THE STRUCTURE AND BIOGENESIS OF NIDULIN

Thesis by

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ABSTRACT

The structure of nidulin, the major metabolic product of Aspergillus nidulans, is demonstrated beyond reasonable doubt to be that which was set forth recently by other workers. In support of the structure, a xanthene was isolated and characterised. It was also discovered that nidulin suffered a remarkably facile dechlorination in the presence of hydrobromic acid and red phosphorous.

Preliminary tracer experiments with labelled acetate were performed, and the expected pattern of incorporation was observed. Isoleucine was likewise shown to be incorporated into nidulin. A possible biogenesis for nidulin is discussed which points the way for future research.

ACKNOWLEDGEMENTS

I am particularly indebted to my research director, Professor John H. Richards, for his cheerful availability for counsel at all times.

I am financially indebted to the National Institutes of Health for a predoctoral fellowship (1959-1961) and to the Institute for teaching assistantships (1957-1959).

I am also indebted to my many associates in Crellin who have contributed to this thesis through their discussions, criticisms and suggestions.

Above all, I am grateful to my wife, Karen, who has cheerfully directed all of her energy to making this venture a successful one.

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1. LITERATURE SURVEY

In 1945, Kurung reported (1) Aspergillus ustus as the fourth species of fungus (see Note I) producing a substance which was active in inhibiting in vitro the growth of Mycobacterium tuberculosis and M. ranae. He found that the active principle could be extracted from the culture medium with ether and other organic solvents. The active principle was quite stable once separated from the medium, and, in spite of its high toxicity to certain bacteria, it could be tolerated by mice at a high level of dosage.

Working with the same strain of mold, Hogeboom and Craig in 1946 reported (2) the isolation of two crystalline compounds by the technique of countercurrent distribution. Each contained covalent chlorine and appeared to have a biological activity similar to that of the ether extract of Kurung. Compound I, melting at 185-187°, was formulated as $C_{21}H_{17}O_6Cl_3$, and compound II, melting at 214-216°, as $C_{21}H_{18}O_6Cl_2$.

Using a simpler method involving bicarbonate and carbonate extractions, Doering, Dubos, Noyce and Dreyfus (1946) (3) obtained compound I, along with other uncharacterized metabolites, from the mycelial felts and liquors of the same fungus. Concluding that it was

the major metabolite, they gave compound I the name "ustin," and assigned to it the presently accepted empirical formula $C_{19}H_{15}O_5Cl_3$. The workers further characterized ustin by preparing mono- (mp 174°) and dimethyl (mp 147°) ethers and a monoacetyl derivative (mp 212°).

The field remained quiet for seven years until 1953 when Dean, Roberts, Robertson and Raper (4) published a preliminary paper containing a partial proposal of structure without supporting evidence. The writers asserted that the mold previously worked with was a "non-ascosporic strain of Aspergillus nidulans" (see Note II), and that the true major product of metabolism is a compound, mp 180°, for which the name nidulin was proposed. It was shown that nidulin is an O-methylester of ustin, and consequently "nornidulin" was suggested as a replacement for "ustin."

Shortly after the appearance of their preliminary paper, Dean, Roberts and Robertson (5) published the details of the chemical data which led them to postulate I as the partial structure of nidulin. The following is a review of their data and deductions.

Nidulin is a colorless, optically inactive compound, mp 180°, which analyzes for $C_{20}H_{17}O_5Cl_3$. It contains one methoxyl group (by analysis) and one hydroxyl group which is readily acetylated and methylated. The compound gives no color with ferric chloride, is not easily oxidized and does not react with the common carbonyl reagents.

In cold, aqueous alcohol, nidulin behaves as a monobasic acid on titration. The acidic character was attributed to the presence of an acidic hydroxyl rather than a carboxyl group, because under the same conditions, O-acetylnidulin is neutral.

The behavior of nidulin as a dibasic acid under more vigorous treatment with alkali, and the hydrolysis of nidulin to nidulinic acid ($C_{20}H_{19}O_6Cl_3$) without the loss of carbon disclosed the presence of a lactone system. This was confirmed by the formation of a hydroxyester, methyl nidulinate, on the treatment of nidulin with methanolic potassium hydroxide, and the production of nidulinic acid and decarbonidulin ($C_{19}H_{19}O_4Cl_3$), a dihydric phenol, when nidulin was boiled with alkali in aqueous dioxan. Although nidulin reacts with hydroxylamine to form a compound $C_{20}H_{20}O_6NCl_3$, this product does not give the expected positive ferric chloride color. The product was assigned a hydroxamic acid structure, for the lack of color in the ferric chloride test was attributed to the bulkiness of the molecule interfering with the formation of the iron complex.

The chlorine atoms in nidulin are stable to hydrolysis with aqueous or alcoholic sodium hydroxide, hydrogenolysis over platinum or palladium catalysts, or the action of Raney nickel alloy and hot alkali. All three chlorines thus appear to be aromatic halides.

Four of the five oxygen atoms in the molecule are in a lactone,

a methoxyl group and a hydroxyl group. The seemingly inert fifth oxygen atom is apparently part of an ether system.

From its composition and properties, in conjunction with the occurrence of known chlorine-containing depsidones in lichens, it seemed probable that nidulin was a depsidone. This assertion was strongly supported by the isolation of methyl 4, 6-dichloro-o-orsellinate (II) and methyl 4, 6-dichloroeverinate (III) as a result of degradations (6) which were carried out by a sequence of chlorination, reduction and methanolysis devised for the fission of the depsidone diploicin, a product of the lichen Buellia canescens. The remarkably facile fission of nidulin by nitric acid to give a high yield of methyl 4, 6-dichloroeverinate (III) also supported the depsidone hypothesis. The isolation of an A ring derivative in the above cases also served to establish the nature and orientation of the A ring substituents in nidulin.

The proposed depsidone structure implies the presence of a second aromatic ring system in the nidulin molecule. Although they were unable to isolate any fragment of nidulin through degradations that demonstrated the existence of a second aromatic ring, the English workers were able to put forth the following arguments in behalf of its existence. First, all three chlorine atoms are remarkably stable. Since the isolation of the A ring fragment places two of them on that ring, one remains for the rest of the molecule. The stability of the

third chlorine atom suggests its attachment to an aromatic ring. Second, when O-methylnidulin is reacted with methanolic sodium methoxide, methyl O-methylnidulinate is formed. The latter material appeared to be phenolic, since it was insoluble in sodium bicarbonate solution, yet readily soluble in sodium carbonate solution. Since the hydroxyl group freed by the cleavage of the lactone cannot be attached to the A ring, the fact that methyl O-methylnidulinate is phenolic is evidence for a second aromatic system. Third, in the absence of a second aromatic system, nidulin would be highly unsaturated (in the olefinic sense), and should be easily hydrogenated. However, nidulin resisted attempts at hydrogenation with Adams' catalyst or Raney nickel and hydrogen at 60 p.s.i. An additional item of evidence for the existence of a second aromatic system was given in a subsequent paper (7). The ultraviolet spectrum of methyl O-methylnidulinate displays a bathochromic shift when alkali is added to its methanolic solution. Such behavior is characteristic of phenols, and since all hydroxyl groups of the A ring are protected in methyl O-methylnidulinate, the presence of a phenol in the compound implies the presence of a second aromatic ring in nidulin. The existence of the ring is thereby assumed, and henceforth it shall be referred to as the B ring.

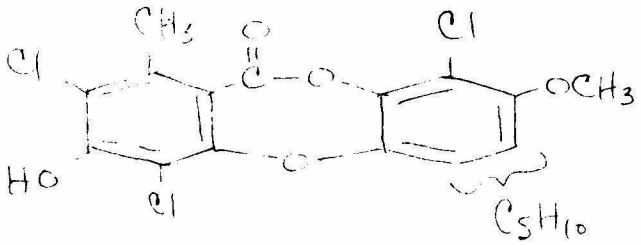
Little, if any, evidence is available for the positioning of the groups (chlorine, methoxyl, and a C_5H_{10} residue) about the B ring.

One might consider that nornidulin's positive reaction in the bleaching powder test implies the presence of a resorcinol system (necessarily associated with the B-ring; nidulin gives no such test). This suggests that the hydroxyl of ring B of nornidulin (and hence the methoxyl group of nidulin) is meta to the lactone bridge, i.e. at positions 4' or 6'. Precedence in known depsidones favors the 4' position for that group.

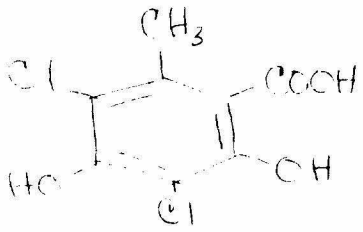
With the above arguments, along with some reasoning on less concrete evidence, Dean, Roberts and Robertson proposed I as nidulin's structure. Structure IV is a more valid conclusion from the published evidence.

In their later paper (7), Dean,Erni and Robertson reported the isolation of a metabolite deficient in chlorine and containing no methoxyl group. The structure of dechloronornidulin was demonstrated as V by the isolation of monochloro A ring fragments. Dechloronornidulin, they said, appeared to be produced when the fungus was grown in a medium deficient in chloride.

The above is a resume of the published literature concerning nidulin and related substances up to 1957. In addition, D. S. Noyce (8) informed us of the successful hydrogenation of nidulin over Adams' catalyst. It was with this information that the study of the structure and biogenesis of nidulin was begun.



I



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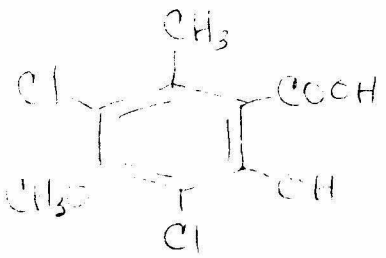

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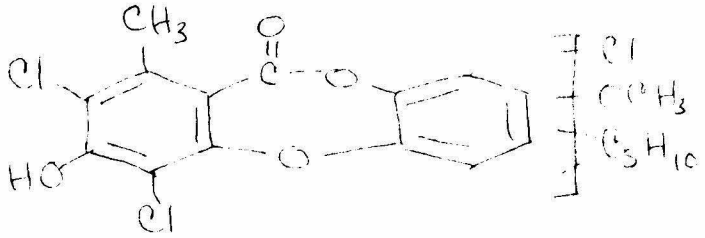

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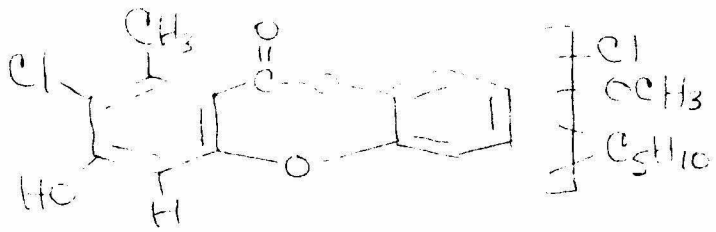


V

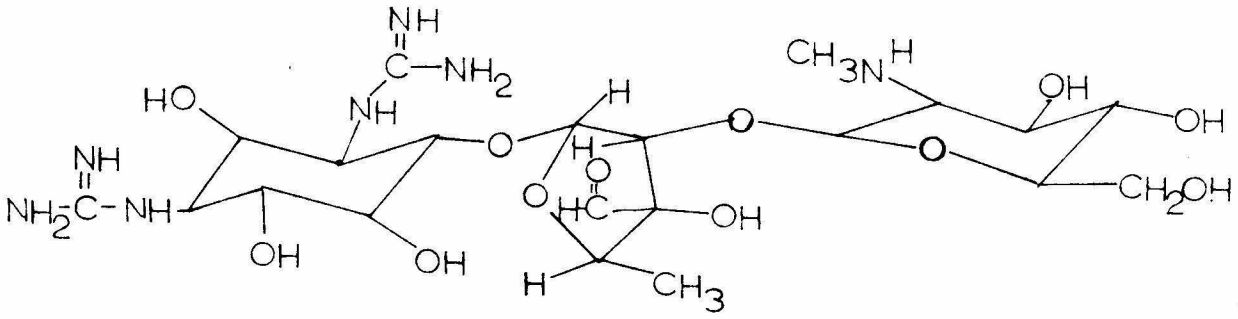
NOTE 1

The three species previously found to have such activity were:

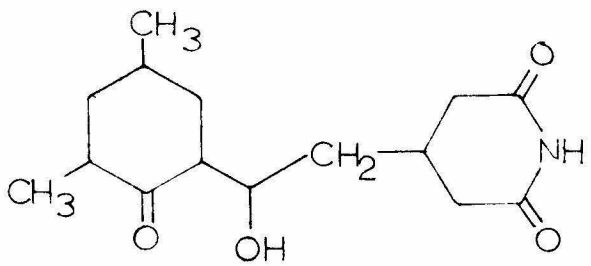
1. Streptomyces (Actinomyces) griseus, presently known as the producer of streptomycin and actidione. Streptomycin (9) was found to be especially active against Mycobacterium tuberculosis, but actidione (10), although possessing a high and useful activity against certain microorganisms, has not been reported to be inhibitory to M. tuberculosis. A third antibiotic (11) of important biological activity (including the inhibition of M. tuberculosis) has been found in a strain of S. griseus which does not produce streptomycin. It has been named grisein, and is thought to be a peptide.

2. An uncharacterized species of fungus, probably a Penicillium, was found to thrive in the presence of, and inhibit the growth of tubercle bacilli (12). While a suspension of the mold was quite effective in the inhibition of M. tuberculosis, a filtrate of the suspension was without effect.

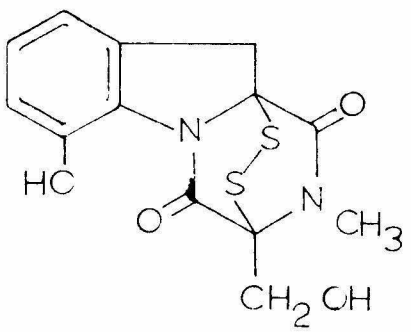
3. Aspergillus fumigatus was also found to produce a substance active against M. tuberculosis (13). Later, it was discovered that A. fumigatus produced an antibiotic similar to (if not identical with) gliotoxin (14). The structure of gliotoxin has only recently been demonstrated (15).



VI. STREPTOMYCIN



VII. ACTIDIONE



VIII. GLIOTOXIN

NOTE II

Kurung's mold was identified as the species Aspergillus ustus by C. Thom, while the later correction to a "non-ascosporic strain of Aspergillus nidulans" was made by K. B. Raper. Thom and Raper are the co-authors of A Manual of the Aspergilli (16), which is the major work relating to the genus; hence their classification must be regarded as authoritative. Aside from the particular species they describe, the names A. ustus and A. nidulans apply to two groups of species within the genus; a group being a number of species having characteristics in common. According to Thom and Raper (17), the A. ustus and A. nidulans groups have five out of six distinguishing characteristics in common, supposedly differing only in that the A. nidulans group possesses ascospores,* while the A. ustus group does not. Yet the classification of non-ascosporic species (and strains) to the A. nidulans group is not without precedent (cf. A. unguis and A. caespitosus (18)). The system of classification is somewhat puzzling to the novice in this respect.

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*(Greek: askos, bladder) -- spores contained in an ascus, a sac-like cell, in addition to the conidia, or spores-on-a-stalk.

2. THE CONTRIBUTION OF N-M-R SPECTROSCOPY TO THE STRUCTURE OF NIDULIN

The N-M-R Spectrum of Nidulin

The experimental results favoring IV as a partial structure for nidulin were presented in the preceding portion of this thesis. Since the character of the alkyl residue on ring B is a major point of interest in the completion of the structural problem, nuclear magnetic resonance (N-M-R) spectroscopy is uniquely endowed as a means of approaching the problem.

The 60-mc N-M-R spectra (HR-60)* of nidulin, O-methyliso-nidulin, and the product of the successful hydrogenation of nidulin, dihydronidulin, are reproduced in figure 1. The nature of the alkyl residue on ring B of nidulin may be determined by examining these spectra.

The appearance of two unsplit methyl peaks in the aromatic methyl (ca. 250 cps) region of the spectrum of each of these compounds can mean only that in nidulin there are two aromatic methyl groups. Previously, the existence of one such methyl group on ring A had been demonstrated (5). Since this is the only methyl group on the A ring,

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*See Experimental. Chemical shifts reported here are related to a benzene external reference sample.

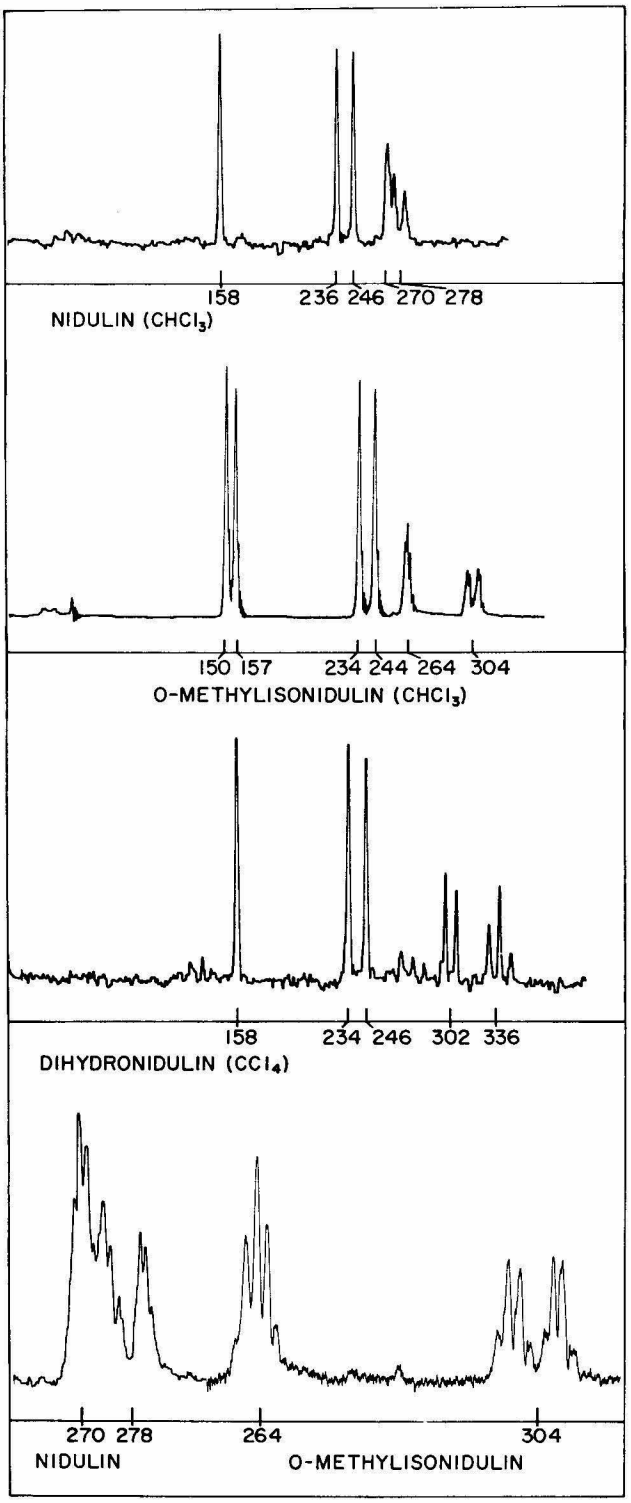

158
236 246 270 278
NIDULIN ($CHCl_3$)
150 157
234 244 264 304
O-METHYLISONIDULIN ($CHCl_3$)
158
234 246 302 336
DIHYDRONIDULIN (CCl_4)
270 278
264
304
NIDULIN
O-METHYLISONIDULIN

FIGURE 1

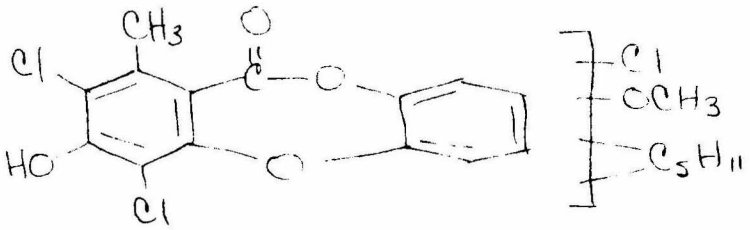
the second aromatic methyl group must be attached to the B ring. Hence, the previously undetermined five-carbon residue consists of a methyl group and a C_4H_7 fragment.

The presence of a double bond in the C_4H_7 fragment, necessitated by the single degree of unsaturation and the unlikelihood of the existence of a cyclic system, is substantiated by a quartet-of-quartets signal, proportional to a single proton in area, in the vinyl hydrogen region of the spectrum. Although the fine structure of this signal can be observed only on slow, high-gain sweeps, its position can be seen in figure 1 below the chloroform spinning side-band in the O-methyl*iso*-nidulin spectrum. In agreement with the presence of an olefin are the two three-proton signals in the vinyl methyl regions of the spectra of nidulin (270 and 278 cps) and O-methyl*iso*nidulin (264 and 304 cps). From the information given thus far, it is deduced that the C_4H_7 residue is a 2-but-2-enyl system, and that the sole difference between nidulin and O-methyl*iso*nidulin, aside from the methylated hydroxyl group of the A ring, is that of a *cis-trans* isomerization about the double bond in the butenyl system. Further support for the presence of a secondary butenyl side chain on nidulin's ring B is given by the N-M-R spectrum of dihydronidulin which is clearly diagnostic of the presence of a secondary butyl side chain.

Recent measurements (19, 20) on 2-butenyl systems have disclosed the generalization that the spin-spin coupling constants linking

vinyl methyl groups across a double bond are greater in magnitude when the methyls are trans- to one another than when they are cis-disposed. Such spin-spin splittings were observed in the N-M-R spectra of nidulin and O-methylisonidulin, and were measured as they appear in the vinyl methyl doublet-of-quartets of the nidulin (278 cps) and O-methylisonidulin (304 cps) spectra by averaging many sweeps through the signal. The values for the splitting constant thus obtained were 1.11 ± 0.04 cps for nidulin and 1.52 ± 0.05 cps for O-methylisonidulin. The major (doublet) splitting constants for the two compounds were nearly identical: nidulin, 6.77 ± 0.08 cps; O-methylisonidulin, 6.76 ± 0.08 cps. These results may be viewed qualitatively in a slow sweep of the vinyl region of each spectrum in figure 1. For reasons which will be developed in the next section of this thesis, the butenyl side chain of the nidulin molecule is assigned the cis-configuration, and the structure of nidulin is accordingly best represented by partial structure VI.

That the ultraviolet spectrum of nidulin remains unchanged upon the saturation of a double bond (supposedly adjacent to a benzene system) is easily justified on the grounds of the overwhelming steric requirements of the substituents ortho to the butenyl group in nidulin, making it impossible for the olefin to become coplanar and therefore in conjugation with the aromatic B ring. Such a suggestion of steric inhibition to resonance is not without precedent (21).



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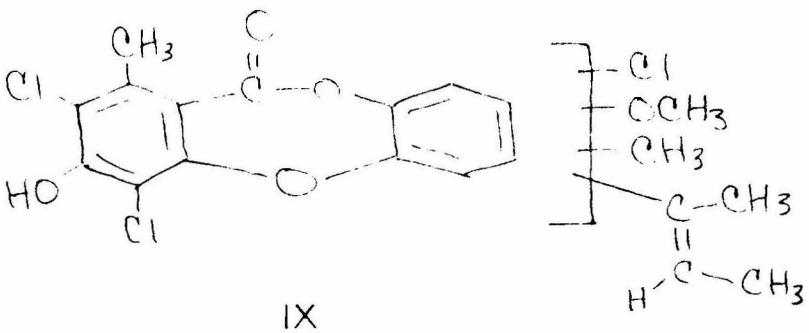


IX

3. A STUDY OF THE LONG-RANGE SPIN-SPIN COUPLINGS IN 2-BUTENES

The isomerization of O-methylnidulin to O-methyl*iso*nidulin was interpreted by means of N-M-R spectroscopy as a *cis-trans* isomerization about the 2-but-2-enyl double bond. The remaining problem was the assignment of *cis* and *trans* configurations to the side chains of the compounds.

The complexity of the rest of the molecule eliminated the possibility of obtaining a reliable configurational assignment based on the classical techniques of physical properties and reactivity (22). A new technique for distinguishing between certain *cis* and *trans* isomers, however, was developed.

The vinyl methyl doublets in the N-M-R spectrum of nidulin and its methylated isomer were split secondarily into quartets. The secondary splitting can be explained only by an interaction through the double bond with the protons of the α-methyl group. Since there was a distinguishable difference between the splittings in nidulin (1.11 ± .04 cps) and O-methyl*iso*nidulin (1.52 ± .05 cps), it was advantageous to ascertain the generality of this phenomenon in variously substituted 2-butenes, and perhaps to be able to assign a *cis* or a *trans* structure to the 2-butenyl group in nidulin.

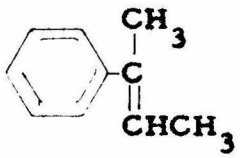
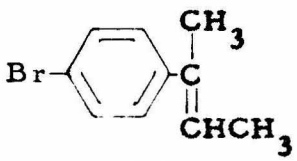
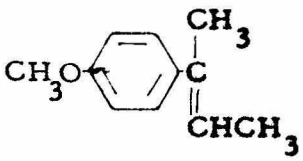
The results of the N-M-R spectral measurements are presented in tables 1, 2, and 3. In table 2 are the results of primary interest in this work. J_{AB}, the magnitude of the spin-spin interaction between the α- and the β-methyl groups of the system, is astonishingly invariant for each isomer among the compounds studied. The compounds themselves represent a wide variation of electronic and substituent effects on the 2-butene system. If an electrostatic effect is to be observed, it should have revealed itself among the measurements made on these compounds. It can therefore be concluded from these results that the five-bond splitting constant for the 2-butene system is a reliable indication of the molecular geometry.

The work of Frazer (19) with the splittings in tiglic and angelic acids (cis and trans 2-carboxy-2-butene, respectively) bear out the assertion stated above. The splitting constants in these compounds are reported as being:

		J_{AB} (cps)
2-carboxy-2-butene	trans	1.46 ± .06
	cis	1.17 ± .06
2-carbomethoxy-2-butene	trans	1.51 ± .05
	cis	1.20 ± .04

J_{AX}, the four-bond splitting constant, on the other hand, appears to be sensitive neither to geometry nor to substitution. This

Table 1. CHEMICAL SHIFTS

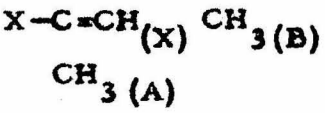
Compound		Vinyl proton	Vinyl methyl protons	
			α	β
$Br-C(CH_3)=CHCH_3$ (HR-60)*	trans (neat)	48 (348)	262 (134)	296 (100)
	cis	46 (350)	266 (130)	300 (96)
 (A-60)*	trans (10%, CCl_4)	331	121	95
	cis	347	121	108
 (A-60)*	trans (10%, CCl_4)	334	121	96
	cis	349	121	109
 (A-60)*	trans (10%, CCl_4)	329	121	97
	cis	343	120	108

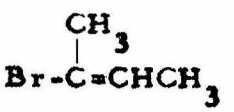
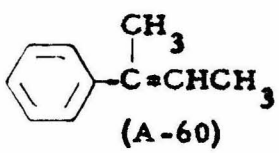
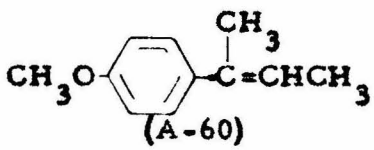
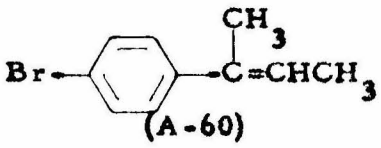
HR-60: Figures represent cps above the signal of an external benzene reference sample. Figures in parentheses represent corresponding numbers on the tetramethylsilane scale, assuming that TMS (5% CCl_4) - benzene (neat) = 396 cps.*

A-60: Figures are in cps below tetramethylsilane (5% in CCl_4) as an external standard.

*See Experimental.

Table 2. SPIN-SPIN SPLITTING CONSTANTS (cps)

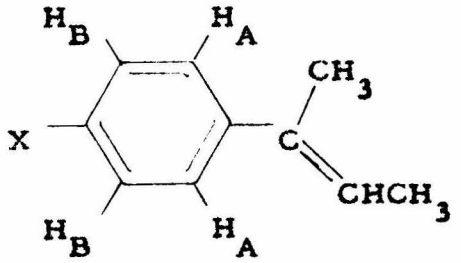


Compound		J_{AB}	J_{AX}	J_{BX}
 (HR-60)	trans	1.59 ± .03	1.36 ± .06	6.39 ± .04
	cis	1.12 ± .04	1.42 ± .05	7.06 ± .06
 (A-60)	trans	1.57 ± .01	1.51 ± .01	6.97 ± .02
	cis	1.11 ± .01	1.42 ± .01	6.93 ± .02
 (A-60)	trans	1.56 ± .01	1.48 ± .01	7.00 ± .01
	cis	1.08 ± .01	1.39 ± .01	6.94 ± .01
 (A-60)	trans	1.56 ± .01	1.51 ± .01	7.03 ± .01
	cis	1.13 ± .01	1.38 ± .01	6.92 ± .01

The measurements were made on neat samples. The values reported are in cps.

Table 3. SPIN-SPIN SPLITTING CONSTANTS

Para substituent	-Br		-OCH_3	
Geometry	trans	cis	trans	cis
Solvent	neat	neat	50% CCl_4	neat
$\delta\nu_o$, Chemical shift	23.4	12.9	16.4	28.5
$J_{AA} = J_{BB}$	2.3	2.5	2.7	2.6
J_{AB}	8.6	9.0	9.1	9.0
Solvent	all 10% in CCl_4			
Chemical shift of the center of the signal (cps below TMS)	435	437	416	418
Positions of A and B absorptions, in cps below TMS	447 423	443 431	424 408	432 404



X

Four types of splitting are possible: J_{AA}, J_{BB}, J_{AB} (ortho), and J'_{AB} (para). J'_{AB} (para) is assumed to be zero, and J_{AA} and J_{BB} are presumed equal.

sort of invariance is also observed in the three-bond splitting constant, J_{BX}.

The chemical shift relationships between the isomers give additional support to the assignment of the geometry about the double bond.

In the bromobutene case, the cis isomer's vinyl hydrogen is unshielded, as expected, due to its proximity to the bromine atom. In the dimethylstyrenes, the cis isomer's vinyl hydrogen is also unshielded, but in this case the unshielding is due to its nearness to the aromatic ring's π-electron system.

The α-methyl group occurs in all instances at a lower field (less shielded) position than the β-methyl group. In the dimethylstyrenes, the chemical shift of the α-methyl group (observed in a 10% solution in carbon tetrachloride) is independent of the substitution of the aromatic nucleus. The chemical shift position of the β-methyl groups in the dimethylstyrenes appears to be anomalous, for the trans isomer's β-methyl group falls at a higher field (more shielded) than that of the cis isomer, in spite of the nearness of the trans isomer's β-methyl group to the aromatic ring. If it is considered that the α-methyl group is almost directly above the plane of the ring, however, the ring current effect may be invoked to explain the shielding observed. It will be noted that in the bromobutenes, where no benzene ring is

involved, the β-methyl group of the trans isomer is shielded with respect to the cis isomer. This may be explained easily on the basis of its distance from the bromine atom.

The interpretation of the ring proton absorptions of the substituted α,β-dimethylstyrenes presents an interesting sideline to these experiments. The various splitting parameters (table 3) are similar to those observed in related systems (57). It is the chemical shift of each type of ring proton which is enlightening.

The methoxyl group has a major effect on the chemical shift of the B protons. By its electron donating resonance interaction with the ring, it raises the electron density at the carbon atoms to which they are bonded, and has a shielding effect on them. The bromine atom also affects primarily the B protons. Being an electronegative atom, it withdraws electrons from the ring, unshielding those atoms which are closer to it.

The chemical shift of the A protons, in each case, is affected primarily by the geometry of the 2-but-2-enyl group. When the geometry is cis, the electronegative double bond exerts an unshielding effect on these nuclei. When the group is trans, however, the β-methyl group intervenes, and the A protons are effectively insulated from the electronegative effects of the double bond (see figure 2).

If the higher field absorption of the methoxyl systems (trans,

408 cps; cis, 404 cps) is assigned to the B protons, the A protons' absorption positions are in accordance with the predicted shielding character of the cis olefin (trans, 424 cps; cis, 432 cps). Likewise, if the lower field (unshielded) absorption of the bromo system (trans, 447 cps; cis, 443 cps) is assigned to the B protons, the A protons' absorption positions are also in accord with the predictions (trans, 423 cps; cis, 431 cps).

In agreement with the predicted unshielding effects of a cis-2-but-2-enyl group with respect to the trans isomer, the ring hydrogens of the unsubstituted α,β-dimethylstyrenes are at lower fields in the cis isomer (434 cps) than in the trans isomer (430 cps). These chemical shifts, of course, are measured at the central positions of the finely split multiplet which represents all of the ring protons, and do not accurately reflect the true differences in environment of the two types of ortho protons.

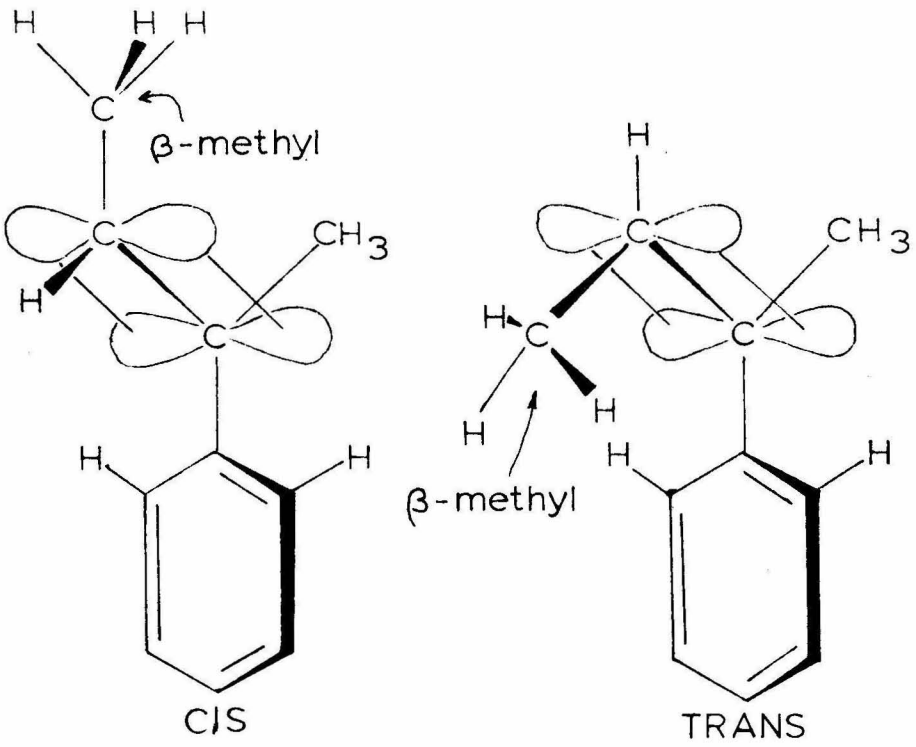


FIGURE 2

The A protons are those nearest the butene side chain. In the cis isomer, with the β-methyl group directed away from the ring, the A protons feel the full effect of the electronegative, unshielding π-electron system of the double bond. In the trans isomer, the nearness of the β-methyl group to the A protons mitigates the effect to some extent. Thus, all other things being equal, the A protons of the cis isomer should absorb at a lower field than those of the trans isomer.

4. THE INFRARED SPECTRUM OF NIDULIN AND SOME OF ITS PHENOLIC DERIVATIVES IN THE THREE MICRON REGION

With the disclosure of the nature of the alkyl groups on nidulin's B ring, the only remaining particular that must be settled for an establishment of nidulin's structure is the disposition of groups about the B ring.

It has been known for some time that phenolic hydroxyl groups are capable of interaction with neighboring electronegative centers (23). The formation of a hydrogen bond between the hydrogen atom of a hydroxyl group and a neighboring electronegative center effects a weakening in the oxygen-hydrogen bond. This, in turn, is observed in a lowering of the bond's force-constant as reflected in its stretching frequency in the 3 μ region of the infrared spectrum. More recently, the quantitative aspects of such bathochromic shifts have been examined (24, 25, 26). Consequently, it is reasonable to expect to gain information about the structure of a complex phenol by examining its infrared spectrum.

Nidulin has a single, sharp hydroxyl band at 3516 cm^{-1}. It must be assigned to the A ring phenolic hydroxyl group which is known to be flanked by two ortho-chlorine substituents. That its frequency is 15-20 cm^{-1} lower than that expected (24) for O-chlorophenols, is undoubtedly the result of the acid strengthening lactone carbonyl group in the para position (25).

The spectrum of decarbonidulin has absorption maxima of approximately equal intensity at 3532 cm^{-1} and 3556 cm^{-1}. The former is due to the hydroxyl in ring A, raised to the normal frequency (24) because of the loss of the lactone carbonyl group. The latter absorption must be assigned to the new hydroxyl group in ring B which was freed by the scission of the lactone bridge. Its frequency, 3556 cm^{-1}, is that expected for an o-phenoxy phenol (26). The spectrum of methyl O-methylnidulinate has a single, symmetrical absorption at 3550 cm^{-1}. Because this compound has only the 2' hydroxyl, the assignment of bands in decarbonidulin is given added support. Since a chlorine atom is at least as effective as an oxygen atom in the role of a hydrogen bond proton acceptor,* the absence of any appreciable OH...Cl absorption (which should appear at ca. 3532 cm^{-1} in the spectrum of methyl O-methylnidulinate) excludes the possibility that a chlorine atom is attached at the 3' position in nidulin.

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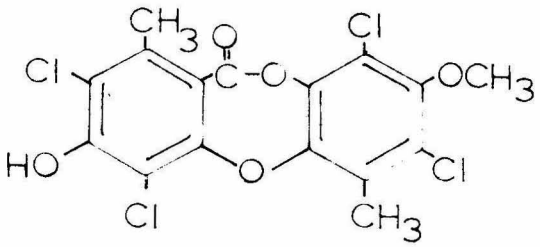
*According to Badger's rule, the energy of a hydrogen bond increases with the bathochromic shift of the frequency of the OH stretching vibrations (23, 27).

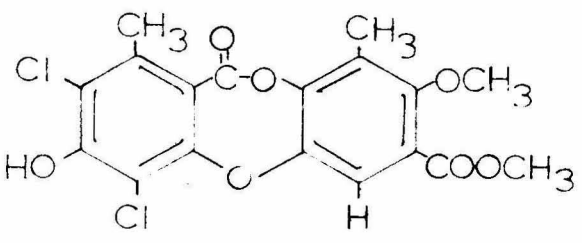
5. A STUDY OF THE DECHLORINATION OF NIDULIN

Although the ability of hydrogen halide acids to cleave aromatic carbon-chlorine bonds has been casually mentioned (28), there are no examples in the literature of this event which could be attributed solely to the action of these reagents.

It was, at one point, desirable to prepare a derivative of nidulin possessing a 4'-hydroxyl group. Such derivatives of diploicin (XI) (6) and gangaleoidir (XII)(29), which are depsidones originating in lichens, have been prepared by Nolan and his co-workers by refluxing the depsidone in glacial acetic acid in the presence of hydrobromic acid and red phosphorous for eight or nine hours. Under conditions strong enough to cleave the ether, hydrolysis of the lactone and decarboxylation of the resulting acid also occur, as would be expected. Neither diploicin nor gangaleoidin loses any chlorine under these conditions.



XI. DIPLOICIN



XII. GANGALEOIDIN

Dihydronidulin was decided upon as the starting point for the preparation of the demethylated derivative because it was feared that the olefinic group of nidulin might be capable of reacting under the vigorous ether cleavage conditions. When the reaction was run for the first time, an apparently pure solid which melted over a narrow range was recovered and analyzed. The chlorine analysis gave 24.6% chlorine, which appeared convincing enough for the presence of three chlorine atoms in the molecule that a structural argument was based on the presence of a chlorine atom on the B ring of this compound.

In repeating the experiment, however, the chlorine analysis for an otherwise similar product revealed the presence of only 19.0% chlorine. The first chlorine analysis was shown to be greatly in error, and this fact in turn completely invalidated the previous structural argument (56).

Certain information concerning the reactivity of the various sites in dihydronidulin can be inferred from the experiments described here. First, 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, being only one-half complete at a reaction time of two hours.

Assigning a structure to demethyldecarbodihydronidulin is

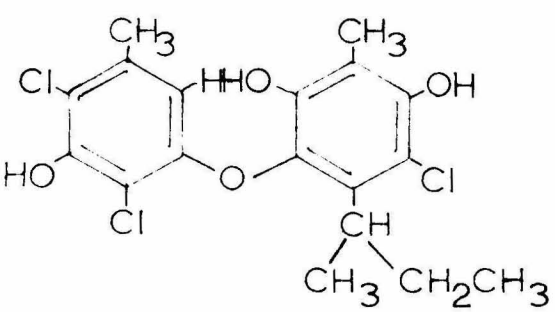
trivial. Its analysis implies an empirical formula of $C_{18}H_{19}O_4Cl_3$. The lack of a methoxyl signal in its N-M-R spectrum shows that it is unmethylated. Hence, its structure can be none other than XIII.

The assignment of a structure to dechlorodemethyldecarbodihydronidulin, the analysis of which implies an empirical formula of $C_{18}H_{20}O_4Cl_2$, is more difficult. The problem is which of the three chlorine atoms is removed. If the missing chlorine was originally one of the A ring chlorine atoms, it would then be expected that after the first chlorine of ring A was removed, the second chlorine would be even easier to remove, because the presence of electronegative substituents on a benzene ring lessens the system's vulnerability to electrophilic attack. Hence, a product containing only one chlorine atom on the A ring would not be produced by any type of electrophilic substitution reaction. Since the dechloro product contains two chlorines, they must be the two chlorines of the A ring, and the chlorine atom that is removed in the dechlorination reaction must be the B ring chlorine.

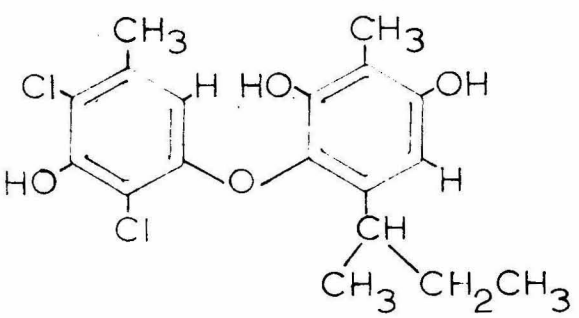
An independent argument for the identity of the missing chlorine atom is as follows. Demethyldecarbodihydronidulin, judging from its infrared spectrum, contains three hydroxyl groups; two of which are hydrogen bonded to chlorines (3534 cm^{-1}), and one of which is hydrogen bonded to an oxygen (3570 cm^{-1}). Dechlorodemethyldecarbodihydronidulin also has three hydroxyl groups; one hydrogen bonded to a chlorine (3533 cm^{-1}), one to an oxygen (3566 cm^{-1}) and one which is

free (3616 cm^{-1}). It can be safely assumed that no gross structural change has occurred and that the positions of the three hydroxyl groups are the same in each case. The net change in the formation of the dechloro compound is that one hydroxyl group is no longer adjacent to a hydrogen bonding proton acceptor. While the loss of one chlorine from the A ring would not produce the observed change in the infrared spectrum, the removal of the sole chlorine next to a hydroxyl group in the B ring would.

The structure of dechlorodemethyldecarbodihydronidulin must be XIV.



XIII



XIV

The dechlorination of nidulin is regarded as an electrophilic attack of hydrogen ion at the ring carbon to which the chlorine atom is bonded, followed by elimination of the positive chlorine species. The positive chlorine species does not remain in the oxidized form, but is soon reduced by the phosphorous. The process might be compared to its antithesis: aromatic chlorination. The same features of nidulin

that would favor its being chlorinated (the many hydroxyl groups it contains) aid in its dechlorination. Just as a hydroxyl activates the positions ortho and para to it for chlorination, a chlorine atom on a ring having ortho or para hydroxyl substituents is vulnerable to electrophilic substitution. This is due, of course, to the stabilisation of the transition state common to both reactions:

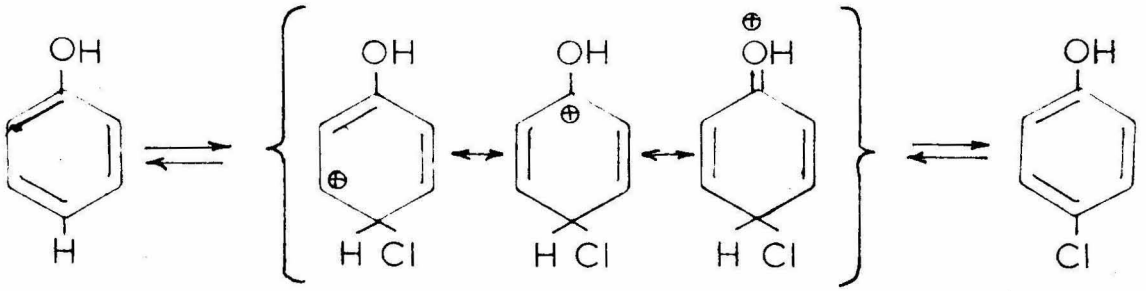


Figure 3

The presence of a second chlorine atom on the ring apparently deactivates the system effectively enough that no dechlorination takes place at all. Clearly, the attenuation of reactivity is due to the inductive effect of the electronegative substituent (chlorine) withdrawing electrons from the ring. In substantiation of this generalisation, the inertness of the A rings of diploicin, gangaleoidin and nidulin, and the B ring of diploicin is cited.

6. A STUDY OF THE FORMATION OF A XANTHENE FROM NIDULIN

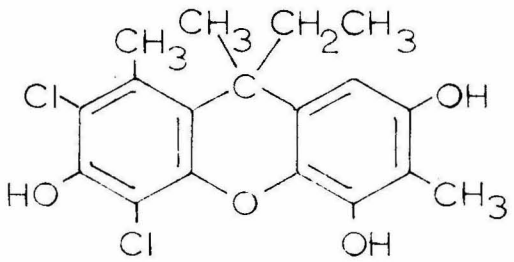
Dean, Deorha, Erni, Hughes and Roberts reported (28) the isolation of ethylmethylmalonic acid from the amorphous substance obtained by heating nidulin or O-methylisonidulin with hydriodic acid in glacial acetic acid solution. Ethylmethylmalonic acid contains one more carbon atom than can be accounted for solely in terms of the B ring of nidulin. To account for the isolation of ethylmethylmalonic acid, they proposed that in addition to the expected series of reactions (hydrolysis, decarboxylation and demethylation), an acid catalyzed internal Friedel-Crafts reaction is effected by the olefinic side chain of the B ring at a vacant site on the A ring. This event would give rise to a xanthene, which, although it was neither isolated nor characterized, could produce ethylmethylmalonic acid upon oxidation.

The English authors' ethylmethylmalonic acid isolation inspired the investigation of the action of hydrobromic acid and phosphorous on nidulin, with the hope of isolating compounds of the xanthene family.

From the reaction of nidulin for various lengths of time with hydrobromic acid in the presence of red phosphorous in acetic acid solution, the following observations concerning the relative reactivities of the various sites in the molecule were made. Hydrolysis of the lactone and decarboxylation of the resulting acid proceeded rapidly,

being essentially complete within twenty minutes. Demethylation is also quite rapid, being about two-thirds complete in twenty minutes and totally finished within one hour. Both the loss of chlorine and the xanthene formation proceeded at about the same rate, and were about one-half and one-third complete, respectively, after three hours' boiling.

The xanthene structure XV is assigned to the ultimate reaction product, mp 171.5-173°, primarily on the basis of its N-M-R spectrum and its microanalysis, which suggests an empirical formula of $C_{18}H_{18}O_4Cl_2$. Outstanding features of its N-M-R spectrum (A-60)* include the methyl protons of the 9-ethyl group, which fall as a 7 cps triplet at 35 cps; the 9-methyl group, which falls at 112 cps as an unsplit peak; and the 1- and 6- aryl methyl groups which fall at 137 and 160 cps. No attempt will be made to assign the latter signals specifically to the

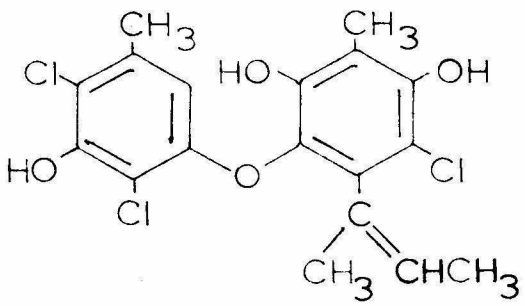


XV

*See Experimental.

methyl groups involved here. It is interesting to note that in the more rigid systems (i.e., xanthene and depsidone), the aryl methyl signals occur father apart (ca. 23 cps in xanthenes, ca. 12 cps in depsidone) than they do in the dephenyl ether system (ca. 3 cps). Although it probably has little to do with the rigidity of the systems involved, the relative chemical shifts of the aryl methyl protons do provide an auxiliary method of monitoring the amounts of the different systems in a mixture. The infrared spectrum of the product also agrees with the structural assignment. The three peaks may be assigned to each of the three different hydroxyl groups: to the 3-hydroxyl, the band at 3533 cm^{-1} (OH...Cl); to the 5-hydroxyl, the band at 3567 cm^{-1} (OH...O); and to the 7-hydroxyl, the band at 3619 cm^{-1} (free OH).

The diphenyl ether structure XVI is assigned to the intermediate

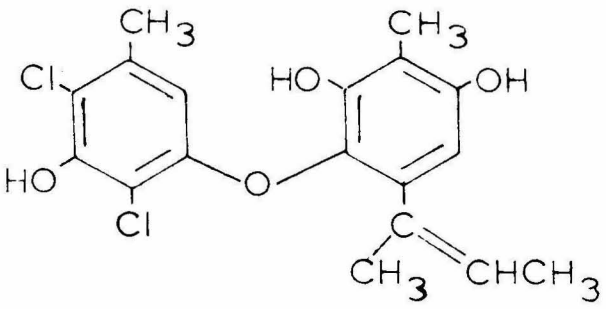


XVI

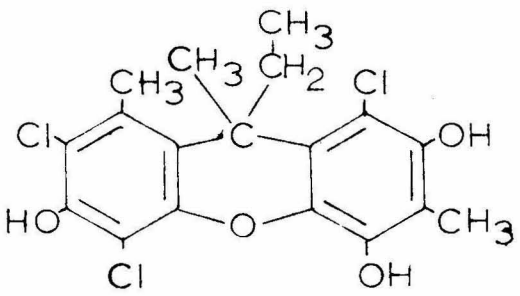
reaction product, mp 141.5-143°, on the basis of its analysis, which

suggests the empirical formula of $C_{18}H_{17}O_4Cl_3$; and its N-M-R spectrum, which indicates the presence of two aryl methyl groups at positions 3 and 3' by sharp peaks at 140 and 143 cps. The complex signal of the vinyl methyl protons indicated that the intermediate product is actually a 2:1 mixture of trans : cis isomers in the olefinic side chain.

Other products, such as the dechlorinated diphenylether XVII or the chlorinated xanthene XVIII, might be expected to form under these reaction conditions. Since chromatography separates so easily the di- and tri-chloro species, the presence of either XVII or XVIII could



XVII



XVIII

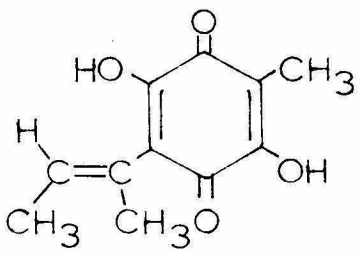
easily be detected. The fact that neither were found in the reaction mixture is significant. That no XVIII was observed indicates that the olefinic side chain is so modified by the adjacent chlorine substituent that no xanthene formation is possible, an event which might have been anticipated. That no XVII was observed means it is converted as rapidly

as it is formed into the observed xanthene (XV). The implication here is that actual rate of the xanthene formation reaction is greater than the actual xanthene formation rate. The rate of xanthene formation is regulated by the progress of the dechlorination reaction, a much slower process than the internal Friedel-Crafts alkylation. The discrepancy between the extent of dechlorination as observed in the infrared and the extent of xanthene formation as observed in the N-M-R spectrum might be cited as an indication of the presence of XVII. The reliability of these measurements, however, is poor, as they were intended only to indicate in a general way the composition of the reaction mixture.

7. THE STRUCTURE OF THE DIHYDROXYBENZOQUINONE ISOLATED FROM NIDULIN'S B RING

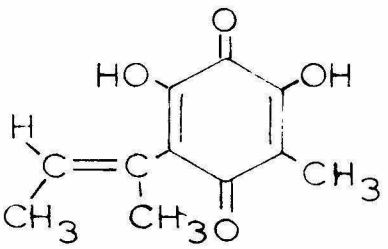
Dean, Deorha, Erni, Hughes and Roberts have recently reported (28) the isolation of a substance, $C_{11}H_{12}O_4$, obtained from nidulin by oxidizing the product of the action of hydriodic acid on nidulin in an alkaline methanolic solution with air. They believe this material to be a B ring residue because of its empirical formula and its physical resemblance to known dihydroxybenzoquinones.

The English authors assigned structure XIX to the compound because its ultraviolet spectrum in "neutral ethanol" bore a close resemblance to that of 3-methyl-2,5-dihydroxybenzoquinone.

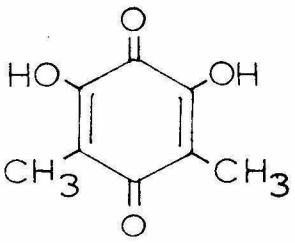


XIX

This structural assignment is premature. The data presented do not eliminate the possibility of structure XX. The sole example of a 2,6-dihydroxybenzoquinone (XXI) in the literature is apparently quite similar to 2,5-dihydroxybenzoquinone in its physical properties.



XX



XXI

Furthermore, it dissolves in alkali to give a bright blue solution, a reported property of the nidulin degradation product, but not a general characteristic of 2,5-dihydroxybenzoquinones. Unfortunately, no ultraviolet spectra are available in the literature for the 2,6-system. It would be expected, however, that structures XIX and XX would give rise to similar ultraviolet absorption spectra.

Discussion

As classes of compounds, 2,5- and 2,6-dihydroxybenzoquinones are distinguishable on the basis of their second acid ionization constants. The 2,5-dihydroxybenzoquinones, in going from the mono- to the dianion form, loose a proton from a system possessing resonance stabilization of the vinylogous carboxylate type. This is the same type of resonance stabilization that is invoked upon removal of the first proton from either the 2,5- or the 2,6- systems. Hence, the ionization constants of a 2,5-dihydroxybenzoquinone would be expected to be rather

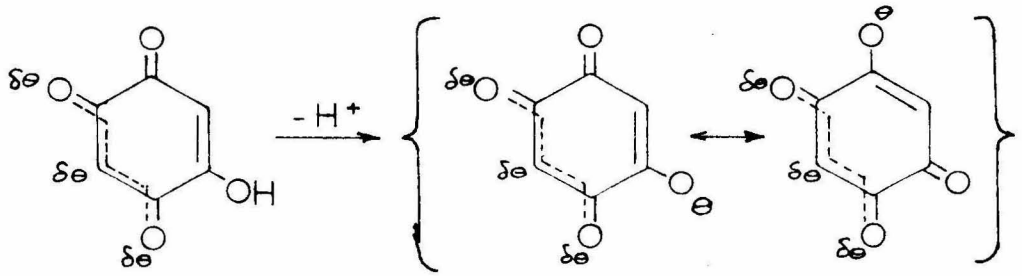


Figure 4

close together, and approximately the same as those of a dicarboxylic acid such as phthalic acid. The second ionization constant of a 2,6-dihydroxybenzoquinone, however, is expected to be comparable to that of a phenol. On loosing its second proton, the 2,6- system does not have available to it the vinylogous carboxylate resonance. The only stabilization it can gain is an enolic resonance.

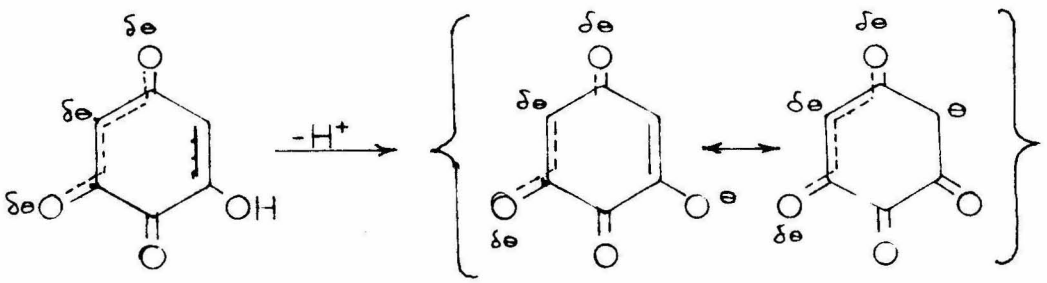
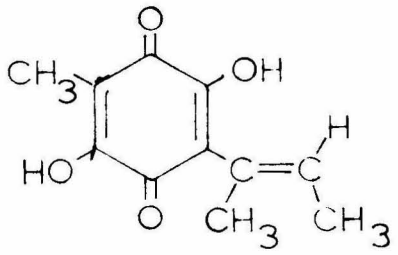


Figure 5

In order that the above conclusions might be verified, the second ionization constants of 2,5-dihydroxybenzoquinone and 3,5-dimethyl-

2,6-dihydroxybenzoquinone were determined by the procedure described in the experimental part of this section. For the 2,5- system, a value of 5.2 was obtained as the pK_a (2). This compared favorably with the value of 5.18 Schwarzenbach and Suter (30) obtained from redox potential measurements, and was expected for the pK_a (2) from the above analysis. For the 2,6- system, a value of 8.8 was observed for pK_a (2) which was also in line with the prediction.

The compound $C_{11}H_{12}O_4$, obtained by degrading nidulin, was observed to have a pK_a (2) of 4.8. Hence, the question of its structure is settled, for this measurement clearly assigns structure XIX, a 2,5- system, to the compound.

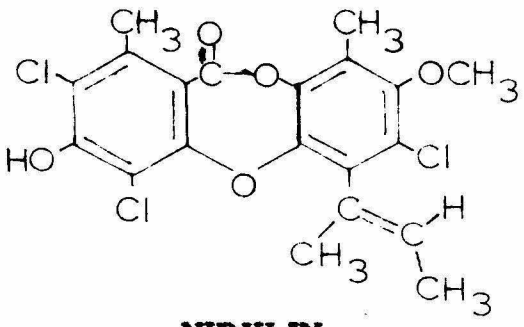


XIX

8. THE STRUCTURE OF NIDULIN

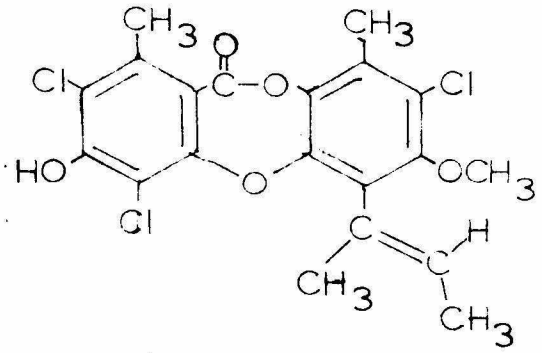
The previous sections of this thesis provide a sound basis for accepting structure XXII for nidulin which was recently proposed by Dean, Deorha, Erni, Hughes and Roberts (28). The study of splitting constants in 2-butene systems provides a solid basis for assigning a cis configuration to the 2-but-2-enyl side chain of nidulin. The compound $C_{11}H_{12}O_4$, obtained by the English workers by degrading nidulin, is shown to be definitely a 2,5-dihydroxybenzoquinone, thus establishing the relative orientation of the B ring alkyl groups. The isolation and characterization of the xanthene resulting from nidulin has provided a sound explanation for the isolation of ethylmethylmalonic acid from nidulin and has fixed the position of the 2-but-2-enyl side chain as being adjacent to the diphenyl ether oxygen.

Although structures XXII and XXIII cannot be distinguished on the basis of the foregoing evidence, structure I is favored because of the positive bleaching powder test (for resorcinol nuclei) that nornidulin (XXV) has been observed to give (5). A second argument for preferring XXII is based on precedent. Of all of the known depsidones, only one, variolaric acid (XXIV), has a group other than hydroxyl or methoxyl at position 4'. It should be noted that even in variolaric acid, the oxygen function of the B ring is in the 6' position and meta to the lactone bridge.

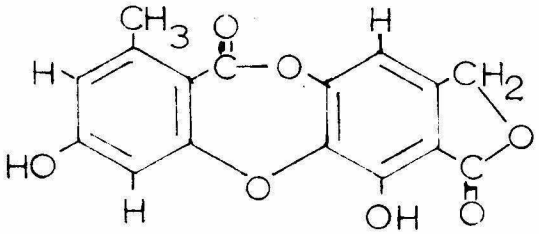


NIDULIN

XXII

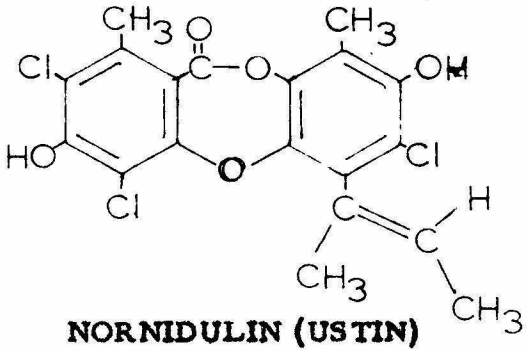


XXIII



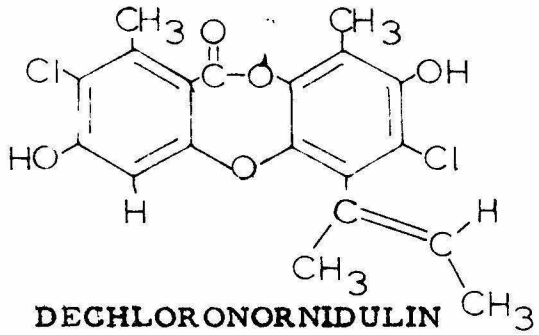
VARIOLARIC ACID

XXIV



NORNIDULIN (USTIN)

XXV



DECHLORONORNIDULIN

XXVI

The structures of the other depsidone metabolites of A. nidulans are now clear. Nornidulin (5) (ustin (3), or compound I of Hogeboom and Craig (2)) must have structure XXV and dechloronornidulin (7) (compound II of Hogeboom and Craig (2) must have structure XXVI. It is interesting to note that while earlier workers (2, 3) isolated large quantities of nornidulin and dechloronornidulin but no nidulin, more recent workers (5, 7) have observed increasing nidulin production and a vanishing output of nornidulin and dechloronornidulin. The isolation of the mixture nornidulin and dechloronornidulin reported in the experimental section of this thesis establishes that the production of these two metabolites is less than one percent of the production of nidulin. Perhaps this reflects a slow mutation of the fungal strain.

9. A PRELIMINARY STUDY OF THE BIOGENESIS OF NIDULIN:

A. The Acetate Experiment

Although in 1907 (31) Collie first introduced the idea that the biosynthesis of many phenolic plant products may occur by the head-to-tail linkage of acetic acid units, this concept remained dormant until within the past decade it has been vigorously advanced by Birch. After setting forth a rather comprehensive treatise on the scope of the acetate theory (32), Birch immediately demonstrated its value by selecting probable structures for many phenolic natural products (33). In most instances, he supported the predictions by synthesis.

In 1955, the acetate theory gained considerable stature when Birch (34) showed that Penicillium griseofulvum, when fed sodium acetate-^{14}C, produced 6-methylsalicylic acid which contained the radiocarbon label only in the positions which are predicted by the acetate theory. Birch has subsequently traced the biogenesis of other metabolites of P. griseofulvum (35), and has established the coexistence of poly-β-keto acid and isoprenoid pathways within the same organism.

In 1958, Birch (36) published the results of a study of the incorporation of labelled acetate into penicillic acid by the organism Penicillium cyclopium. From the observed distribution of the label, he suggested that the biosynthesis of penicillic acid proceeded through orsellinic acid via an oxidative ring opening:

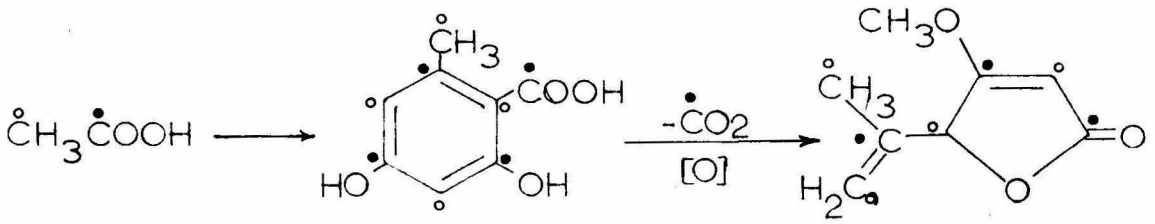


Figure 6

Confirming the prediction of the intermediacy of orsellinic acid, Bently and Keil (37) successfully isolated orsellinic acid from cultures of P. cyclopium by paper chromatography. They also showed by labelling experiments that the methoxyl carbon atom of penicillic acid can be derived from formate or methionine S-methyl. Furthermore, they demonstrated that while penicillic acid is formed from four acetate units, their experiments with 2-C^{14} malonate show that only three units of malonate are incorporated, position 7 being found to be inactive. Recent thinking on the biogenesis of fatty acids (58) has favored the idea that the chain extension actually takes place with malonate, not acetate, as the nucleophilic reagent which attacks the coenzyme A thiol ester end of the chain. The terminus of the chain, however, is acetyl coenzyme A thiol ester. Although the incorporation of acetate into all parts of such chains implies a ready conversion of acetate to malonate,* the reverse route apparently does not occur to any great extent. The

*In certain systems, an enzyme has been shown to be present (by isolation) which performs the carboxylation function in the presence of adenosine triphosphate and manganous ion (58a).

biogenesis of orsellinic acid is quite similar, then, to that of fatty acids. They differ in principle only in that the fatty acid biosynthesis involves a reduction of the many keto groups along the chain perhaps by the reversal of the β-oxidation cycle (58b).

The end group hypothesis was fortified by the recent work of Bu'lock and Smalley (38). Orsellinic acid was obtained from the organism P. urticae which was fed either sodium acetate-1-^{14}C or diethyl malonate-2-^{14}C. Kuhn-Roth C-methyl oxidations were performed on the orsellinic acid from each of these sources. That which arose from labelled acetate produced acetic acid of a specific activity of one-fourth of that of the original orsellinic acid. That which arose from labelled malonate gave acetic acid which was inactive.

The end group hypothesis has led to the proposal for the biogenesis of orsellinic and 6-methylsalicylic acids which is presented in figure 7.

Sodium acetate-1-C^{14} was incorporated into the nidulin produced by A. nidulans to the extent of 1.6%. This value, although slightly lower than is customarily observed in similar systems, strongly implies a direct biosynthetic pathway between the substrate and the metabolite.

In order to check the incorporation of activity into the A ring, the degradations outlined diagrammatically in figure 8 were performed. Decarbonidulin was found to have 84% of the activity of nidulin, indicating that 16% of nidulin's activity is located in its lactone carbon atom. The

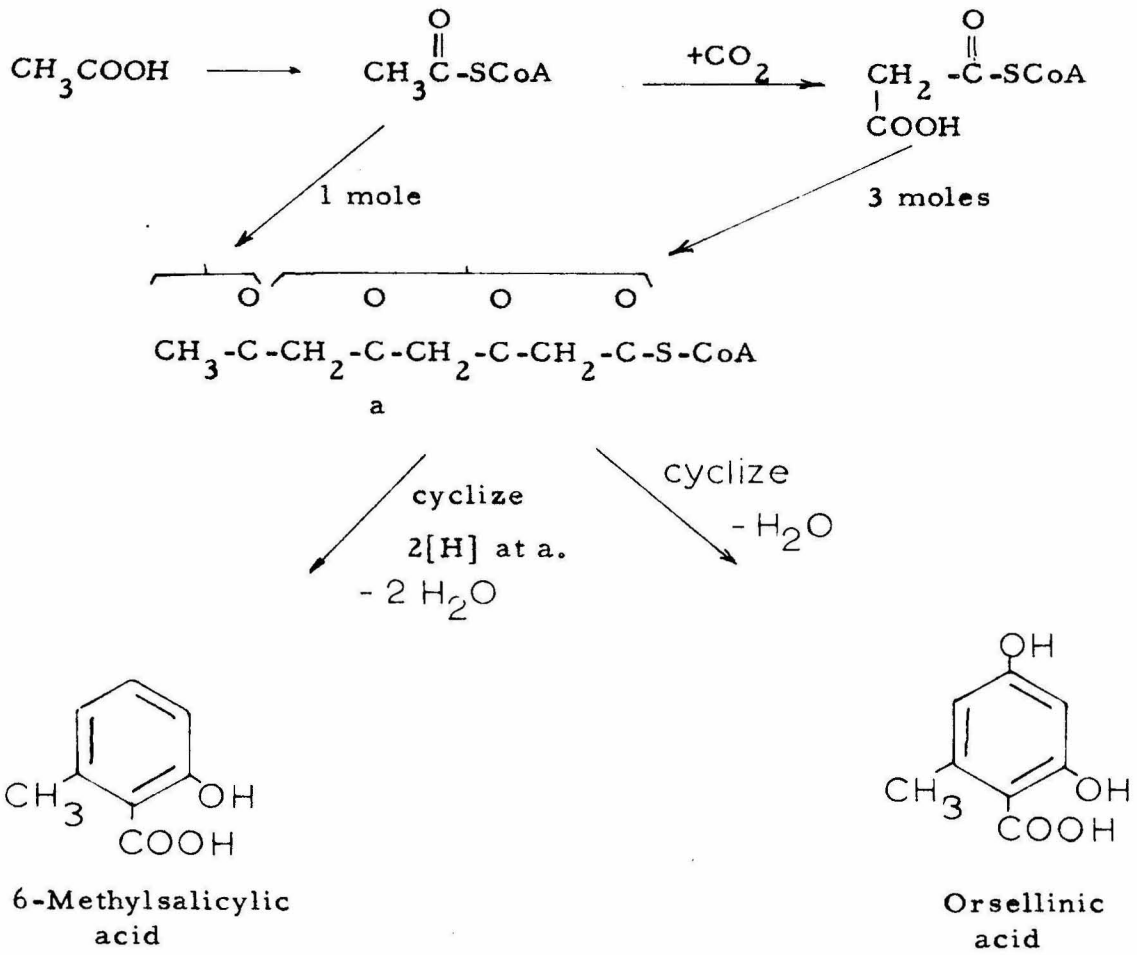
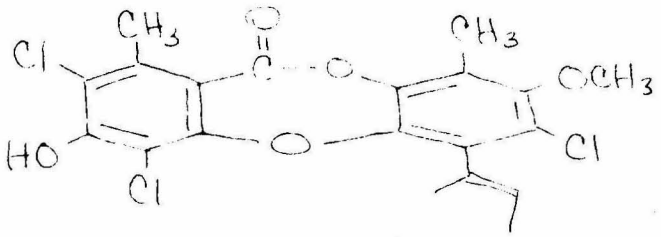
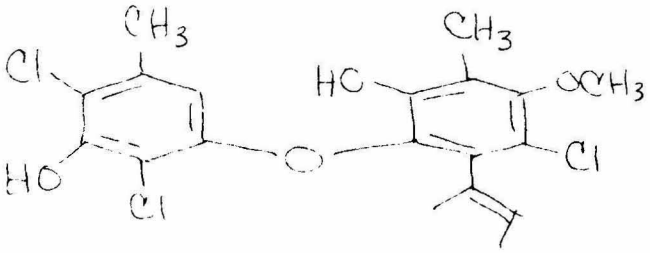

CH3COOH
CH3C-SCoA
+CO2
CH2 -C-SCoA
COOH
1 mole
3 moles
CH3-C-CH2-C-CH2-C-CH2-C-S-CoA
a
cyclize
2[H] at a.
- 2 H2O
cyclize
- H2O
CH3
OH
COOH
6-Methylsalicylic acid
OH
CH3
OH
COOH
Orsellinic acid

Figure 7

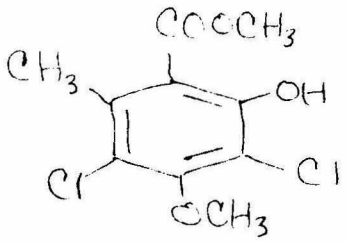
FIGURE 8.



NIDULIN (100%)



DECARBONIDULIN (84%)



METHYL 4,6-DICHLORO-
EVERINATE (49%)

activity located in the A ring carbon atoms was determined by isolating the ring as methyl 4, 6-dichloroeverinate. It was determined that 49% of the molecule's activity was located in the A ring.

Although the observed lactone carbon activity is more than the expected value of one-fourth that of the entire A ring, the values obtained do indicate that the orsellinate system is formed by the same route present in the related systems discussed earlier in this section. Since the value of 16% observed for the lactone carbonyl was determined by subtracting the activity of decarbonidulin from the activity of nidulin, it could be greatly in error. Assuming a 3% error in each measurement, the lactone carbonyl activity is 16 $\pm$ 4%, while the activity in the A ring is 49 $\pm$ 2%.

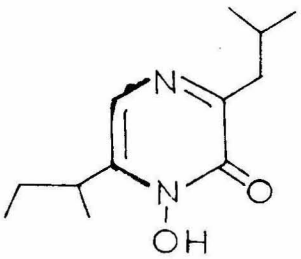
B. The Isoleucine Experiment

The acetate experiment of the preceding section of this thesis leaves little doubt that ring A of nidulin, an orsellinic acid system, arises biosynthetically by the condensation of four acetate units. The origin of the remainder of the molecule, especially of the 2-butenyl side chain, is not, however, immediately apparent.

A 2-butenyl side chain on an aromatic system has no precedent in nature. There are, however, certain examples of secondary four-carbon side chains in nature that might suggest a possible biogenetic pathway for the novel side chain in nidulin.

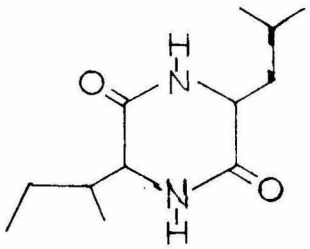
Aspergillus flavus produces an acidic nitrogen-containing metabolite that has been given the name aspergillic acid (39). This substance (XXVII) will be readily recognized as an oxidized form of the mixed amino acid anhydride (diketopiperazine) (XXVIII) of isoleucine (XXIX) and leucine (XXX). Identified as one of the metabolites produced simultaneously with aspergillic acid is hydroxyaspergillic acid (XXXI) (40). If the latter substance were to loose one mole of water from its secondary four-carbon side chain, a system resembling that found in nidulin would result. Unfortunately, no such metabolite of A. flavus has been characterized.

Because isoleucine is almost certainly the precursor of a potential 2-butenyl side chain in the aspergillic acid series, the incorporation of isoleucine into the nidulin skeleton by Aspergillus nidulans was investigated.

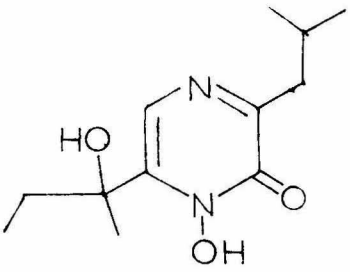


XXVII

Aspergillic Acid

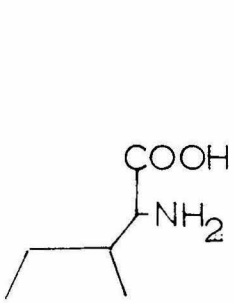


XXVIII



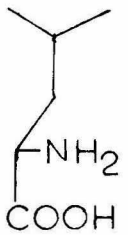
Hydroxyaspergillic Acid

XXXI



Isoleucine

XXIX



Leucine

XXX

It is observed in this experiment that when uniformly labelled isoleucine-C^{14} is fed to the mold Aspergillus nidulans, the nidulin that is produced by the mold is efficiently labelled (4% incorporation of activity). Thus isoleucine is shown to be a biogenetic precursor of nidulin. Furthermore, 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 molecule's radioactivity is concentrated in the B ring.

If isoleucine's role in the biogenesis of nidulin were solely to provide the basis for a 2-butenyl side chain on ring B, there would be no activity in ring A. However, isoleucine can be degraded in vivo into acetate, which the previous experiment has shown to be a precursor of the orsellinic acid A ring of nidulin. It is well known that the metabolism of isoleucine is both glycogenic and ketogenic (41); in other words, that it produces both pyruvate and acetate fragments, which are capable of being transformed by the standard biochemical routes into glucose and acetoacetate, respectively. A study of the enzymic extracts of pig heart and rat liver (42) has disclosed several of the intermediates in the metabolism of isoleucine, along with the fact that acetyl coenzyme A thiolester is one of the metabolism end products.

In figure 9 is presented diagrammatically the interrelationships of leucine, acetate and nidulin as detected by this experiment. If it is

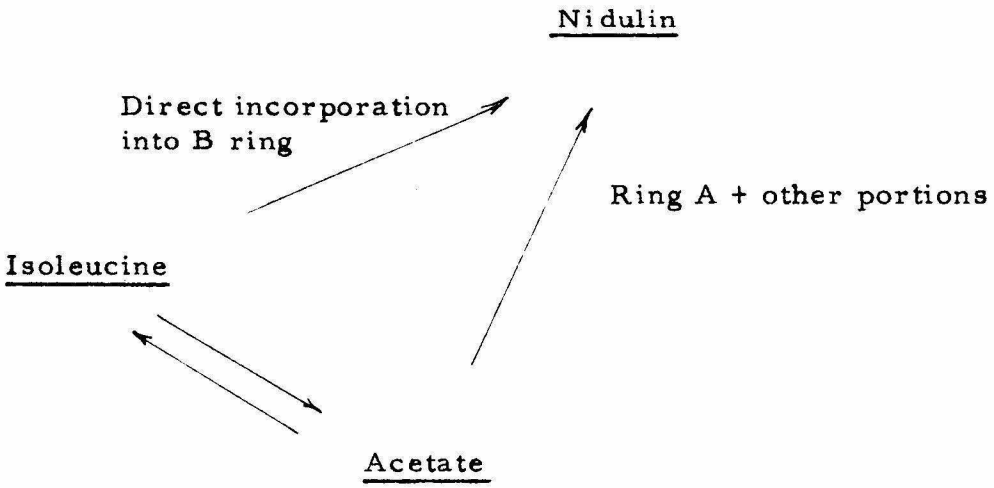
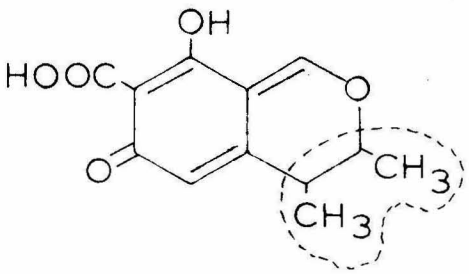

Nidulin
Direct incorporation into B ring
Ring A + other portions
Isoleucine
Acetate

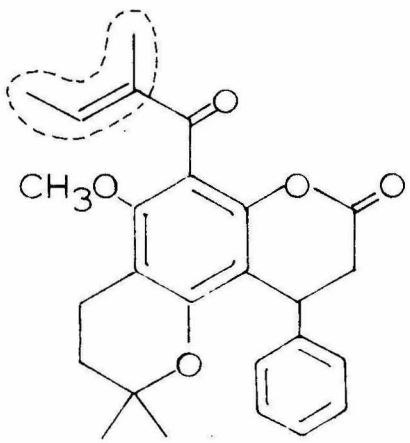
Figure 9

assumed that all of the carbons of nidulin's skeleton come from either acetate or isoleucine, and furthermore that all carbons coming from the same source are equally labelled, then with the further assumption that only one isoleucine unit is incorporated in the B ring skeleton, it is calculated that the carbon atoms which arise from isoleucine are approximately nine times more radioactive than those arising from acetate.

In their most recent publication (28), Dean,Deorha, Erni, Hughes and Roberts set forth some thoughts concerning how the B ring of nidulin might be assembled in nature. They cited structural similarities between nidulin's side chain and parts of the metabolites citrinin (XXXII) (44) and calophyllolide (XXXIII) (45), and proposed that



XXXII



XXXIII

the 2-butenyl systems in each of these metabolites might arise from acetate plus a one-carbon unit, such as is diagrammed in figure 9.

The results of the isoleucine experiment, however, suggest a different kind of biogenesis for nidulin. In the following discussion an overall biogenetic pathway is suggested for the purpose of inspiring future biosynthetic work. The discussion is intended to indicate generally what sort of intermediates are thought to be involved in the biosynthesis of nidulin, but is not to be taken as a mechanism for the transformation.

Isoleucine may be incorporated in the form of its corresponding aldehyde, 2-methylbutyraldehyde. The formation of the B ring might proceed as diagrammed in figure 10 by the condensation of 2-methylbutyraldehyde with three acetate units, followed by an oxidation preparatory to ring closure. With the loss of one mole of water, the ring becomes aromatic.

The source of the 3' methyl group should be a one-carbon unit such as "formaldehyde" or methionine's S-methyl. C-Methylation might occur either before or after aromatization (59).

The double bond in the side chain might be formed in at least two ways. After the aromatic nucleus is formed, an oxidation to an alcohol might occur at the tertiary carbon of the side chain followed by elimination of water to form the olefin. This appears to be the route to hydroxyaspergillic acid. Alternately, the isoleucine unit used in the initial condensation might have been tiglic acid (cis-2-carboxy-2-butene) or tiglaldehyde. The latter proposal is not without support. Tiglic acid

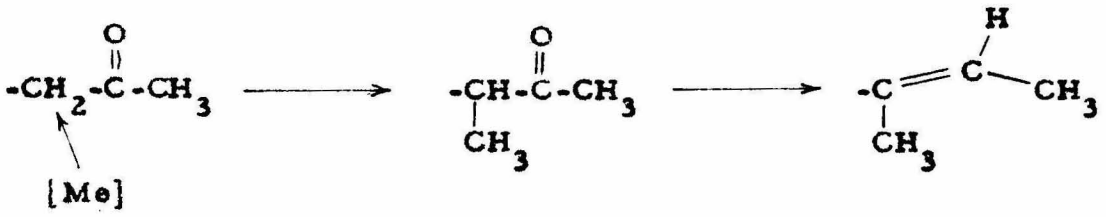


Figure 10

Coenzyme A thiol ester has been identified as an intermediate in the metabolism of isoleucine by the enzymic extracts of rat liver and pig heart (42).

A molecule of orsellinic acid, synthesized from acetate units at another site, would esterify the 2' hydroxyl group, forming a depside which would be capable of coupling via a one-electron oxidation to form the depsidone diphenylether linkage (60).

Chlorination of the system at this stage would proceed quickly under very mild laboratory conditions, so it is not unreasonable to propose that chloride ion, in the presence of an oxidizing enzyme, would provide nidulin with its chlorine atoms. When o-hydroxybenzoic acids are treated with chlorine at room temperature in water, carbon dioxide is liberated quantitatively, and chlorophenols are formed. Kolthoff used the quantitative gas evolution with bromine-water as a basis for a determination of salicylic acid in the presence of other phenols (45). Thus the presence of a B ring chlorine atom is explained.

Aromatic chlorination is retarded by the presence of electronegative substituents on the nucleus to be chlorinated. Therefore, if a dichloro compound is formed, the missing chlorine will be absent from the A ring.

The methylation of the hydroxyl groups is apparently one of the last steps of the biosynthesis. It is interesting to note that the least acidic hydroxyl group of nidulin is the one that is methylated. When nornidulin is treated with precisely one mole of diazomethane, the A ring hydroxyl group is methylated while the B ring hydroxyl group is untouched. The mechanism of biochemical methylation in A. nidulans must therefore be quite unlike that of the diazomethane methylation .

It is of great interest at this point to test the above biogenetic scheme against the acetate experiment already performed. The biogenesis of isoleucine has been worked out for the organism Escherichia coli (56). It is assumed that the same scheme (outlined in figure 11) holds also for the biosynthesis of isoleucine in A. nidulans. The conclusion one must come to concerning the distribution of the radioactive label in nidulin grown on sodium acetate-1-^{14}C is that both rings should be equally labelled. This deduction agrees well with the observed value of 49% of the activity in the A ring. Further experiments, including the incorporation of "formaldehyde" into nidulin and degradations of the B ring of nidulin are now in order.

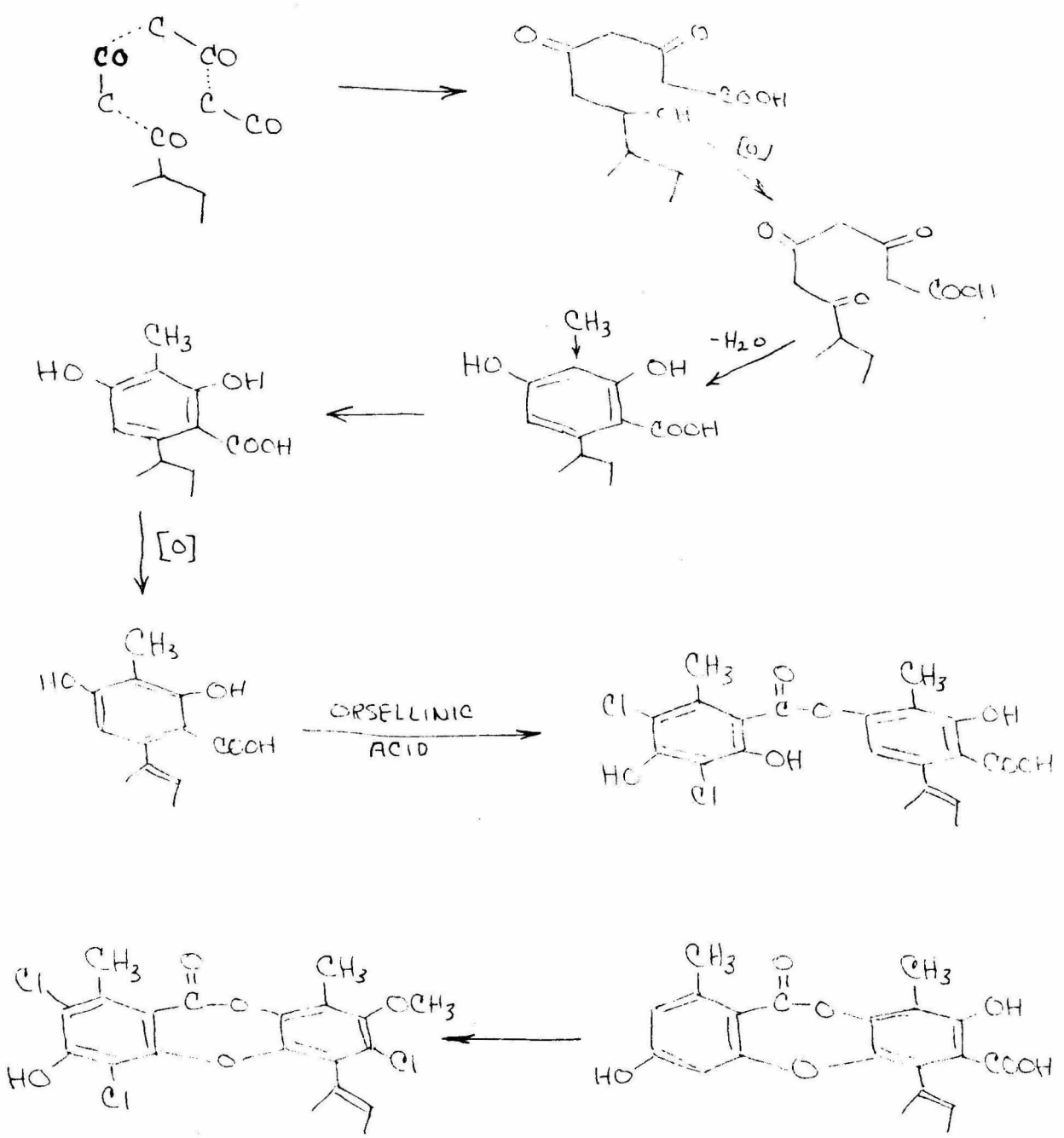
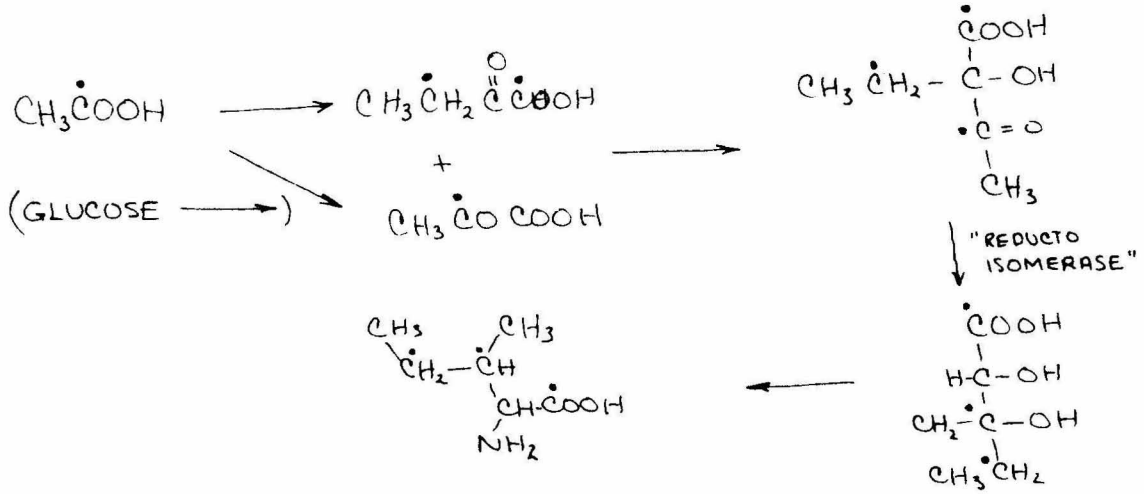

CO
CO
CO
CO
CO
COOH
[O]
$-H_2O$
CH_3
HO
OH
COOH
[O]
HO
OH
COOH
ORSELLINIC
ACID
CH_3
Cl
HO
Cl
OH
COOH
CH_3
OCH_3
Cl
HO
Cl
OH
COOH

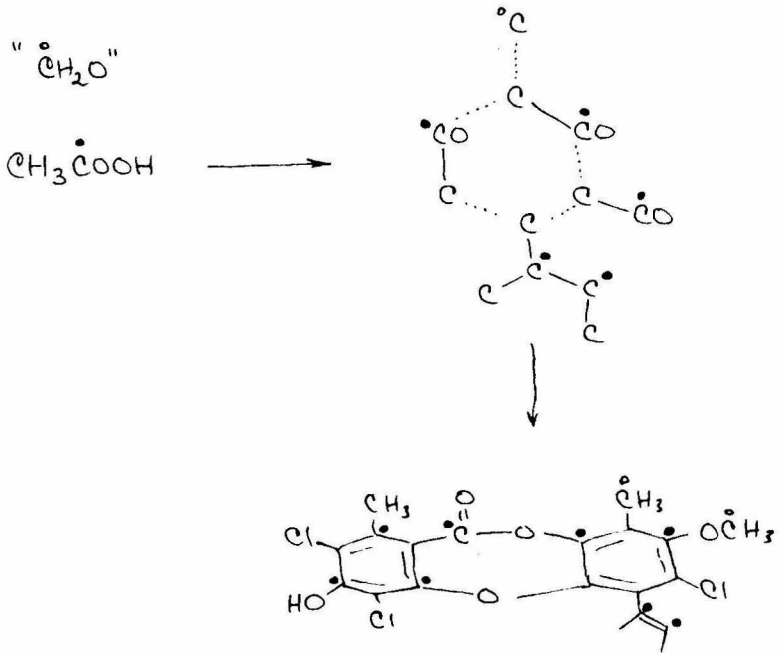
Figure 11

FIGURE 12

Biogenesis of isoleucine:



Implications for nidulin biosynthesis:



EXPERIMENTAL

Special Services and Equipment. The microanalyses reported here were performed by either Dr. Adalbert Elek, Elek Microanalytical Laboratories, Los Angeles, California (E); the Schwarzkopf Microanalytical Laboratories, Woodside, New York (S), or the Spang Microanalytical Laboratories, Ann Arbor, Michigan (Sp). All melting points are uncorrected.

The infrared spectra were observed on a Beckman grating spectrometer, Model IR-7. The ultraviolet absorption spectra were obtained using a Cary recording spectrophotometer, Model 11M.

The nuclear magnetic resonance spectra (N-M-R) were observed at 60 Mc on either of two Varian Associates machines. Data marked HR-60 were obtained with the Varian Model V4300D spectrometer equipped with a Super Stabilizer, constant-temperature magnet cooling and, in all but the spectra for the first structural interpretations on nidulin, field homogeneity controls and an integrator. Chemical shifts were measured by the audio sideband technique with a Hewlett-Packard Model 521 C frequency counter and Model 200 AB audio oscillator. Chemical shifts are quoted in cycles per second (cps) upfield from the signal of an external benzene reference sample. Data marked A-60 were observed on a Varian A-60 analytical N-M-R spectrometer. Chemical shifts were measured directly from the recorder chart, and were reported in cps downfield from the signal of an external reference sample

of a five percent solution of tetramethylsilane in carbon tetrachloride. For purposes of comparing the two scales, the benzene signal is observed at 396 cps downfield from the tetramethylsilane signal. The recorder chart positions, when checked with the calibration apparatus mentioned above, were found to be reliable to within one percent at the 50 cps sweep width position.

Vapor phase chromatographs were obtained with a Perkin-Elmer Vapor Fractometer, Model 154-B. All preparative runs were made on a Beckman Megachrom.

Acknowledgements: The work of Leland Hartwell, who carried out much of the synthetic work associated with the α,β-dimethylstyrenes; Gary Chamness, who did some pioneering work in the same area; and Bob Ross, who prepared 2,5-dihydroxybenzoquinone, is gratefully acknowledged.

Isolation of Nidulin.

Stock cultures of Aspergillus nidulans, NRRL No. 2006, obtained from the U. S. Department of Agriculture, 1815 N. University St., Peoria 5, Illinois, were kept on malt agar slants. For long-term storage, the stock cultures were wrapped in a water-tight Saran Wrap and aluminum foil jacket and kept in the freezing compartment of a refigerator. About one-half dozen stock cultures were kept on hand to ensure the purity of the strain.

The mold was grown for the production of nidulin on a Czepek-Dox liquid medium of the following formulation:

Glucose	7.0%	KCl	0.050%
$NaNO_3$	0.20%	$FeSO_4$-$7H_2O$	0.0010%
KH_2PO_4	0.10%	*Marmite	0.10%
$MgSO_4$-$7H_2O$	0.050%		

The nutrient solution was poured in 350 ml. portions into one-liter erlenmeyer flasks. The flasks were stoppered with gauze-and-cotton plugs, and were autoclaved for about 20 min. at full pressure. It was customary at this point to set the flasks aside for one or two weeks to check for contamination.

Those flasks which showed no signs of contamination were innoculated with a suspension of spores in sterile water from the stock culture slants. The growth of the mold was considerably slower than was expected, the optimum yield of mycelium occurring after two months' growing time at room temperature. At this point the mycelial felts had changed in appearance from the rather pleasant-looking, mossy green (characteristic of new cultures of the species) to an ugly, wrinkled brown and white.

In the most convenient harvesting technique, the mycelial felts from approximately ten flasks were filtered from the liquors through a one-foot square piece of cheese cloth. The corners of the cloth were

*Marmite is an English brand of protein hydrolysate derived from yeast. It is sometimes available at better groceries in this country, but, if a substitution is necessary, an American product, Vegex, has been found to be equivalent to Marmite.

then turned up, holding the felts in a "sack" for drainage. After a few days' air drying, the mycelia were crisply dry. At this point they were broken up with a blunt instrument and pulverized (dry) in a Waring Blendor.

The dried, pulverized mycelia, averaging (in a good batch) 3.2 grams per flask, were extracted with hexane in a Soxhlet apparatus. It was sometimes necessary to stop the extraction two or three times to remove nidulin which had precipitated in the boiling flask. The solid which was obtained by this method was usually quite pure, although brown in color, and often amounted to as much as 10% of the total weight of the dried mycelial felts. Additional nidulin could be obtained by concentrating the boiling-flask liquors.

The crude nidulin was purified by successive crystallizations from ethanol (as stout prisms) and n-heptane (as long, slender, shining rods). The purest nidulin thus obtained had mp 180-1/2 - 181-1/2°. Its ultraviolet absorption spectrum is: λ_{max} (acidic ethanol) 268 mμ (log ϵ 3.95); λ_{max} (alkaline ethanol) 323 mμ (log ϵ 4.27). In the infrared, nidulin absorbs in the three micron region at 3516 cm^{-1}. (Calculated for $C_{20}H_{17}O_5Cl_3$: C, 54.13; H, 3.86; Cl, 23.97. Found (E): C, 54.22; H, 3.77; Cl, 23.91.)

O-Methylnidulin

This substance was prepared according to either procedure of Dean, Roberts, and Robertson (5); i.e., by using either diazomethane or methyl iodide and potassium carbonate in acetone as methylating agents. Our material had mp 143-145° (lit. (5), 144-145°).

O-Methylisonidulin

The isomerization of O-methylnidulin was effected by treatment for a short time with nitric acid in acetic acid solution while warming on a steam bath (5). After repeated crystallizations from methanol-chloroform mixtures, the pure material had mp 172-173° (lit. (28), 174°). Found (E): C, 55.28; H, 4.21; Cl, 23.11. $C_{21}H_{19}O_5Cl_3$ requires: C, 55.10; H, 4.18; Cl, 23.24.

Dihydronidulin

Nidulin (340 mg., 0.78 mmols) was dissolved in 20 ml of glacial acetic acid in the flask of a small hydrogenation apparatus. Platinum dioxide catalyst (20 mg) was added, and the ensuing reaction was followed eudiometrically at room temperature and atmospheric pressure. After the initial uptake of the expected catalyst blank, 0.78 mmol of hydrogen gas was absorbed by the substrate, and the rate of hydrogen uptake fell off 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 being dried with anhydrous magnesium sulfate,

the ethereal solution was taken to dryness. The resulting residue was crystallised from hexane, giving dihydronidulin as long, transparent needles of mp 147-150°. The ultraviolet spectrum of dihydronidulin in acidified ethanol has an absorption maximum at 268 mμ (log ε 3.95). In basic ethanol, this band is replaced by one at 322 mμ (log ε 4.27). Found (S): C, 53.73; H, 4.19; Cl, 23.60. $C_{20}H_{19}O_5Cl_3$ requires: C, 53.89; H, 4.30; Cl, 23.86.

Decarbonidulin

This substance was prepared by the method of Dean, Roberts and Robertson (5), and melted at 145-146.5° (lit. (5): two crystalline forms are reported, one having mp 148-149°, the second having mp 117-118°). (Calculated for $C_{19}H_{19}O_4Cl_3$: C, 54.63; H, 4.58; Cl, 25.47. Found (E): C, 54.59; H, 4.59; Cl, 25.44.)

Methyl O-Methylnidulinate

This material arose from the action of diazomethane on nidulinic acid, the product of a gentle alkaline hydrolysis of nidulin. This phenol-ester was demonstrated to be identical with that formed by the treatment of O-methylnidulin with methanolic KOH. The identity of the free hydroxyl group was thus confirmed. One batch of this substance was actually obtained by allowing a methanolic solution of O-methylnidulin to stand at room temperature for a few weeks. Large, well-formed crystals of methyl O-methylnidulinate appeared at the bottom of the flask. (Calculated for $C_{22}H_{23}O_6Cl_3$: C, 53.95; H, 4.73; Cl, 21.72.

Found (Sp): C, 54.06; H, 4.76; Cl, 21.90.) Methyl O-methylnidulinate has mp 157-161° (lit. (5): 161°, 162°).

Nornidulin and dechloronornidulin: All residues of the hexane extracts of the mycelia of Aspergillus nidulans (from which nidulin had been crystallized) were combined in one-half liter of ether. The ethereal solution was extracted with 10% aqueous sodium carbonate (2 x 100 ml). The alkaline solution was acidified immediately. The liberated acidic solids were extracted into fresh ether, which upon being evaporated left a yellow gum (2 gm). The gum was redissolved in ether and extracted successively with water and 10% aqueous sodium bicarbonate solution, the latter being immediately acidified and re-extracted into fresh ether. After several repetitions of this procedure, the ethereal solution of the liberated acids deposited on evaporation a yellow solid (190 mg).

The yellow solids thus obtained were chromatographed on a 100 gm silicic acid-Supercel (7:3) column using methylene chloride as an eluent and following the elution in the infrared. A rather sharp band containing solely lactonic (ca. 1750 cm^{-1}) absorption in the six micron region was collected and crystallized from hot heptane (5 ml). A crystalline product was thus obtained having mp 158-159°. According to its N-M-R spectrum, this crystalline solid contained no methoxyl methyl protons. Otherwise, its N-M-R spectrum was identical to that of nidulin. These colorless needles were recrystallized from a benzene-

heptane mixture, producing short, stout prisms of mp 181-184°. The melting point was unaffected by further recrystallization. (Analysis of these crystals: C, 54.64; H, 3.72; Cl, 23.43. A mixture of 25% dechloronornidulin and 75% nornidulin requires C, 54.80; H, 3.70; Cl, 23.31.) The melting points in the literature for nornidulin and dechloronornidulin are 185-186° (5) and 212-214° (7), respectively.

The preparation of substituted cis- and trans-2-butenes:

dl-2,3-Dibromobutane (46): A 150 ml, three-necked flask equipped with a thermometer, a gas inlet tube, a dry ice condenser, a magnetic stirring bar and a dropping funnel was rigged so that its contents might be kept at -20° by prudent immersions in a dry-ice-acetone cooling bath. A quantity of cis-2-butene (Phillips) in excess of 20 gm (0.36 mole) was condensed in the reaction flask. While the flask's contents were maintained at -20°, liquid bromine (18 ml, 57 gm, 0.36 mole) was added dropwise with stirring. After the addition was completed, the contents of the reaction flask were distilled in vacuo (bp 50°/20 mm Hg). Vapor phase chromatography verified the homogeneity of the product, dl-2,3-dibromobutane.

trans-2-Bromo-2-butene (46): A 100 ml reaction flask was equipped with a dropping funnel, a mechanical stirrer, a thermometer and a Claisen distilling head with a condenser and a take-off system attached. dl-2,3-Dibromobutane (20 gm, 0.094 mole) and ethylene

glycol were charged into the reaction vessel and heated to 110°. While the reaction temperature was maintained, a solution of potassium hydroxide (85%) (6.9 gm, 0.123 mole) in ethylene glycol (20 ml) was added dropwise. The volatile product, along with some moisture, soon distilled over. When the reaction terminated, the distillate was treated with anhydrous calcium chloride and then filtered, giving trans-2-bromo-2-butene, which was shown to be more than 98% pure immediately after preparation by vapor phase chromatography. A slow isomerization was observed, however. After twenty days of refrigeration, the product contained 14% of the cis-isomer.

meso-2,3-Dibromobutane (46): Bromine was added to trans-2-butene by the same procedure as was used in the preparation of the dl-isomer. The resulting product (bp 52°/22 mm Hg) was shown to be homogeneous by vapor phase chromatography. meso-2,3-Dibromobutane was observed to freeze at -23°.

cis-2-Bromo-2-butene (46): Meso-2,3-Dibromobutane was dehydrobrominated by the same procedure used in the preparation of the trans-isomer. Vapor phase chromatography revealed the presence of 6% of the trans-isomer in the freshly distilled product. Within two days at refrigerator temperatures, however, the product contained 48% of the trans-isomer, indicating a facile isomerization from cis to trans .

α,β-Dimethylstyrene (47): Magnesium turnings (2.43 gm, 0.10 mole) and 5 ml of anhydrous ether were placed into a 500 ml, three-necked, round-bottomed flask equipped with a stirrer, a reflux condenser and a dropping funnel. The apparatus was flushed with dry nitrogen. A solution of ethyl bromide (13.30 gm, 0.122 mole) in anhydrous ether (70 ml) was added dropwise to the magnesium turnings at such a rate as to maintain gentle refluxing. When all of the magnesium had reacted, a solution of acetophenone (12.01 gm, 0.10 mole) in anhydrous ether (70 ml) was dropped into the Grignard reagent. The reaction mixture was stirred for three hours, and then decomposed with aqueous ammonium chloride (500 ml, 1 N). The aqueous phase was separated and washed with two additional portions of ether. The ethereal extracts were combined and stripped of solvent. Concentrated sulfuric acid (0.07 ml) and picric acid (5 mg) were added to the residue, which was then distilled under nitrogen at atmospheric pressure and a temperature of 120° to effect dehydration. The product was then collected by distilling in vacuo the residue from the dehydration (bp 81-95°, 12-15 mm Hg).

The product was analyzed on a Perkin-Elmer vapor phase chromatograph (column C, 147°), and the presence of four components was revealed. Using information gained from infrared and N-M-R spectra, the components were identified as:

1. trans-α,β-dimethylstyrene, (37%).
2. acetophenone, (8%).
3. α-ethylstyrene, (6%).
4. cis-α,β-dimethylstyrene, (49%).

p-Methoxyacetophenone (48): A 500 ml, three-necked, round-bottomed flask was fitted with an efficient mechanical stirrer, a condenser and a 125 ml dropping funnel. The apparatus was flushed with dry nitrogen. Absolute ethanol (8 ml, 0.14 mole) and carbon tetrachloride (0.8 ml) were added slowly to magnesium metal turnings (8.00 gm, 0.329 mole) contained in the flask. The ensuing reaction was allowed to proceed for five minutes. Then anhydrous ether (110 ml) was added cautiously over a period of one-half hour. A mixture of diethylmalonate (52.8 gm, 0.33 mole), absolute ethanol (30 ml, 0.51 mole) and anhydrous ether (38 ml) was dropped into the reaction flask at such a rate as to cause refluxing. The reaction solution was refluxed for an additional 3-1/2 hours, dissolving virtually all of the magnesium metal.

p-Anisoyl chloride (52 gm, 0.303 mole) was dissolved in ethyl ether (50 ml). The resulting solution was dropped into the preparation of the magnesium ethoxy derivative of malonic ester with efficient stirring at the rate required for gentle refluxing. (Toward the end of the addition, the reaction mixture sets up into a solid mass.) When the addition was completed, the reaction mixture was decomposed with

dilute sulfuric acid. The ethereal phase was separated, and the aqueous phase was given two ether washes. The ethereal solution of diethyl anisoylmalonate was stripped of solvent. The residue was combined with glacial acetic acid (45 ml), water (50 ml) and concentrated sulfuric acid (5 ml), and refluxed to complete the decarboxylation. After three hours' refluxing, the solution was chilled with ice and made alkaline with 20% aqueous sodium hydroxide. The product was extracted with ether. The ethereal solution was dried over anhydrous sodium sulfate and stripped of solvent. The resulting residue was distilled in vacuo, giving p-methoxyacetophenone (bp 100°/1.5 mm Hg).

Vapor phase chromatography demonstrated that the liquid was homogeneous. When the product was left at room temperature, or somewhat below, it crystallized. However, no melting point was obtained. (Calculated for $C_9H_{10}O_2$: C, 71.98; H, 6.71. Found: C, 72.02 (E); H, 6.82 (E).)

The dinitrophenylhydrazone derivative was prepared. Crystallization from a chloroform-ethanol mixture produced a red-orange substance of mp 224-227°. (Calculated for $C_{15}H_{14}O_5N_4$: C, 54.53; H, 4.27; N, 16.98. Found: C, 54.80 (E); H, 4.31; N, 17.06 (E).)

p-Methoxy-α,β-dimethylstyrene: In order to minimize the amount of the acetophenone in the final product, the procedure was changed slightly. Magnesium turnings (2.92 gm, 0.125 mole) and anhydrous

ether (20 ml) were placed in the 500 ml apparatus previously described. Ethyl bromide (16.5 gm, 0.15 mole) dissolved in ether (70 ml) was added dropwise over a period of 45 minutes to the magnesium turnings. p-Methoxyacetophenone (8 gm, .055 mole), dissolved in ether (70 ml), was added at the same rate to the Grignard reagent. The reaction mixture was stirred for nine hours and allowed to stand overnight. The reaction mixture was then poured over 75 gm of ice, and the resulting slurry was treated with sulfuric acid (60 ml, 15%). The aqueous layer was separated and washed with two portions of ether, and the ethereal extracts were stripped of solvent. The resulting residue was combined with concentrated sulfuric acid (0.1 ml) and picric acid (5 mg) and dehydrated as before. The product was isolated by vacuum distillation (bp 70-90°, 3 mm Hg).

Vapor phase chromatography of the product mixture revealed the presence of three components (Perkin-Elmer, column C at 160°). In order of their retention times, the peaks were identified as follows:

1. trans-p-methoxy-α,β-dimethylstyrene (42%).
2. p-methoxy-α-ethylstyrene (8%).
3. cis-p-methoxy-α,β-dimethylstyrene (51%).

(Calculated for $C_{11}H_{14}O$: C, 81.44; H, 8.69. Found: C, 81.09 (E); H, 8.73 (E).)

p-Bromo-α,β-dimethylstyrene: The modified procedure of the previous synthesis was used, substituting p-bromoacetophenone as the Grignard substrate. The product mixture had bp 75-83° at 1 mm Hg.

Vapor phase chromatography of the product mixture (Perkin-Elmer, column C, 161°) demonstrated the presence of three components. With the aid of the N-M-R spectrum of the mixture, the three bands were identified as follows:

1. trans-p-bromo-α,β-dimethylstyrene (47%).
2. p-bromo-α-ethylstyrene (5%).
3. cis-p-bromo-α,β-dimethylstyrene (48%).

(Calculated for $C_{10}H_{11}Br$: C, 56.89; H, 5.25; Br, 37.85. Found: C, 56.90 (E); H, 5.43 (E); Br, 37.78 (E).)

Separation of the styrene isomers was achieved with the help of a Beckman Megachrom preparative vapor phase chromatograph employing an apiezon column.

The α,β-dimethylstyrene mixture separated cleanly into three components at a column temperature of 128°. In the order of their elution, the bands were: the trans isomer, a mixture of acetophenone and α-ethylstyrene, and the cis isomer. The isomers of α,β-dimethylstyrene have been examined by Cram (47) who demonstrated that the lower boiling isomer was, indeed, the trans isomer. This was expected in view of the relative compactness of the two molecules.

The p-methoxy-α,β-dimethylstyrene mixture separated partially

on the column at 170°, concentrating what was assigned the trans configuration in the first fraction. The lack of sharpness in the chromatogram is attributed to isomerisation on the column, which is apparently catalysed by a trace of acid in the apiezon column packing material.

The p-bromo-α,β-dimethylstyrene mixture was separated very cleanly at 170° on the apiezon column. The trans configuration was assigned to the lower boiling isomer.

Measurement of the coupling constants.

The spin-spin coupling constants were observed by measuring appropriate splittings in the spectrum. J_{AB}, the five-bond interaction between the methyl groups across the double bond, was measured by the secondary splittings in the β-methyl group's doublet of quartets. J_{AX}, the four-bond interaction between the α-methyl group and the vinyl hydrogen, was measured at the vinyl hydrogen quartet-of-quartets by the secondary splittings. The three-bond interaction of the vinyl hydrogen with the β-methyl group was measured on either signal, or both, when practicable.

The coupling and chemical shift parameters involved in the A_2B_2 system of the ring hydrogens in the para substituted α,β-dimethylstyrenes were determined by fitting calculated spectral lines (49) to the spectra actually observed.

The Dechlorination of Dihydronidulin

The action of hydrobromic acid and phosphorous on dihydronidulin (29): To a boiling solution of nidulin (500 mg, 1.12 mmol) in glacial acetic acid (10 ml), red phosphorous (1 gm) and constant boiling (48%) hydrobromic acid (8 ml) were added. The reaction mixture was refluxed for the desired reaction time.

The reaction mixture was then poured into water (150 ml) contained in a 250 ml separatory funnel. The aqueous slurry was shaken with three portions of ether. The ethereal extracts were filtered free from residual phosphorous, and washed successively with water, saturated aqueous sodium bicarbonate, and once again with water.

The residue remaining after the evaporation of the ether in a stream of nitrogen was taken up in sufficient carbon tetrachloride to make a one-half percent solution. This solution was examined in the three micron and six micron regions of the infrared.

Depsidone assay: The amount of starting material present may be estimated by referring to the carbonyl region of the spectrum. The depsidone carbonyl band is located at 1750 cm^{-1}, and its extinction coefficient is roughly 600, or about three times that of the hydroxyl bands. The twenty minute reaction mixture contained the only detectable amount of depsidone, which was estimated as less than one mole percent.

Nidulinic acid assay: Nidulinic acid, the product of the hydrolysis of the lactone system, would be expected to be extracted into the aqueous bicarbonate solution. However, control experiments have demonstrated that these bicarbonate extractions are inefficient. Hence, some of the nidulinic acid formed is expected to remain in the organic phase. Here it is observed at 1710 cm^{-1}, the stretching frequency of the carboxylic acid carbonyl group. No acids were detected either in the 1710 cm^{-1} region of the reaction mixture solution or upon acidification of the aqueous bicarbonate extracts.

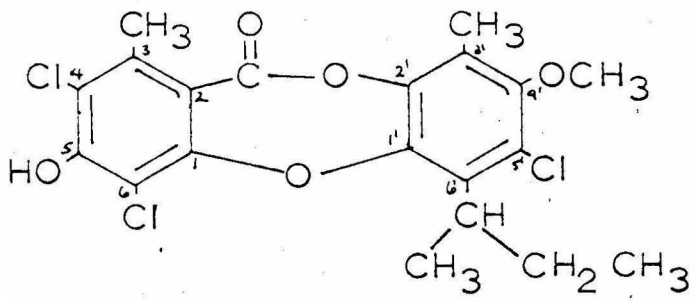
Estimation of the extent of demethylation and dechlorination: The results on the assays of depsidone and acids have shown that the preliminary steps of hydrolysis and decarboxylation are essentially complete after twenty minutes' reaction time. From this point forward, all species have only one hydroxyl group that absorbs at 3573 cm^{-1} (the OH...O frequency): the phenolic hydroxyl that was liberated with the lactone hydrolysis. Consequently, the 3573 cm^{-1} band may be used as a measure of the concentration of the reaction species present. As demethylation proceeds, a third hydroxyl group is liberated, and additional absorption is observed in the infrared spectrum. Either this newly liberated hydroxyl group will be adjacent to the B ring chlorine atom and absorb at 3536 cm^{-1} (OH...Cl); or, if dechlorination has already taken place, it will be "free," and absorb at 3617 cm^{-1} (free OH). As dechlorination progresses, the 4'-hydroxyl group, if

unmethylated, will appear at 3617 cm^{-1}. If demethylation proceeds sufficiently faster than dechlorination, which apparently is the case in this system, the build-up of the 3617 cm^{-1} absorption reflects the progress of the dechlorination reaction.

Table 4 represents the extent of demethylation, as measured by the increase in absorption at 3536 cm^{-1} over that observed in decarbonidulin plus the absorption at 3616 cm^{-1}, and the extent of dechlorination, as measured by the absorption at 3616 cm^{-1}.

Table 4

	Mole Percent:		
Species analyzed for:	20 minutes	1 hour	2 hours
Depsidone	1%	0	0
Acids	0	0	0
Demethylated species	53%	93%	100%
Dechlorinated species	7%	32%	48%



XXXII DIHYDRONIDULIN

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 solution were introduced to the top of the column. Elution was started, and a residue was soon detected in the eluent. Two distinct bands were obtained.

The first band proved to contain decarbodemethyldihydronidulin. After a few recrystallizations from heptane, it appeared as well-formed crystals of mp 127.3-129.7°. Its N-M-R spectrum had no methoxyl methyl absorption. Its infrared spectrum possessed a band at 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. (Calculated for $C_{18}H_{19}O_4Cl_3$: C, 53.29; H, 4.72; Cl, 26.22. Found: C, 52.96 (S), 53.09 (E); H, 4.91 (S), 5.00 (E); Cl, 26.13 (S), 26.42 (E).)

The second band to be eluted from the column contained dechlorodemethyldecarbodihydronidulin. Recrystallization from a mixture of benzene and heptane produced well-formed crystals of mp 157.0-158.0°. The infrared spectrum of this material had three bands of approximately the same intensity at 3533 cm^{-1}, 3566 cm^{-1} and 3616 cm^{-1}. (Calculated 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 (S).) In a preliminary experiment, an analysis of

Cl, 24.58% (E) was obtained for a product otherwise closely resembling dechlorodemethyldecarbodihydronidulin.

The formation of a xanthene from nidulin.

The action of hydrobromic acid and phosphorous on nidulin: Nidulin (500 mg, 1.13 mmol) is treated by exactly the same procedure as was used on dihydronidulin in the dechlorination experiments. After the infrared spectra were taken, the carbon tetrachloride solution was reduced in volume ten-fold for the N-M-R spectra.

Estimation of the degree of demethylation: Species that contain a methoxyl group absorb sharply at about 235 cps in the N-M-R spectrum, while all species contain two aromatic methyl groups which give two unsplit peaks in the 150 cps region. In an unknown mixture, then, the mole percent of the methoxylated species can be calculated from the spectral areas in these two regions. The results for these experiments are displayed in table 5.

Estimation of the degree of dechlorination: Here, the infrared procedure developed for the dechlorination experiment was employed. The results are displayed in table 6.

Xanthene assay: Species of the xanthene type give rise to a triplet absorption in the N-M-R spectrum at 35 cps. This signal provides a means of estimating the mole percent of xanthene species in the reaction mixture. These data are presented in table 7.

Table 5. Methoxyl assay.

Reaction time	% Methoxyl
6 minutes	83%
20 minutes	33%
55 minutes	0%

Table 6. Dechlorination assay.

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

Note: $A(3619\ cm^{-1})/A(3567\ cm^{-1})$ is 0.96 in the xanthane.

Table 7. Xanthane assay.

Reaction time	% Xanthane
20 minutes	0%
55 minutes	16%
3 hours	35%

Isolation of the xanthene and demethyldecarbonidulin: A 100 gm dry-packed chromatographic column containing a 3:7 mixture of Supercel and silicic acid was primed with methylene chloride. The reaction mixture of the three-hour run was placed on the column in a small amount of methylene chloride. Elution with the same solvent produced two bands of material, which were detected by residue weight.

The first band to be eluted contained demethyldecarbonidulin, which, after being crystallized twice from a chloroform-heptane mixture, had mp 141.5-143°. Its infrared spectrum (CCl_4, ca. 0.5%), in the hydroxyl stretching frequency region, possessed a band at 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 ($CHCl_3$, 5%) were two closely spaced unsplit peaks at 140 and 143 cps, and a rather complex series of absorptions between 75 and 120 cps. The latter absorptions, due to vinyl methyl groups, indicated that this substance was actually not a single compound but a mixture of isomers of the 2-but-3-enyl group attached to the B ring. Judging from the relative sizes of the signals, the mixture appears to be about 2:1 :: trans : cis. (Calculated for $C_{18}H_{17}O_4Cl_3$ (Sp): C, 53.55; H, 4.25; Cl, 26.35. Found: 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 mp 171.5-173°. Its infrared spectrum (CCl_4, ca. 0.05%) in the hydroxyl stretching frequency region possessed three bands of approx-

imately equal intensity at 3533 cm^{-1}, 3567 cm^{-1} and 3619 cm^{-1}. The major features of its N-M-R spectrum ($CHCl_3$, ca. 5%) were (a) a triplet at 35 cps, proportional to three protons, (b) a singlet at 112 cps representing three protons, and (c) two singlets, each proportional to three protons, at 137 and 160 cps. (Calculated for $C_{18}H_{18}O_4Cl_2$ (Sp): C, 58.55; H, 4.91; Cl, 19.21. Found: C, 58.61; H, 4.98; Cl, 19.30.)

2,5-Dihydroxybenzoquinone (50): A 500 ml. three-necked flask was provided with an efficient stirrer, a dropping funnel and an air condenser which was to serve primarily as a vent and a thermometer well. A 50% aqueous sodium hydroxide solution (200 gm) was prepared in the flask. After it had cooled, hydroquinone (27.5 gm, 0.25 mole) was added slowly, with stirring, to make a smooth slurry. Hydrogen peroxide (30%) (100 ml) was dropped slowly into the reaction mixture while the temperature of the mixture was regulated within the range 45-50°. The addition was completed within one hour. Stirring was continued for an additional ninety minutes, during which the reaction mixture was maintained at the same temperature.

The reaction mixture, at that time a slurry of the sodium salt of the product, was stirred into cracked ice (500 gm), and acidified with concentrated hydrochloric acid while being chilled with a second portion of ice (300 gm). The resulting yellow solid, only slightly soluble in cold water, was collected on a filter and rinsed free of peroxide with cold water. It was then dried overnight in a vacuum desiccator.

After recrystallization from ethyl acetate, 2,5-dihydroxybenzoquinone appeared as red-orange crystals having a mp of about 215° (the exact range was obscured by darkening that started at about 195°) (lit. (51), 218-220° (d.)). (Calculated for $C_6H_4O_4$: C, 51.44; H, 2.88. Found (Sp): C, 51.41; H, 2.93.)

2,6-Dihydroxy-3,5-dimethylbenzoquinone:

Trinitro-m-xylene: Nitric acid (d 1.42) (36 ml, 51 gm, 0.57 mole) and sulfuric acid (d 1.82) (141 ml, 256 gm) were placed into a 500 ml, three-necked, round-bottomed flask equipped with a dropping funnel, a thermometer, a mechanical stirrer, and an air condenser. m-Xylene (20 gm, 0.189 mole) was dropped into the mixed acids from the dropping funnel at the rate of one drop per second. Throughout this addition the reaction mixture temperature was maintained at 40-45° with the help of an ice bath. When the m-xylene addition was completed, 65% oleum (70 ml) was placed in the dropping funnel and added dropwise to the reaction mixture at a temperature never exceeding 40°. The reaction mixture was stirred for one-half hour after the completion of the oleum addition. It was then heated over a one-half hour period to 110°, and held at that temperature for four hours.

The reaction slurry was cooled to below room temperature and poured over ice in a one liter beaker. The liquid phase was removed by filtration. The crystals of the product, trinitro-m-xylene, were thoroughly washed with water and pressed dry on the filter. The

crystals were then leached in warm (50°) ethanol, filtered and pressed dry. After air-drying, the product melted at 178-181° (lit. (52), 182.2°). The yield was 93% of theory.

Dimethylphloroglucinol (53): Trinitro-m-xylene (20 gm, 0.083 mole), granulated tin metal (180 gm, 1.5 mole), and concentrated aqueous hydrochloric acid (300 ml, 3.6 moles) were charged into a one-liter Erlenmeyer flask. The mixture was warmed on a steam bath to a temperature of 60-70° to start the reduction. After one-half hour of vigorous swirling, the organic material had largely disappeared, leaving a brown-colored supernatant solution over the residual tin metal. The supernatant solution was filtered through a glass wool plug, and the filtrate was chilled to 0° in an ice bath. The filtrate was saturated in the cold with hydrogen chloride gas until a dense white solid precipitated, forming a thick paste in the flask. The precipitate was filtered on a sintered glass funnel and dried at 10 mm Hg over potassium hydroxide pellets. The yield of the stannous chloride double salt of triamino-m-xylene trihydrochloride was nearly quantitative.

The tin double salt was refluxed in water (1500 ml) for one day. The aqueous solution was extracted four times with ether. Evaporation of the ether produced a small quantity of residue, which, when sublimed (120°, 0.1 mm Hg), gave 2,4-dimethylphloroglucinol. Crystallization of the sublimate from toluene produced long, thick needles of mp 163.0-164.3° (lit. (53), 164°). The yield, however, was only 20% of theoretical.

6-Nitroso-2,4-dimethylphloroglucinol (a monoxime of 2,6-dihydroxy-3,5-dimethylbenzoquinone)(54): D methylphloroglucinol (250 mg, 1.62 mmol) was dissolved in water (50 ml) containing nitric acid (d 1.42) (6.3 ml, 100 mmol). The solution was chilled by the addition of ice (ca. 25 gm). Solid sodium nitrite (114 mg, 1.62 mmol) was added to the iced solution in one portion. The solution suddenly turned dark red-brown. Shortly afterwards, a precipitate began to form. As the slurry grew thicker, the dark color of the solution lightened. The orange solid removed by filtration was washed with a little water and dried. It melted at 171-172.5° (decomposition with gas evolution producing a black melt) (lit. (54), 158° (uncorr.)).

2,6-Dihydroxy-3,5-dimethylbenzoquinone (54): 6-Nitroso-2,4-dimethylphloroglucinol (525 mg, 2.87 mmol) was dissolved in a mixture of hydrochloric acid (50 ml) and water (75 ml) by heating on a steam bath,producing a black colored solution. Solid stannous chloride dihydrate (1.43 gm, 6.37 mmol) was added in one portion, bleaching all color from the solution. Stirring was continued for one-half hour to ensure the completeness of the reduction. Solid ferric chloride (1.87 gm, 11.5 mmol) was then added to the colorless solution, and deep color returned, followed immediately by a precipitate of the quinone. After one hour's stirring, the precipitate was isolated by filtration and purified by sublimation (120°, 0.1 mm Hg). The 2,6-dihydroxy-3,5-

dimethylbenzoquinone obtained in this manner had mp 168.5-170.5° (lit. (54), 167°). (Calculated for $C_8H_{10}O_4$: C, 57.14; H, 4.79. Found (Sp): C, 57.26; H, 4.86.)

The ultraviolet spectrum of 2,6-dihydroxy-3,5-dimethylbenzoquinone is presented in figure 12 as it is observed in "neutral ethanol" at various concentrations. In figure 13, the spectrum at a nominal concentration in "neutral ethanol" is compared with the spectrum of the compound $C_{11}H_{12}O_4$ published by Dean, Roberts, et al (28).

Isolation of 2,5-dihydroxy-3-methyl-6-(2-but-2-enyl) benzoquinone from nidulin (28): A solution of nidulin (500 mg, 1.13 mmol) in glacial acetic acid (10 ml) was brought to a boil. Red phosphorous (1.0 gm) and constant boiling hydrobromic acid (8 ml) were added and refluxed for forty minutes.

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

The evaporation residue was taken up into methanol (20 ml). The methanolic solution, along with two pellets of potassium hydroxide, was heated at reflux in a stream of nitrogen for two hours.

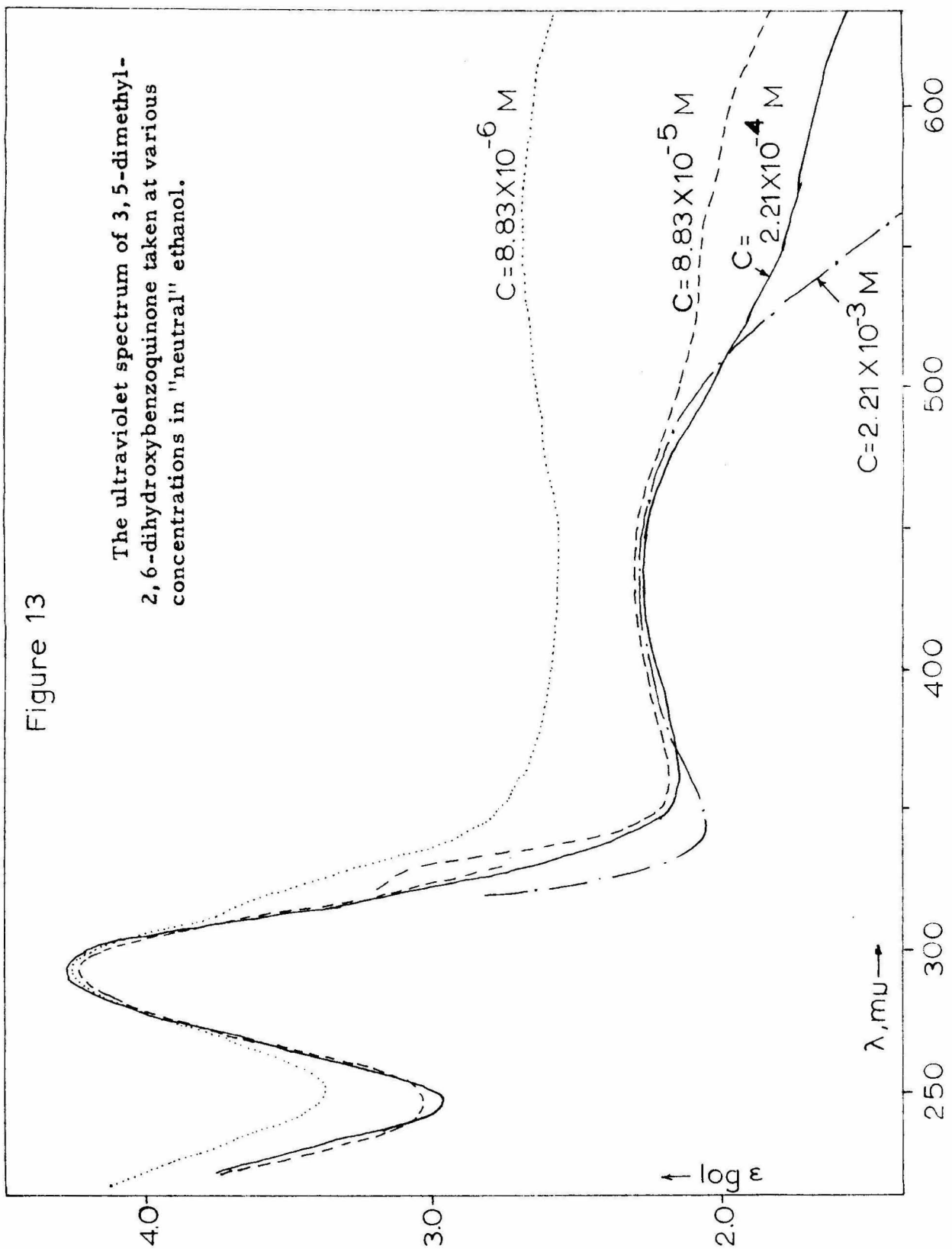
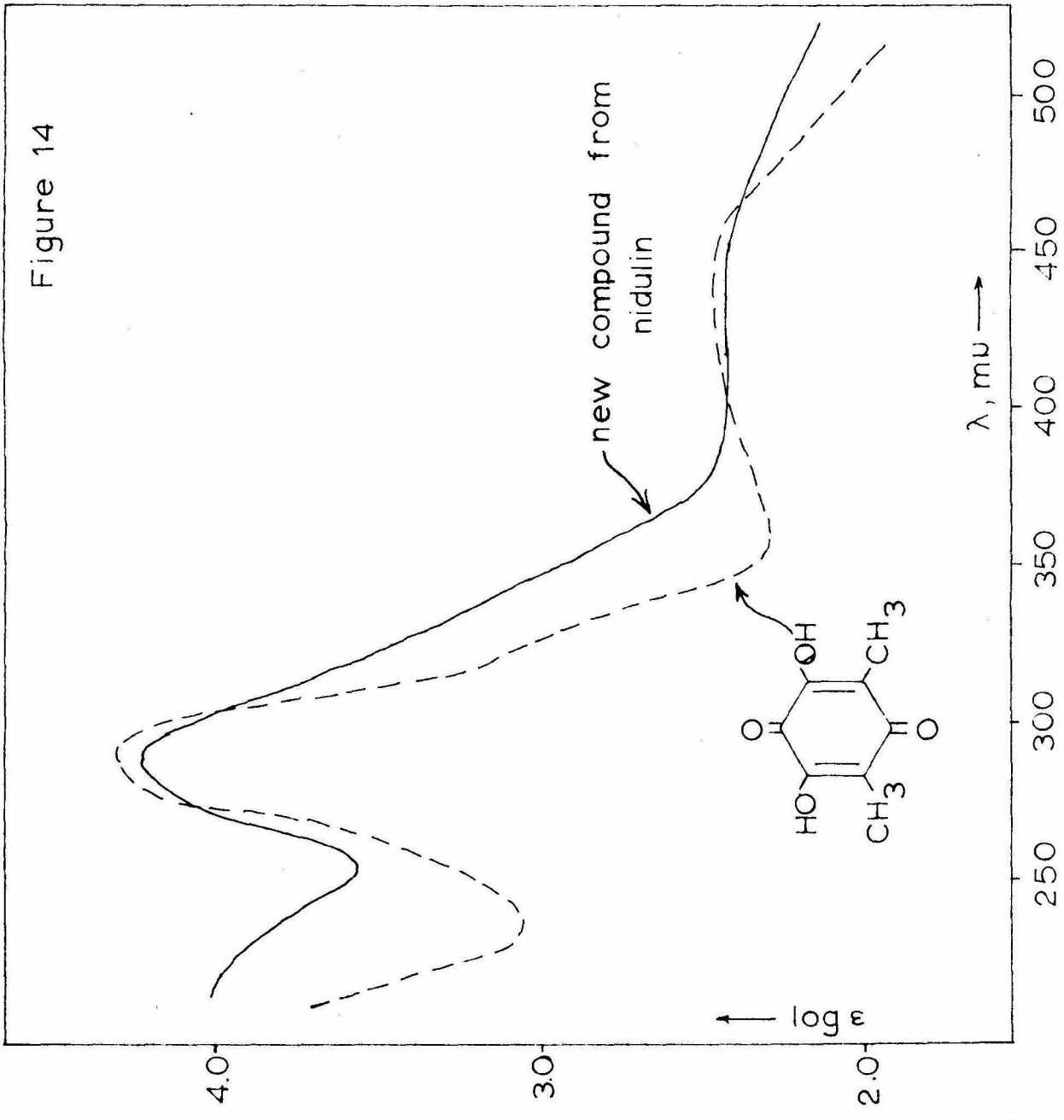


Figure 13
The ultraviolet spectrum of 3, 5-dimethyl-2, 6-dihydroxybenzoquinone taken at various concentrations in "neutral" ethanol.
C=8.83X10-6 M
C=8.83X10-5 M
C= 2.21X10-4 M
C=2.21X10-3 M
4.0
3.0
2.0
log ε
250
300
400
500
600
λ,mu

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.



The dark methanolic solution was poured into water (100 ml). Solid potassium dihydrogen phosphate (3 gm) was added to the solution, bringing its pH to 6.3. The resulting solution was extracted with two volumes of ether. The aqueous phase was then made very acidic with sulfuric acid and again extracted twice with ether. The ethereal solution of the acidic products thus obtained was taken to dryness, and the resulting residue was sublimed many times to give a small amount of the red compound (10 mg), 2,5-dihydroxy-3-methyl-6-(2-but-2-enyl)-benzoquinone, mp 190-197°. (Calculated for $C_{11}H_{12}O_4$: C, 63.45; H, 5.81. Found (Sp): C, 63.21, 63.14; H, 5.69, 5.72.)

Determining $pK_a(2)$ of dihydroxybenzoquinone: Ultraviolet spectroscopy has been used in an elegant manner to determine, for example, both acid constants of compounds such as the phthalic acids and chloranilic acid (55). The requirements of this experiment, however, are quite simple. A relatively crude ionization constant measurement will suffice to distinguish between a 2,5-dihydroxybenzoquinone and a 2,6-dihydroxybenzoquinone nucleus. Hence, for this experiment, the following procedure was developed.

Because of the limited supply of unknown material, a procedure based on preparing a different solution for each pH that was to be examined spectroscopically was impossible. An attractive alternative to this was "sweeping" the pH range by adding acid to an appropriate buffer salt. The use of potassium phosphate as the buffer salt had two

distinct advantages. First, all phosphate ions that would be encountered would be transparent in the portion of the u.v. spectrum that was of interest. Second, the use of phosphate provided a reasonably good control of pH from 12 down to and past 4. Acid had to be added in a small volume, so that the total volume of the solution would not change appreciably over the entire range "swept out." Thus, the use of concentrated acid was dictated. Concentrated sulfuric acid was especially useful here, not only for the reason mentioned above, but also because it provided a divalent anion so that the loss of the trivalent phosphate ion was not felt so strongly in the change of ionic strength throughout the pH range.

The material to be investigated (10 mg) was dissolved in a potassium phosphate solution (0.05 M, 100 ml). The solution, in a 1.00 cm cuvette, was examined with a Cary model 14 recording spectrometer. The pH was made progressively more acidic by adding appropriate amounts of concentrated sulfuric acid and a 1:5 solution of sulfuric acid in water. Any back-titrating that was necessary was done with a 50% aqueous solution of potassium hydroxide. pH measurements were made with a Beckman model G portable pH meter.

Wavelengths whose absorptions appeared to change sharply with pH within certain regions were assumed to be representative of two related species as the pH approached their relating pK_a. The pK_a was determined from a plot of absorption at those wavelengths versus pH.

The graphical determinations of $pK_a(2)$ for 2,5-dihydroxybenzoquinone, 3,5-dimethyl-2,6-dihydroxybenzoquinone, and the compound $C_{11}H_{12}O_4$ isolated from nidulin are reproduced in figures 14, 15, and 16, respectively. In figure 17, a spectrum of a buffered aqueous solution of the nidulin degradation product, $C_{11}H_{12}O_4$, is presented at two different pH's, demonstrating the difference in the mono- and di-anion absorption spectrum.

The Acetate experiment

The growth of Aspergillus nidulans on a medium containing sodium acetate-1-C^{14} and the isolation of the resulting nidulin: Five one liter 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 labelled acetate. The highly active nidulin was diluted with sufficient inactive nidulin that the resultant material was at the relatively safe but nevertheless useful specific activity of $0.4594 \pm .0032$ μc/mmol. The active nidulin, after a number of crystallizations from heptane, was very pure from a chemical standpoint (mp 180.3 - 181.5°).

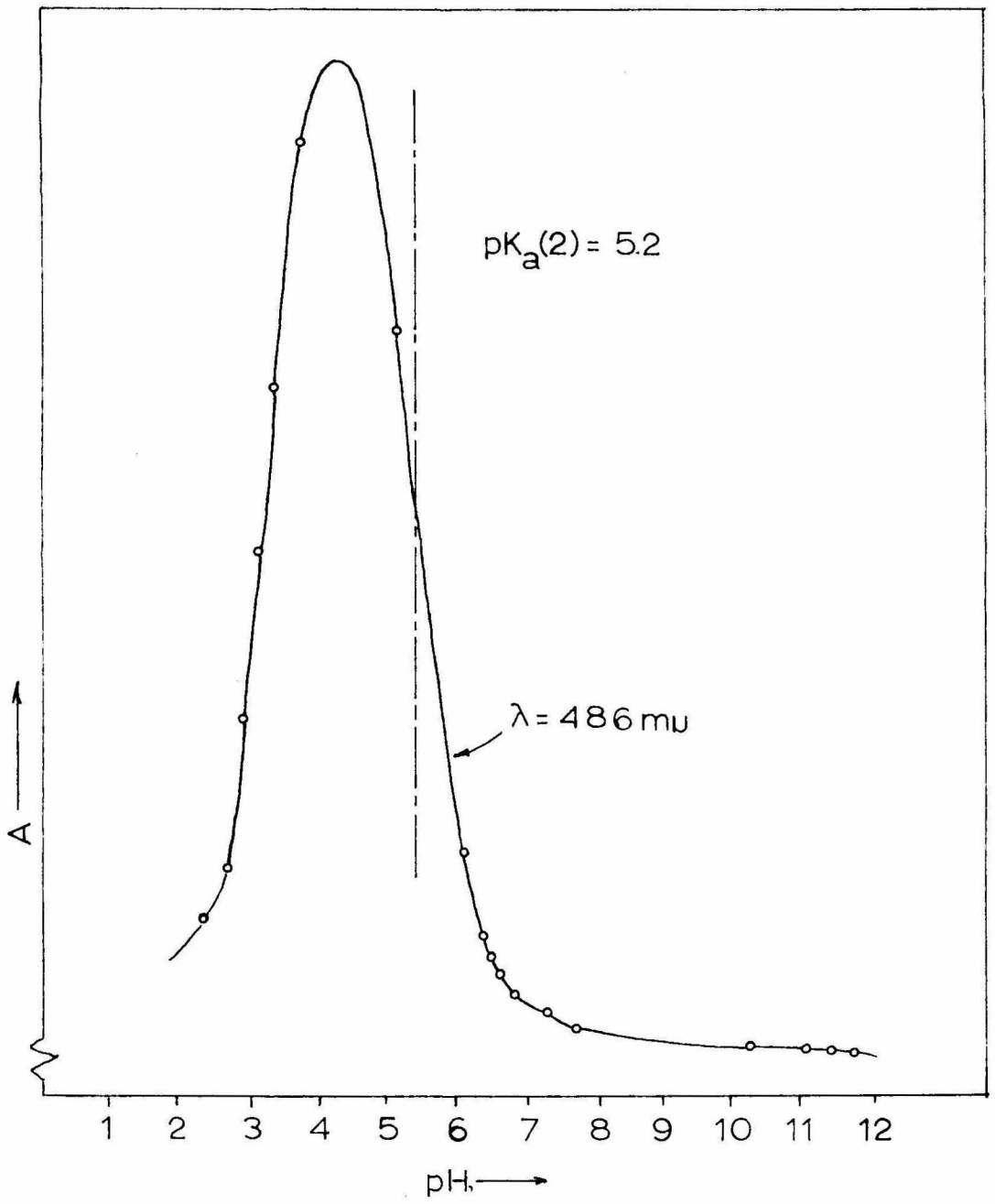


Figure 15.

Determining the second ionization constant of 2,5-dihydroxybenzoquinone.

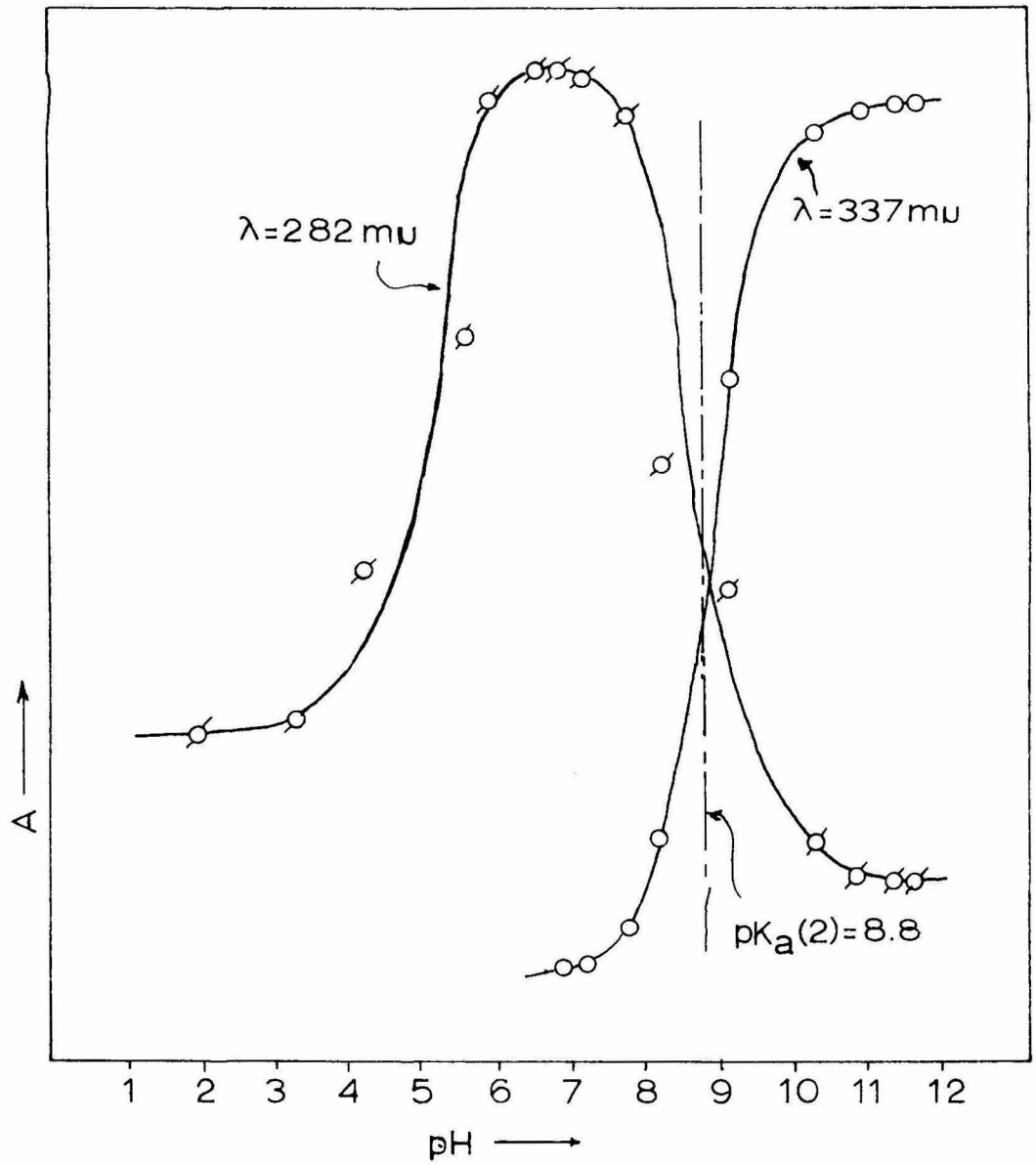


Figure 16.

Determining the second ionization constant of 3,5-dimethyl-2,6-dihydroxybenzoquinone.

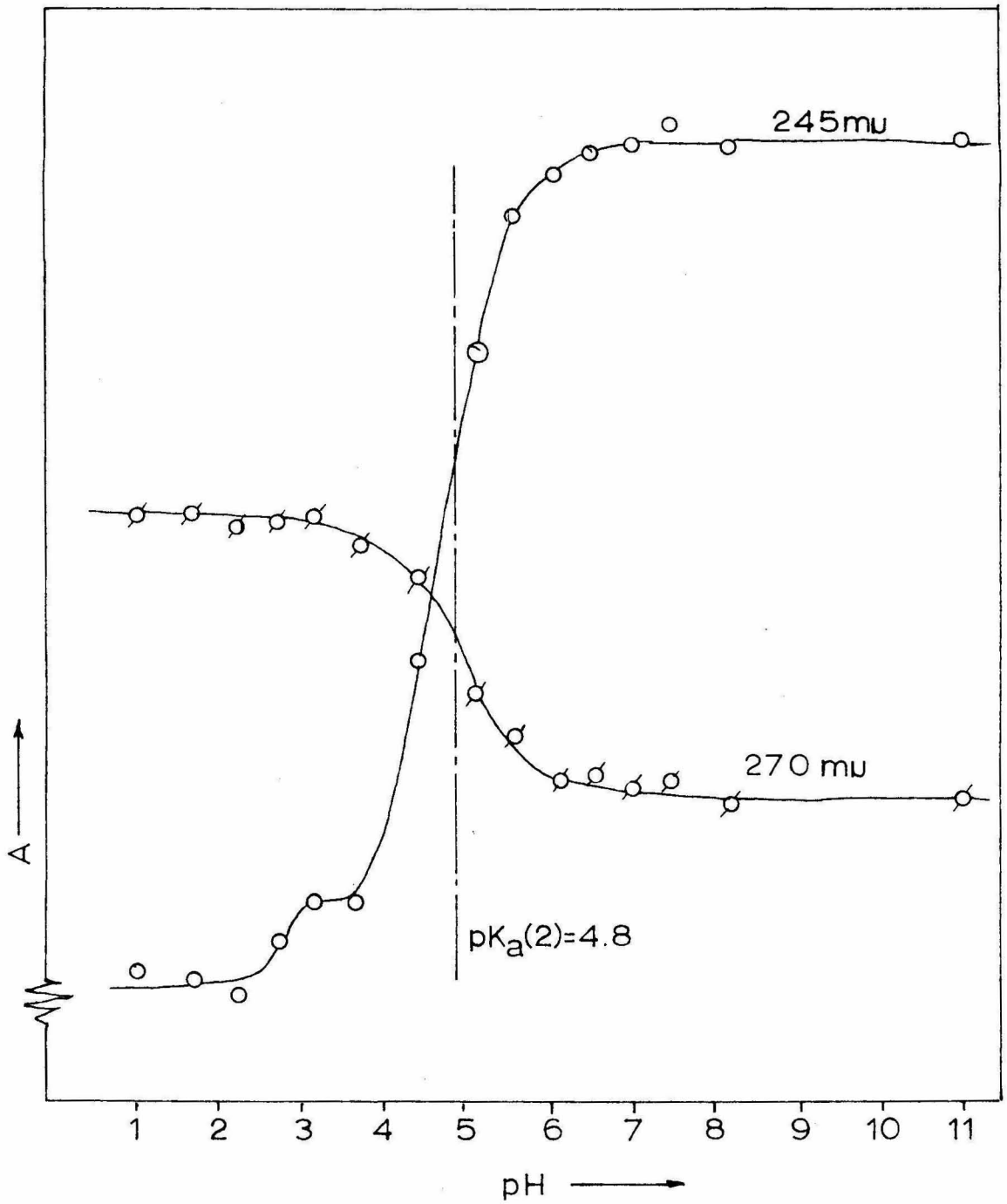


Figure 17.

Determining the second ionization constant of the compound $C_{11}H_{12}O_4$ which results from the degradation of nidulin.

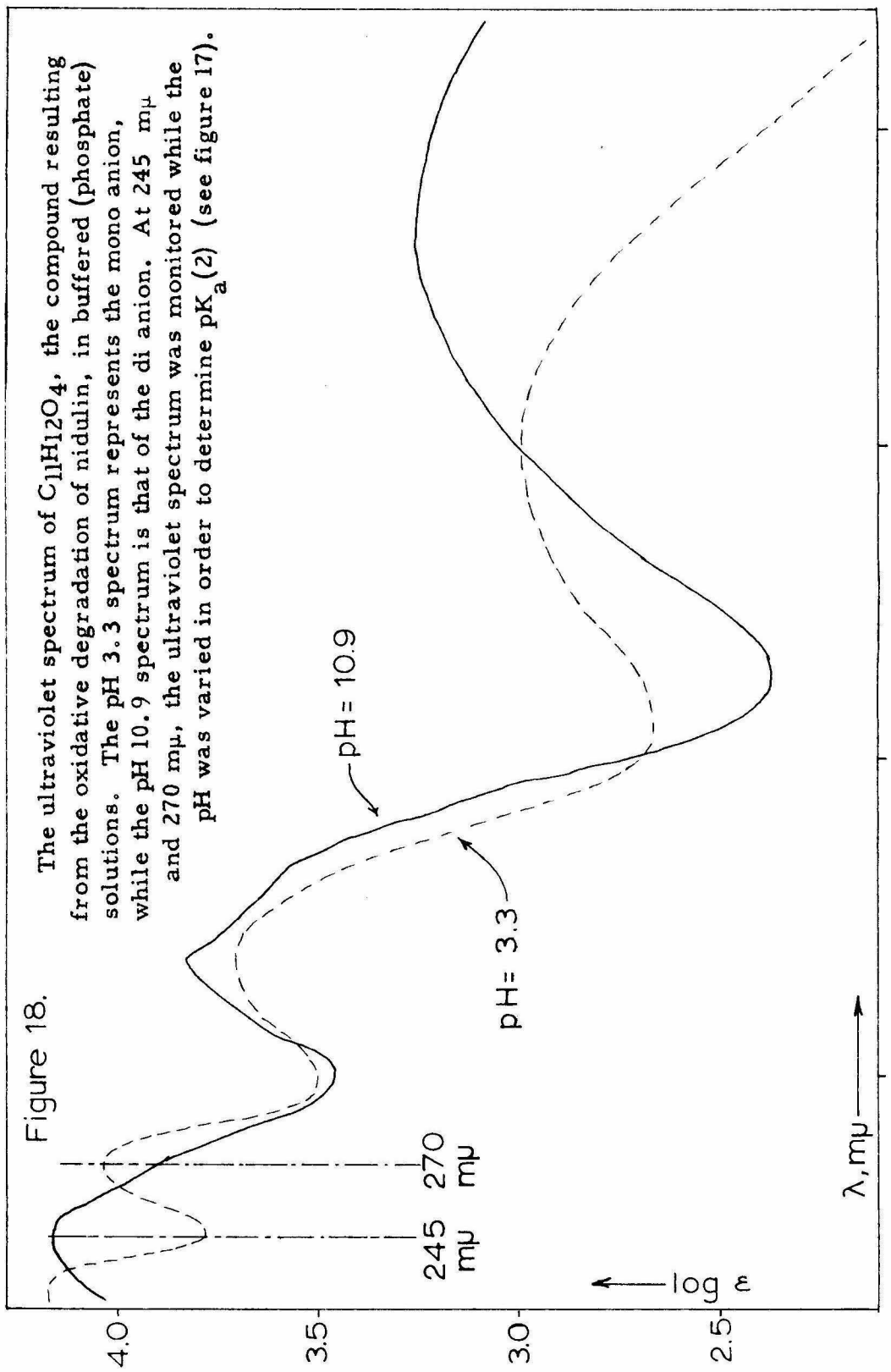


Figure 18. The ultraviolet spectrum of $C_{11}H_{12}O_4$, the compound resulting from the oxidative degradation of nidulin, in buffered (phosphate) solutions. The pH 3.3 spectrum represents the mono anion, while the pH 10.9 spectrum is that of the di anion. At 245 mμ and 270 mμ, the ultraviolet spectrum was monitored while the pH was varied in order to determine $pK_a(2)$ (see figure 17).

Preparation and counting of decarbonidulin: Nidulin (500 mg, 1.13 mmol, 0.4594 ± .0032 μc/mmol) was dissolved in 4 ml of dioxan. The dioxan solution was added to 25 ml of hot aqueous sodium hydroxide (100 ml, 2 N) slowly, so that no permanent precipitate formed. A trap was arranged so that the exit gases from the reaction mixture would be washed in an alkaline solution to remove any radioactive carbon dioxide they might contain before they were vented to the atmosphere. After the alkaline solution was refluxed for eight hours, it was acidified with dilute sulfuric acid and stirred for three hours 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 radioactive decarbonidulin were thus obtained, mp 145.5 - 147.5° (lit. (5), 148-149°). The level of activity in this material was assayed at 0.3872 ± .0039 μc/mmol. Subsequent recrystallizations changed neither the melting point nor the specific activity of this material.

Preparation and counting of methyl 4,6-dichloroeverinate: Nidulin (500 mg, 1.13 mmol, 0.4594 ± .0032 μc/mmol) was dissolved in dioxan (4 ml). The dioxan 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 with a steam bath was continued for five minutes 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 of nidulinic acid was treated with an excess of an ethereal diazomethane solution. After fifteen minutes' standing, a little acetic acid was added to decompose the residual diazomethane, and the solvent was evaporated in a stream of nitrogen.

The residue, presumably all methyl O-methylnidulinate, was taken up in glacial acetic acid (50 ml). Five drops of concentrated nitric acid were added to the solution, the color of which changed within a few minutes through yellow to a bright cherry red. After fifteen minutes 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 (2N). 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 yellowish needles mp 80-1/2 - 81°. Recrystallization raised the melting point to 82-82-1/2° (lit. (5), 82-83°). The specific activity of the recrystallized methyl 4,6-dichloroeverinate was 0.2244 $\pm$ .0052 μc/mmol.

The isoleucine experiment:

The growth of A. nidulans on a medium containing uniformly labelled isoleucine-^{14}C and the isolation of the nidulin so produced:

Four one liter flask colonies of A. nidulans were inoculated with 0.33 mc of uniformly labelled isoleucine-^{14}C (Merck radiochemicals, lab. #59 CD 768). After forty-six days' growth, the mycelia were dried, pulverized and continuously extracted with hexane. The hexane extract residues were combined with non-radioactive nidulin (1.00 gm) and chromatographed on a silicic acid-Supercel (7:3) column, using methylene chloride as an eluent. The central fractions of the nidulin band were combined and crystallized from heptane, giving nidulin having a specific activity of 2.80 μc/mmol. The incorporation of activity was estimated at 4%. A portion of the nidulin thus obtained was diluted with some non-radioactive material to give, after one crystallization from heptane, large glistening needles (mp 177-179°) having a specific activity 0.4306 ± 0.0035 μc/mmol.

Preparation and counting of methyl 4,6-dichloroeverinate:

Nidulin (500 mg, 1.13 mmol, 0.4306 ± .0035 μc/mmol) was hydrolyzed, methylated and cleaved with nitric acid in acetic acid exactly as described previously for the isolation of ring A in the acetate experiment. The purified methyl 4,6-dichloroeverinate (mp 82.0-82.7°) had a specific activity of 0.0576 ± 0.0005 μc/mmol.

REFERENCES

1. J. Kurung, Science, 102, 11 (1945).

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

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

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

5. F. M. Dean, J. C. Roberts and A. Robertson, J. Chem. Soc., 1954, 1432.

6. T. J. Nolan, J. Algar, E. P. McCann, W. A. Manahan and N. Nolan, Sci. Proc., Roy. Dublin Soc., 24, 319 (1948).

7. F. M. Dean, A. D. T. Erni and A. Robertson, J. Chem. Soc., 1956, 3545.

8. D. S. Noyce, personal communication.

9. W. B. Emery, Chem. & Ind., 1952, 254 (a review).

10. E. C. Kornfeld, R. G. Jones and T. V. Parke, J. Am. Chem. Soc., 71, 150 (1949).

11. N. A. Krasnil'nikov, et al., Mikrobiologia, 26, 418-25 (1957).

12. D. K. Miller and A. C. Rekate, Science, 100, 172 (1944).

13. I. N. Asheshov and F. Strelitz, Science, 101, 119-120 (1945).

14. J. G. Kidd, Science, 105, 511 (1947).

15. R. B. Woodward, J. R. Johnson, et al., J. Am. Chem. Soc., 80, 1001 (1958).

16. C. Thom and K. B. Raper, A Manual of the Aspergilli, The Williams and Watkins Co., Baltimore (1945).

17. Ibid., p. 87.

18. Ibid., p. 156.

19. R. R. Fraser, Can. J. Chem., 38, 549 (1960).

20. J. H. Richards and W. F. Beach, J. Org. Chem., 26, 623 (1961).

21. E. A. Braude and F. C. Nachod, Determination of Organic Structures by Physical Methods, Academic Press, Inc., New York (1955), p. 175.

22. C. S. Marvel, Organic Chemistry, an Advanced Treatise, H. Gilman, ed., John Wiley & Sons, Inc., New York (1943), p. 444.

23. L. Pauling, The Nature of the Chemical Bond, Cornell University Press, Ithaca, N. Y. (1960), 3rd edition, p. 490.

24. a) A. W. Baker, J. Am. Chem. Soc., 80, 3598 (1958).
b) A. W. Baker and W. A. Kaeding, J. Am. Chem. Soc., 81, 5904 (1959).

25. N. A. Puttnam, J. Chem. Soc., 1960, 5101.

26. A. W. Baker and A. T. Shulgin, J. Am. Chem. Soc., 80, 5358 (1958).

27. R. M. Badger, J. Chem. Phys., 5, 857 (1937).

28. F. M. Dean, D. S. Deorha, A. D. T. Erni, D. W. Hughes and J. C. Roberts, J. Chem. Soc., 1960, 4831.

29. V. E. Davidson, J. Keane and T. J. Nolan, Sci. Proc., Roy. Dublin Soc., 23, 160 (1943).

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

31. J. N. Collie, J. Chem. Soc., 91, 1806 (1907).

32. A. J. Birch and F. W. Donovan, Australian J. Chem., 6, 360 (1953).

33. (a) A. J. Birch and F. W. Donovan, Australian J. Chem., 6, 373 (1953);
(b) A. J. Birch, A. R. Penfold and P. Elliott, Australian J. Chem., 7, 169 (1954).
(c) A. J. Birch and F. W. Donovan, Australian J. Chem., 8, 529 (1955).
(d) A. J. Birch, L. Bauer and A. J. Ryan, Australian J. Chem., 8, 534 (1955).
(e) A. J. Birch and R. A. Massy-Westropp, J. Chem. Soc. 1957, 2215.

34. A. J. Birch, R. A. Massy-Westropp and C. J. Moye, Australian J. Chem., 8, 539 (1955).

35. (a) A. J. Birch, R. A. Massy-Westropp and R. W. Rickards, J. Chem. Soc., 1956, 3717.
(b) A. J. Birch, R. J. English, R. A. Massy-Westropp and H. Smith, J. Chem. Soc., 1958, 369.
(c) A. J. Birch, R. J. English, R. A. Massy-Westropp, M. Slaytor and H. Smith, J. Chem. Soc., 1958, 360.
(d) A. J. Birch, R. A. Massy-Westropp, R. W. Rickards and H. Smith, J. Chem. Soc., 1958, 360.

36. A. J. Birch, G. E. Blance and H. Smith, J. Chem. Soc., 1958, 4582.

37. R. Bentley and J. G. Keil, Proc. Chem. Soc., 1961, 111.

38. J. D. Bu'Lock and H. M. Smalley, Proc. Chem. Soc., 1961, 209.

39. G. T. Newbold, W. Sharp and F. S. Spring, J. Chem. Soc., 1951, 2679.

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

41. A. White, P. Handler, E. L. Smith and D. Setton, Principles of Biochemistry, The Maple Press Co., York, Pa., (1954), p. 578.

42. W. G. Robinson, B. K. Bachawat and M. J. Coon, J. Biol. Chem., 218, 391 (1956).

43. A. J. Birch, P. Fitton, E. Pride, A. J. Ryan, H. Smith and W. B. Whalley, J. Chem. Soc., 1958, 4576.

44. J. Polonsky, Bull. soc. chim. France, 1957, 1079.

45. I. M. Kolthoff, Pharm. Weekblad, 69, 1159 (1932); cf. C.A. 27, 280.

46. F. G. Bordwell and P. S. Lands, J. Am. Chem. Soc., 79, 1596 (1957).

47. D. J. Cram, J. Am. Chem. Soc., 74, 2137 (1952).

48. H. G. Walker and C. R. Hauser, J. Am. Chem. Soc., 68, 1386-88 (1946).

49. (a) J. D. Roberts, An Introduction to Spin-Spin Splitting in High Resolution Nuclear Magnetic Resonance Spectra, W. A. Benjamin, Inc., New York (1961), Chapter 4.
(b) J. A. Pople, W. G. Schneider and H. J. Bernstein, High-resolution Nuclear Magnetic Resonance, McGraw-Hill Book Company, Inc., New York (1959), pp. 138-151.

50. R. G. Jones and H. A. Shonle, J. Am. Chem. Soc., 67, 1034 (1945).

51. V. Ettel, J. Pospisil and J. Bauer, Chem. Listy, 51, 505 (1957); cf. C. A., 51, 10413i (1957).

52. L. A. Burkardt, J. Phys. Chem., 61, 502 (1957).

53. H. Brunmayr, Monatsch., 19, 237 (1898).

54. H. Brunmayr, Monatsch., 21, 1 (1900).

55. B. J. Thamer and A. F. Voigt, J. Phys. Chem., 56, 225 (1952).

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

57. For a recent commentary with references, see R. A. Hoffman, Arkiv. for Kemi, 17, 1 (1961).

58. (a) S. J. Wakil, J. Am. Chem. Soc., 80, 6465 (1958).
(b) S. J. Wakil, L. W. McLain and J. B. Warshaw, J. Biol. Chem., 235, PC31 (1960).
(c) S. J. Wakil and J. Ganguly, J. Am. Chem. Soc., 81, 2597 (1959).
(d) R. O. Brady, Proc. Nat. Acad. Sci., 44, 993 (1958).

59. A. J. Birch, R. J. English, R. A. Massy-Westropp, M. Slaytor and H. Smith, J. Chem. Soc., 1958, 365.

60. D. H. R. Barton, A. M. Deflorin and O. E. Edwards, J. Chem. Soc., 1956, 530.

PROPOSITION 1

Asperthecin (1, 2), a pigment isolated from two strains of Aspergillus quadrilineatus, is a particularly interesting substance because the color of its solution in aqueous 0.5 M sodium bicarbonate closely resembles the characteristic purple-red color of the perithecia and ascospores of A. quadrilineatus, and, for that matter, a number of the other species of the A. nidulans group.

Asperthecin, $C_{15}H_{10}O_8$, does not melt below 370°, and contains neither methoxyl nor C-methyl groupings. It forms a hexaacetate by treatment with acetic anhydride and a trace of sulfuric acid. Prolonged methylation with methyl sulfate and potassium carbonate in acetone affords the pentamethyl derivative, which is insoluble in alkali. Asperthecin dissolves readily in aqueous bicarbonate, although it is nearly insoluble in water. Alkaline solutions of asperthecin are increasingly sensitive to oxidative decomposition as the alkalinity is increased: the initially purple solution in aqueous sodium hydroxide fades in a few minutes to orange, becoming colorless overnight.

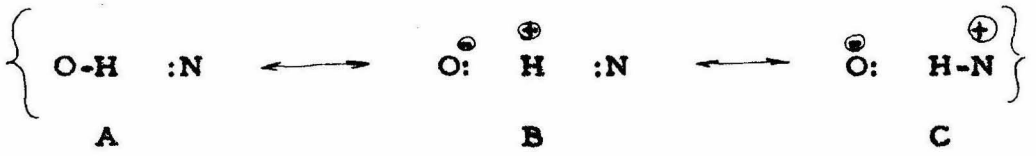
When asperthecin is subjected to a zinc dust distillation, 2-methylanthracene is recovered, which contains all of the original carbon. The origin of the 2-methyl group is interpreted as being a methylol ($-CH_2OH$) group, since the sixth hydroxyl group of asperthecin can be acetylated but cannot be methylated.

Upon treatment of asperthecin with hydriodic acid in acetic acid solution in the presence of red phosphorous, a reduction product is formed which gives emodin (I) on oxidation with chromic acid in acetic acid solution.

Oxidation of the pentamethyl ether of asperthecin with potassium permanganate produced a white crystalline material which was proven to be 5-carboxy-3,4-dimethoxyphthalic anhydride (II) by synthesis. This experiment fixes the position of one of the remaining hydroxyl groups, and leaves a two-fold ambiguity of III and IV for the structure of asperthecin.

Recent observations (3) in the hydroxyl stretching frequency region (three microns) of the infrared have indicated that the absorptions of a molecule in this region are an excellent indication of the surroundings of the phenolic groups that it contains. For example, a hydroxyl adjacent to an oxygen function such as an ether or another hydroxyl group absorbs at about 3570 cm^{-1}. On the other hand, the much stronger hydrogen bonding of a hydroxyl group to a carbonyl oxygen atom is eflected in a much greater bathochromic shift in the OH stretching frequency: 3,6-dimethyl-2,5-dihydroxybenzoquinone absorbs at 3380 cm^{-1} and 3,6-diethyl-2,5-dihydroxybenzoquinone absorbs at 3360 cm^{-1}(4).

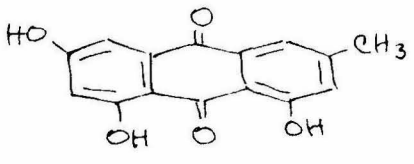
The hydrogen bond may be thought of as involving valence-bond resonance forms of the following kinds (5):



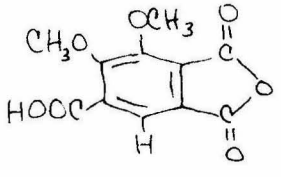
A B C

If the hydrogen bond proton acceptor atom (:N) is acting as such for two hydroxyl groups, the degree with which it interacts with each hydroxyl group will be lessened due to the charge transfer resonance form C. Thus the hydroxyl stretching frequency of 3,5-dimethyl-2,6-dihydroxybenzoquinone would be expected to be higher than that of its 2,5-isomer. The observed frequency is 3473 cm^{-1}(4).

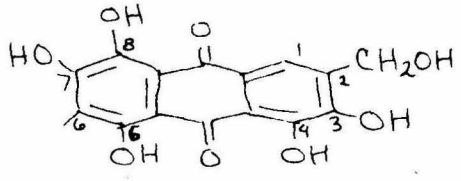
It is proposed that the infrared spectrum of asperthecin be examined. Structures III and IV should be readily distinguishable in that IV should have no absorption in the 3370 cm^{-1} region, while III should absorb near that frequency due to its 8 hydroxyl group.



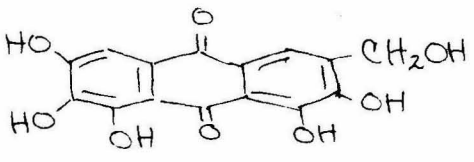
I. Emodin



II



III



IV

References:

1. S. Neelakantan, A. Pocker and H. Raistrick, Biochem. J., 66, 234 (1957).

2. B. H. Howard and H. Raistrick, Biochem. J., 59, 475 (1955).

3. For leading references, see this thesis, part 4.

4. W. Beach, unpublished data.

5. L. Pauling, The Nature of the Chemical Bond, Cornell University Press, Ithaca, N. Y., 3rd edition (1960), p. 452.

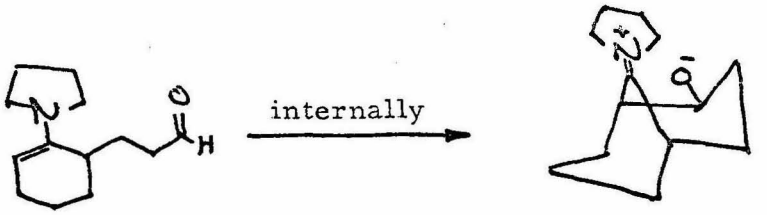
PROPOSITION 2

Stork (1) has described a novel ring enlargement sequence involving the reaction of the pyrrolidine enamines of cyclic ketones with carolein. The product of this reaction is a keto-amine which is converted via a Hofmann elimination to a cycloalkene carboxylic acid, thus expanding the ring by two carbon atoms.

The structure proposed by Stork for the keto-amine is I. The evidence presented in his note favored this structure, and this structure would lead directly to the observed cyclooctenecarboxylic acid (from cyclohexanone). No argument with the assignment will be attempted here.

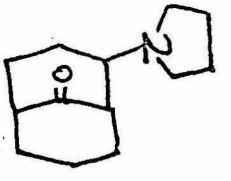
It is well known that enamines are carbon nucleophiles. They react in most cases as if $\{\overset{+}{N}=C-\overset{-}{C}\}$ were the major resonance form. Keeping this fact in mind, along with the assigned structure of the reaction product, we are led through the following reaction sequence:

1. 

2. 

The product of the second step is a Zwitterion which cannot enolize, for each of the α-carbon atoms is a bridgehead carbon. The conversion of the Zwitterion into I is not easily explained. Topologically speaking, the path must belong to one of two distinguishable mechanistic types. In the first, the heterofunctions would exchange positions. Alternatively, there is the possibility that a skeletal rearrangement occurs, leaving the heterofunctions attached to their original carbon atoms. The first type of mechanism would predict that the keto carbon atom of cyclohexanone is not converted into the carboxylate carbon atom of the product, while the second type of mechanism would not lead to this prediction of exchange.

It is proposed that the reaction be investigated with the use of cyclohexanone-C^{14}. If the carboxylate group of the product is radioactive, the interchanging of heterofunctions during the reaction is eliminated as a possible mechanism.

I 

References

1. G. Stork, J. Am. Chem. Soc., 78, 5129 (1956).

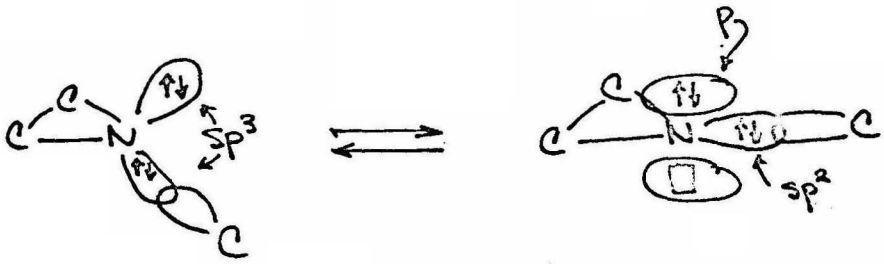
PROPOSITION 3

The energy barrier associated with the inversion of the amine nitrogen atom can be explored rather uniquely by NMR spectroscopy. If the rate of inversion is measured as a function of temperature, the activation energy for the inversion is derived from its temperature dependence by the usual methods (1, 2, 3, 4). This technique has been used with some success as refinements in instrumentation have been made available.

Aziridines were the first class of compounds to be examined by this technique because it was felt that the strain associated with the three-membered ring might increase the energy barrier of inversion. The magnitude of the barrier proved insufficient to allow the resolution of an aziridine into its optical antipodes.

Many oxaziranes have been successfully prepared by Emmons (5). Oxaziranes are the class of compounds which contains the three-membered carbon-oxygen-nitrogen ring. It is proposed that the inversion of the nitrogen atom in certain oxaziranes be studied. The results of such a study would be especially meaningful when they are compared with the results obtained with the aziridine system.

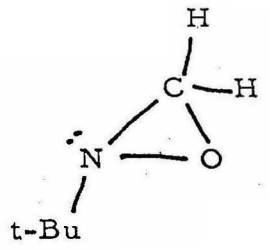
A plausible mechanism for the inversion of a nitrogen atom may be expressed as follows:



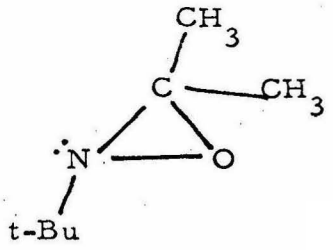
If either of the other members of the three-membered ring possesses electrons in p-orbitals, an interaction may occur which may change the energy of the transition state with respect to the ground state. For example, in the compound methylene aziridine, the electrons of the planar transition state can be delocalized in a molecular orbital which is isoelectronic with the allyl anion. Therefore the transition state is stabilized and the barrier to inversion is lowered. The prediction is in agreement with experimental observation (1). On the other hand, if the ring contains an oxygen atom, such as is found in the oxaziranes, the simple molecular orbital treatment gives the prediction that no stabilization of the transition will occur, for the pi-electron system of the planar transition state is isoelectronic with the ethylene dianion. Hence it is predicted that the barrier to inversion in oxaziranes should not be lowered due to the presence of oxygen in the ring.

A study of the barrier to nitrogen inversion in oxaziranes might be investigated with either I or II initially, each of which are known compounds (5). Compound I is known to possess (at room temperature) an inversion rate that is slower than the NMR "shutter speed" (5). This is in general agreement with the above elementary molecular orbital

predictions, and will serve as a starting point for further investigations.



I



II

References

1. A. Loewenstein, J. Neumer and J. D. Roberts, J. Am. Chem. Soc., 82, 3599 (1960).

2. J. D. Roberts and A. T. Bottini, J. Am. Chem. Soc., 80, 5203 (1958).

3. J. D. Roberts and A. T. Bottini, J. Am. Chem. Soc., 78, 5123 (1956).

4. H. S. Gutowsky, Ann. N. Y. Acad. Sci., 70, 786 (1956).

5. W. D. Emmons, J. Am. Chem. Soc., 79, 5739 (1957).

6. See A. Streitwieser, Molecular Orbital Theory, J. Wiley and Sons, New York (1961), pp. 117-135, for the treatment of heterofunctions.

PROPOSITION 4

Calophyllium inophyllium (1) is a medium-sized tree indigenous to lands in and surrounding the South Pacific. Sometimes known as Alexandrian laurel, it bears a small fruit containing a large pit. It is the pit or nut which is of considerable economic importance in India, for it is the source of domba oil, which is used extensively for lighting purposes. The oil of the nut has also been used by the natives of Madagascar in the treatment of leprosy (2b).

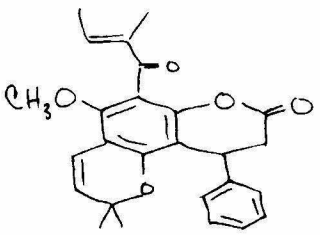
From the nut has been isolated three substances: calophyllolide, inophyllolide and calophyllic acid. Their structures, variations on the same theme, have been demonstrated to be I, II, and III, respectively, largely due to the work of Polonsky (2).

The structure of calophyllolide suggests an interesting biogenesis. The central phloroglucinol nucleus is formed in the usual manner. Owing to its tautomeric keto forms, phloroglucinol may act as a nucleophile at each of the un-oxygenated ring carbons. It is quite probable, then, that the non-aromatic side chains are attached by the nucleophilic attack of phloroglucinol on an oxidized form of isoleucine and leucine at the position which was the α-carbon of the original amino acid.

The attachment of the third side chain is a little more obscure. Perhaps a product of the amino acid phenylalanine is attacked at the α-carbon nucleophilically by phloroglucinol. The usual function of amino

acids in biogenesis is supplying the corresponding aldehyde or amine through decarboxylation (3). In this case, however, the carboxylate group is apparently retained.

In order that the above ideas be tested, it is proposed that the biogenesis of calophyllolide be studied by the use of appropriate labelled substrates.



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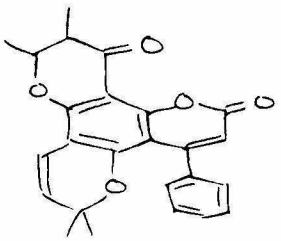

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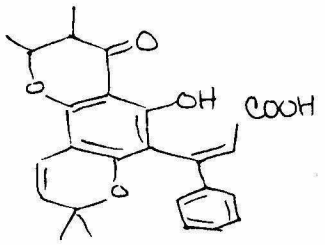


III

References:

1. The Standard Cyclopedia of Horticulture, L. H. Bailey, Ed., The Macmillan Co., New York (1939), p. 636.

2. a) J. Polonsky and Z. Baskevich, Bull. Soc. Chim. de France, 1958, 929.
b) J. Polonsky, Bull. Soc. Chim. de France, 1957, 456.
c) " " " " " " " 1957, 1079.
d) " " " " " " " 1956, 914.
e) " " " " " " " 1955, 541
f) J. Polonsky and E. Lederer, Bull. Soc. Chim. de France, 1954, 924.
g) P. Dietrich, E. Lederer and J. Polonsky, Bull. Soc. Chim. de France, 1953, 546.
h) A. Ormancy-Potier, A. Busas and E. Lederer, Bull. Soc. Chim. de France, 1951, 577.

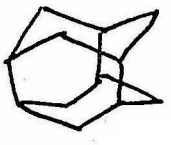
3. K. W. Bentley, The Alkaloids, Interscience Pub. Co., Inc., New York (1957), Chapter 11.

PROPOSITION 5

The presently unknown tetracyclic hydrocarbon I should be of substantial interest to those who investigate the chemistry of bridgehead carbon atoms. There is little doubt that it could exist, for models of the system appear to be practically strain free. Its synthesis would place it beside adamantane (II), which presently is the sole member of the class of highly symmetric, saturated, polycyclic hydrocarbons.

The recent synthesis of II (1, 2) by the isomerization of tetrahydrodicyclopentadiene (III) with a Lewis acid under the proper conditions might lead one to consider a similar route to I. However, a tetracyclic C_{12} system of the type that could be isomerized to I might well be more difficult to prepare than I itself.

A more promising route to I is based on the results derived from a study of the condensation of allene dimer with maleic anhydride (3, 4). Allene dimer, or 1,2-dimethylenecyclobutane (IV), will condense with two moles of maleic anhydride to produce a good yield of Δ-9,10-octahydro-2,3,6,7-naphthalenetetracarboxylic acid dianhydride (V).



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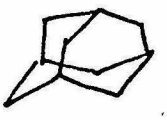

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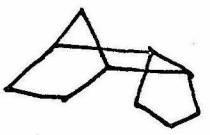
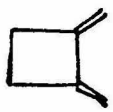


III

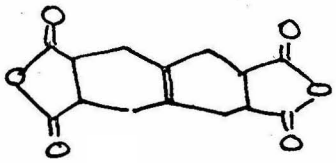
The formation of V in this manner might lead to either of two

isomeric forms: cis and trans. There is no reason to expect the predominance of either isomer, based on the currently accepted reaction mechanism. Whether the molecule's configuration is cis or trans, its most stable conformation is expected to be the linear extension (Va, b). Therefore, no difficulty is expected to be encountered in the hydrogenation of the 9, 10 double bond of V, the first uninvestigated step of the proposed synthesis of I. Catalytic hydrogenation of double bonds is well known to proceed in such a way as to give cis ring junctures in similar systems (5).

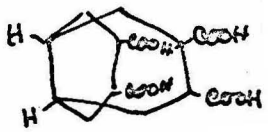
It is further proposed that the hydrogenated dianhydride VI be converted into the tetracycle VII by means of a metallic salt pyrolysis. Such pyrolyses, unlike the operationally equivalent Dieckman condensation, are not dependent on the formation of a β-keto ester which can produce a stable anion by enolization. The pyrolysis is driven to completion by removal of the reaction product from the reaction medium, an end which is usually effected by decarboxylation of the β-ketoacid formed in the condensation. In the case of VI, however, decarboxylation is not likely to occur, for it would require that a double bond be formed at a bridgehead, and because of this constitutes a violation of Bredt's rule (7). In this case, the removal of the pyrolysis product of VI from the reaction mixture will proceed in a novel manner: by the formation and volatilization of VII.



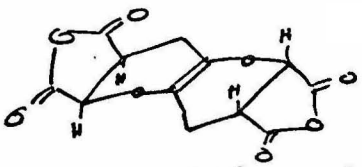
IV



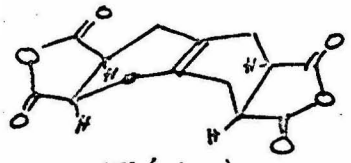
V



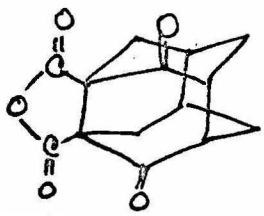
VI



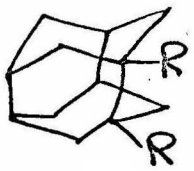
Va(trans-)



Vb(cis-)



VII



VIII a - R=COOH

b - R=Br

The tetracyclic pyrolysis product can be converted into experimentally useful compounds after the keto functions are removed reductively. Using the Hunsdeicker reaction (8) one can convert the dianhydride VII through the diacid VIIIa into the dibromide VIIIb. If the hydrocarbon is desired, it may also be easily obtained by a hydrogenation (Raney nickel) of the dibromide.

One of the major interests in this ring system would be for the study of reactions at bridgeheads (10). The formation of a carbonium ion

at a bridgehead in I should be more favorable than that in bicyclo(2,2,1)-heptane or even in bicyclo(2,2,2)octane. The latter system has been judged to have 22.5 Kcal./mole of strain in its carbonium ion, while the bicycloheptane system is thought to have a more strained carbonium ion. The more flexible system I should have considerably less strain in its carbonium ion than the bicyclooctane system, causing reactions which must proceed through the carbonium ion to be more rapid. A study of this sort would begin with an evaluation of the dibromide's reactivity toward two-step displacement reagents, such as aqueous silver nitrate. The significance of such a study would be to provide an example of a less-strained bridgehead carbonium ion to round out the studies which have been completed thus far.

References

1. P. von R. Schleyer and M. M. Donaldson, J. Am. Chem. Soc., 82, 4645 (1960).

2. Belgian patent (583,579) to the duPont Company.

3. A. T. Blomquist and J. A. Verdol, J. Am. Chem. Soc., 78, 109 (1956).

4. K. Alder and O. Ackermann, Ber., 87, 1567 (1954).

5. R. P. Linstead, W. E. Doering, S. B. Davis, P. Levine and R. R. Whetstone, J. Am. Chem. Soc., 64, 1985 (1942).

6. R. C. Fuson, Organic Chemistry, an Advanced Treatise, Ed. by H. Gilman, J. Wiley and Sons, Inc., New York (2nd ed.) (1953), p. 81.

7. H. H. Wasserman, Steric Effects in Organic Chemistry, Ed. by M. S. Newman, J. Wiley and Sons, Inc., New York (1956), p. 353.

8. Reference 7, p. 354.

9. C. A. Grob, M. Ohta, E. Renk and A. Weis, Helv. Chim. Acta, 41, 1191 (1958).

10. D. E. Applequist and J. D. Roberts, Chem. Reviews, 1954, 1065-1089.

11. P. D. Bartlett and L. H. Knox, J. Am. Chem. Soc., 61, 3184 (1939).