrected. Microanalyses are by Elek Microanalytical Laboratories, Los Angeles, Calif., (E), Schwarzkopf Microanalytical Laboratories, Woodside, N. Y. (S), or Spang Microanalytical Laboratories, Ann Arbor, Mich. (Sp).

Isolation of 2,5-Dihydroxy-3-methyl-6-(2-but-2-enyl)benzoquinone from Nidulin.—A solution of nidulin (500 mg., 1.13 mmoles) in glacial acetic acid (10 ml.) was brought to a boil. Red phosphorus (1 g.) and constant boiling hydrobromic acid (8 ml.) were added and the mixture was refluxed for 40 min.

The reaction mixture was then poured into water (150 ml.) and the resulting slurry was extracted several times with ether. The ether extracts were filtered free from residual phosphorus and washed successively with water, saturated aqueous sodium bicarbonate, and again with water. The ether was removed in a stream of nitrogen.

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The residue was taken up in methanol (20 ml.) and the methanolic solution, along with two pellets of potassium hydroxide, was heated at reflux for 2 hr. in a nitrogen atmosphere.

The dark solution was poured into water (100 ml.). Solid potassium hydroxide (3 g.) was added to the solution bringing its pH to 6.3. The resulting solution was extracted with two volumes of ether, and the aqueous phase was then made highly acidic with sulfuric acid and again extracted twice with ether. The ethereal solution of the acidic products, thus obtained, was evaporated and the resulting residue was sublimed several times to give a small amount of a red compound (10 mg.), 2,5-dihydroxy-3-methyl-6-(2-but-2-enyl)benzoquinone, m.p. 196–197° (lit.[12] m.p. 198°).

Anal. Calcd. for $C_{11}H_{12}O_4$: C, 63.45; H, 5.81. Found (Sp): C, 63.21; H, 5.72.

Determination of pK_a (2) for Dihydroxybenzoquinones.—The second acid dissociation constants were determined by observing the change in the intensity ultraviolet absorption of the derivative in question at an appropriate wave length as a function of pH. The wave lengths used in mμ and the experimentally determined pK_a (2) values (in parentheses) are: 2,5-dihydroxybenzoquinone, 486 (5.2); 3,5-dimethyl-2,6-dihydroxybenzoquinone, 282 and 337 (8.8 at both wave lengths); nidulin degradation product 3-*cis*-(2-but-2-enyl)-6-methyl-2,5-dihydroxybenzoquinone, 245 and 270 (4.8 at both wave lengths).

Dihydronidulin.—Nidulin (340 mg., 0.78 mmole) was dissolved in glacial acetic (20 ml.) acid, and platinum dioxide (20 mg.) was added. The mixture was hydrogenated at room temperature and atmospheric pressure. After consumption of about one mole of hydrogen, the rate of hydrogen uptake fell to essentially zero. The reaction solution was diluted with water and extracted with ether. The ethereal solution was washed with water, saturated aqueous sodium bicarbonate, and again with water. After drying the ethereal solution over anhydrous magnesium sulfate, the ether was distilled. The residue was crystallized from *n*-hexane giving long transparent needles (280 mg., 82%) of dihydronidulin, m.p. 147–150°.

Anal. Calcd. for $C_{20}H_{19}O_5Cl_3$: C, 53.89; H, 4.30; Cl, 23.86. Found (S): C, 53.73; H, 4.19; Cl, 23.60.

The Dechlorination of Dihydronidulin. The Action of Hydrobromic Acid and Red Phosphorus on Dihydronidulin.—To a boiling solution of dihydronidulin (500 mg., 1.12 mmoles) in glacial acetic acid (10 ml.), red phosphorus (1 g.) and constant boiling (48%) hydrobromic acid (8 ml.) were added. The reaction mixture was refluxed for the desired reaction time, after which it was poured into water (150 ml.) contained in a separatory funnel. The aqueous slurry was shaken with three portions of ether. The ethereal extracts were filtered free from residual phosphorus, and washed successively with water, saturated aqueous sodium bicarbonate, and once again with water.

The ether was evaporated and the residue was dissolved in sufficient carbon tetrachloride to make a 0.5% solution. This solution was then examined in an infrared spectrophotometer.

Depsidone Assay.—The amount of starting material present may be estimated by the intensity of the depsidone carbonyl band at 1750 cm.$^{-1}$. Its extinction coefficient is roughly 600 or about three times that of the hydroxyl bands.

Estimation of the Extent of Demethylation and Dechlorination.—Results on the assays of depsidone showed that the preliminary steps of hydrolysis and decarboxylation are essentially completed after 20-min. reaction time. From this point forward, all species have only one hydroxyl group, the phenolic hydroxyl that was liberated in the lactone hydrolysis, that absorbs at 3573 cm.$^{-1}$. As demethylation proceeds the third hydroxyl group is liberated, and additional absorption is observed in the infrared spectrum. This newly liberated hydroxyl group may be adjacent to the B-ring chlorine atom and absorb at 3536 cm.$^{-1}$, or, if dechlorination has already taken place, it will be unassociated and absorb at 3617 cm.$^{-1}$. Thus by observing the intensity of the hydroxyl bands at 3573, 3536, and 3617 cm.$^{-1}$, one has a measure of the relative amounts of demethylated and dechlorinated species.

The results of these experiments are tabulated in Table I.

Isolation of Demethyldecarbodihydronidulin and Dechlorodemethyldecarbodihydronidulin.—A chromatographic column of 3.5-cm. diameter was prepared by dry-packing a mixture of Supercel and silicic acid (3:7 by weight). The column was primed with methylene chloride. The combined reaction mixtures in methylene chloride solutions were introduced at the top of the column. The first band eluted was decarbodemethyldihydronidulin. After a few recrystallizations from heptane, it appeared as well formed crystals, m.p. 127.5–129.5°. Its n.m.r. spectrum had no methoxyl methyl absorption. Its infrared spectrum possessed a band of 3534 cm.$^{-1}$ which was roughly twice as intense as a band at 3570 cm.$^{-1}$. There was no absorption in the 3617-cm.$^{-1}$ region.

TABLE I

	Mole %		
	20 min.	1 hr.	2 hr.
Species analyzed for			
depsidone	1	0	0
acids	0	0	0
Demethylated species	53	93	100
Dechlorinated species	7	32	48

Anal. Calcd. for $C_{18}H_{19}O_4Cl_3$: C, 53.29; H, 4.72; Cl, 26.22. Found (E): C, 53.09; H, 5.00; Cl, 26.42.

The second band to be eluted from the column contained dechlorodemethyldecarbodihydronidulin. Recrystallization from a mixture of benzene and heptane produced well formed crystals of m.p. 157.0–158.0°. Infrared spectrum of this material had three bands of approximately the same intensity at 3533, 3566, and 3616 cm.$^{-1}$.

Anal. Calcd. for $C_{18}H_{20}O_4Cl_2$: C, 58.23; H, 5.43; Cl, 19.10. Found (S): C, 58.13; H, 5.57; Cl, 18.95. In a previous analysis a value of Cl, 26.48%, was obtained for this identical product.

The Formation of Xanthene from Nidulin. The Action of Hydrobromic Acid and Red Phosphorus on Nidulin.—Nidulin (500 mg., 1.13 mmoles) was treated by an identical procedure as was used with dihydronidulin in the foregoing dechlorination experiments. After the infrared spectra were taken, the carbon tetrachloride solution was reduced in volume tenfold for n.m.r. spectra determination.

Estimation of the Degree of Demethylation.—Species that contain a methoxyl group absorb sharply at about 235 c.p.s. in the n.m.r. spectrum, while all species contain two aromatic methyl groups, which give two unsplit peaks in the 150-c.p.s. region. The mole % of the methoxylated species can thus be calculated from the areas in these two regions.

Estimation of the Degree of Dechlorination.—In this case the infrared procedure developed for the foregoing dechlorination experiment was again employed.

Xanthene Assay.—Species of the xanthene type give rise to a triplet absorption in the n.mr. spectrum at 35 c.p.s. This signal provides a means of estimating the mole % of xanthene species in the reaction mixture. The data obtained after various reaction times are presented in Table II.

TABLE II

Methoxyl assay

Reaction time	% Methoxyl
6 min.	83
20 min.	33
55 min.	0

Dechlorination assay

Reaction time	A(3619 cm.$^{-1}$)/A(3567 cm.$^{-1}$)	% Dechlorination
20 min.	0.09	9
55 min.	0.22	23
3 hr.	0.47	49

Xanthene assay[a]

Reaction time	% Xanthene
20 min.	0
55 min.	16
3 hr.	35

[a] A(3619 cm.$^{-1}$)/A(3567 cm.$^{-1}$) is 0.96 in the xanthene.

Isolation of the Xanthene and Demethyldecarbonidulin.—A dry-packed chromatographic column of a 3:7 mixture of Supercel and silicic acid (100 g.) was primed with methylene chloride. The reaction mixture of the 3-hr. run was placed on the column in a small amount of methylene chloride. Elution was carried out with the same solvent.

The first band to be eluted contained demthyldecarbonidulin, which, after being crystallized twice from a chloroform–heptane mixture, had m.p. 141.5–143°. Its infrared spectrum (carbon tetrachloride, *ca.* 0.5%), in the hydroxyl stretching frequency

region, possessed a band of 3537 cm.$^{-1}$, which was about twice the optical density of a shoulder at *ca.* 3561 cm.$^{-1}$. The major features of its n.m.r. spectrum (chloroform, 5%) were two closely spaced unsplit peaks at 140 and 143 c.p.s., and a rather complex series of absorptions between 75 and 120 c.p.s. The latter absorptions, due to vinyl methyl protons, indicated that the substance was actually not a single compound but a mixture of isomers of the 2-but-2-enyl group attached to the B ring. Judging from the relative sizes of the signals, the mixture appears to have a *trans–cis* ratio of about 2:1.

Anal. Calcd. for $C_{18}H_{17}O_4Cl_3$: C, 53.55; H, 4.25; Cl, 26.35. Found (Sp): C, 53.59; H, 4.41; Cl, 26.42.

The second band eluted from the column contained the xanthene, which, after being crystallized twice from carbon tetrachloride, had m.p. 171.5–173°. Its infrared spectrum (carbon tetrachloride *ca.* 0.05%) in the hydroxyl stretching frequency region possessed three bands of approximately equal intensity at 3533, 3567, and 3619 cm.$^{-1}$. The major features of its n.m.r. spectrum (chloroform *ca.* 5%) were (a) a triplet at 35 c.p.s., proportional to three protons, (b) a singlet at 112 c.p.s., representing three protons, and (c) two singlets, each proportional to three protons, at 137 and 160 c.p.s.

Anal. Calcd. for $C_{18}H_{18}O_4Cl_2$: C, 58.55; H, 4.91; Cl, 19.21. Found (Sp): C, 58.61; H, 4.98; Cl, 19.30.

The Incorporation of Acetate-1-C^{14} in the Nidulin by *A. Nidulans*.—Five 1-l. flask colonies of *A. nidulans* were inoculated with a total of one mc. of sodium acetate 1-C^{14} (Merck). After thirty-days' growth, the felts were processed in the customary manner to give a hexane solution of crude nidulin. The crude mixture was chromatographed on a silicic acid–Supercel (7:3) column with a 2% solution of ether in hexane. The total recovery of nidulin was estimated at 200 mg., and its total activity represented an incorporation of 1.6% of the labeled acetate. The highly active nidulin was diluted with sufficient inactive nidulin that the resultant material possessed an activity of 0.4594 ± 0.0032 μc./mmole. This active nidulin, after a number of crystallizations from heptane, had m.p. 180.5–181.5°.

C^{14}-Decarbonidulin.—Nidulin (500 mg., 1.13 mmoles, 0.4594 ± 0.0032 μc./mmole) was dissolved in 4 ml. of dioxane. The dioxane solution was added to 25 ml. of hot aqueous sodium hydroxide (100 ml., 2 *N*) slowly, so that no permanent precipitate formed.

After the alkaline solution was refluxed for 8 hr., it was acidified with dilute sulfuric acid and stirred for 3 hr. in a stream of nitrogen to ensure the removal of any radioactive gases. The solid product was collected and purified by crystallization from aqueous methanol. White crystals of C^{14}-decarbonidulin were thus obtained, m.p. 145.5–147.5° (lit.[9] m.p. 148–149°). This material had specific activity of 0.3872 ± 0.0039 μc./mmole. Subsequent recrystallizations changed neither the melting point nor the specific activity of this material.

C^{14}-Methyl 4,6-Dichloroeverinate.—Nidulin (500 mg., 1.13 mmoles, 0.4594 ± 0.0032 μc./mmole) was dissolved in dioxane (4 ml.). The dioxane solution was added to a warm aqueous solution of sodium hydroxide (25 ml., 2 *N*) at such a rate that no permanent precipitate formed. Heating on a steam bath was continued for 5 min. after the completion of the addition. The resulting solution was chilled with ice and acidified with concentrated sulfuric acid. The liberated solid was extracted into ether, and the ethereal extract was in turn washed twice with water.

The resulting ethereal solution was treated with an excess of diazomethane and ether. After 15-min. standing, a little acetic acid was added to decompose the residual diazomethane, and the solvent was evaporated.

The residue was taken up in glacial acetic acid (50 ml.). Five drops of concentrated nitric acid was added to the solution, the color of which changed within a few minutes through yellow to a bright cherry red. After 15 min. at room temperature, the reaction mixture was poured into water (600 ml.). The slurry thus produced was extracted into ether as a cherry red solution, which was successively washed with water, saturated aqueous sodium bicarbonate solution, and sodium hydroxide solution (2 *N*). The sodium hydroxide fraction, when acidified, produced a solid which, after being allowed to digest for a few hours, was separated by filtration, washed well with water, and sucked dry. This solid was crystallized from aqueous methanol to give yellow needles, m.p. 80.5–81°. Recrystallization gave material melting at 82–82.5° (lit.[9] m.p. 82–83°). The specific activity of the recrystallized methyl 4,6-dichloroeverinate was 0.2244 ± 0.0052 μc./mmole.

Nidulin from Uniformly Labeled Isoleucine.—Nidulin was isolated from a culture of *A. nidulans* to which had been added 0.33 mc. of uniformly labeled isoleucine. Nidulin was extracted in the usual fashion and had a specific activity of 2.80 μc./mmole. It was estimated that 4% of the added isoleucine had been incorporated into the nidulin. A portion of the nidulin thus obtained was diluted with some nonradioactive material to give, after one crystallization from heptane, large glistening needles (m.p. 177–179°) having a specific activity 0.4306 ± 0.0035 μc./mmole. The specific activity was not changed on further recrystallization.

Preparation of C^{14}-Methyl 4,6-Dichloroeverinate.—Nidulin (500 mg., 1.13 mmoles, 0.4306 ± 0.0035 μc./mmole) was hydrolyzed, methylated, and cleaved with nitric acid in acetic acid exactly as described for the isolation of ring A. The purified methyl 4,6-dichloroeverinate (m.p. 82.0–82.7°) had a specific activity of 0.05778 ± 0.0005 μc./mmole.