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DEFENSIVE CHEMISTRY OF NAVANAX AND RELATED OPISTHOBRANCH MOLLUSCS

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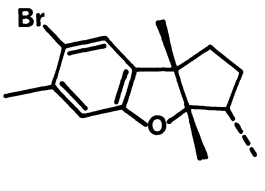
Abstract - The strategies of chemical defense in marine opisthobranchs are reviewed, emphasizing the accumulation of secondary compounds through the food chain and the production of defensive compounds in specialized glands. The structures of several new compounds from Aplysia brasiliana, Bursatella leachii, and Navanax inermis are presented.

INTRODUCTION

It seems clear that the majority of the marine molluscs (Phylum Mollusca) owes its enhanced survival to the evolution of protective outer shells. A subclass of the molluscs, the opisthobranchs (Opisthobranchia), has, however, evolved as uncoiled soft-bodied invertebrates, lacking the characteristic external shell but frequently possessing a minor internal shell vestige. In lieu of physical protection, many of the opisthobranchs appear to have evolved to use toxic and/or noxious organic compounds as defensive agents. Two strategies seem to exist, one involving the use of compounds produced by marine algae and concentrated in the animal through intensive grazing (Ref. 1), and the other a specific glandular production, storage, and secretion (Ref. 2).

CONCENTRATION OF SECONDARY COMPOUNDS THROUGH THE FOOD CHAIN

The herbivorous sea hares (opisthobranchs of the Order Anaspidea) are now well known to prefer grazing on marine algae which produce unusual secondary metabolites. Early work in Japan with the digestive gland components of Aplysia kurodai resulted in the isolation and structure elucidation of the brominated compounds aplysin (1) and aplysin-20 (2) (Refs. 3,4). Taken with the early work of Irie, this observation suggested that A. kurodai had been consuming red algae of the genus Laurencia. Subsequent studies with



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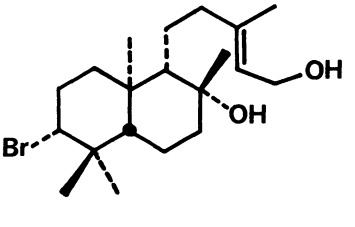

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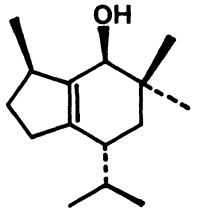
various Aplysia species, particularly the investigation of A. californica by Stallard and Faulkner (Refs. 5,6), have confirmed both a distinct feeding preference for Laurencia and Plocamium species and a very efficient accumulation in the digestive gland of the halogenated metabolites from these sources. Table 1 summarizes the subsequent studies of sea hares which concentrate red algal metabolites as apparent defensive strategies (Ref. 1).

TABLE 1. Anaspideans (Sea Hares) which concentrate red algal metabolites

Anaspidean (location)	Compounds observed	Apparent dietary sources
Aplysia californica (Calif.) (Refs. 5,6)	halogenated mono- and sesquiterpenoids	Laurencia and Plocamium spp.
A. kurodai (Japan) (Refs. 3,4)	halogenated sesqui- and diterpenoids	Laurencia spp.
A. dactylomela (Carib.) (Refs. 7,8,9)	halogenated C_{15} non-terpenoids and rearranged sesquiterpenoids	Laurencia spp. L. poitei
A. limacina (Med.) (Ref. 10)	halogenated monoterpenoids	Plocamium cartilagineum
A. brasiliana (Carib.) (Refs. 11,12)	irregular sesquiterpenoids and halogenated C_{15} non-terpenoids	Laurencia spp. L. obtusa
A. parvula (Gulf of Calif.) (Ref. 13)	halogenated diterpenoids	L. irieii

While field observations indicate that sea hares lack significant predators, it remains to be proven that their enhanced survival is a consequence of the digestive gland components acting as defensive chemicals. In recent work, we and the Cornell group (Refs. 11,14) have investigated the digestive gland components of Aplysia brasiliana, collected both in the Florida Keys and in Southern Texas (Ref. 14). A. brasiliana was a logical choice for study, since this swimming sea hare is conceptually easily preyed upon, but has been reported to lack significant predators (Ref. 15). From the animals collected in Texas, we have isolated the rearranged sesquiterpenoids brasilenol (3) and epibrasilenol (4) (Ref. 12), as well as the new 9-membered ring ether brasilenyne (5) (Ref. 14).



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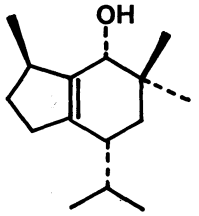

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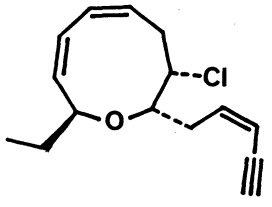

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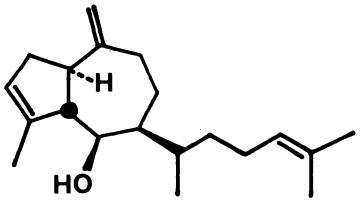
Eisner and his colleagues have subsequently shown that brasilenyne (5), as well as many of the Laurencia-derived compounds concentrated by Aplysia spp., is a potent feeding deterrent to fresh water fish.

Certain anaspideans also appear to exercise feeding preferences for the brown algae and particularly for those members of the family Dictyotaceae which are known to produce non-halogenated diterpenoids. Vanderah and Faulkner (Ref. 16) have isolated the diterpenoid pachydictyol A (6) from digestive glands of the pacific sea hare Aplysia vaccaria. It appears that A. vaccaria concentrates this diterpenoid by grazing on the brown alga

Pachydictyon coriaceum or on various related Dictyota species known to contain pachydictyol A (Ref. 17). A similar situation exists in the Mediterranean Sea with A. depilans, which grazes upon Dictyota dichotoma and concentrates several diterpenoids, the major component being the pachydictyol A-related structure dictyol A (7) (Refs. 18,19).



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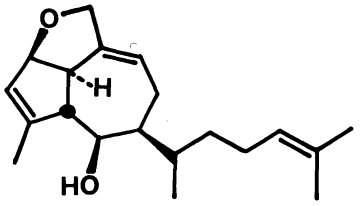

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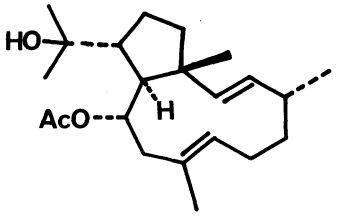
Another group of anaspideans of the genus Dolabella has been the subject of two recent and independent studies. From the digestive glands of the Gulf of California sea hare D. californica, Ireland and Faulkner isolated and described a series of new diterpenoids (the dolabelladienes), based upon an X-ray structure elucidation of compound 8 (Refs. 20,21). From D. auricularia from the Indian Ocean, the Pettit group isolated and described the related, but tricyclic, compound dolatriol 6-acetate (9), as well as the corresponding triol (10) (Ref. 22). Based upon the feeding behaviors of other anaspideans, the sources of 8 - 10 could be predicted as marine algae, but despite many studies, compounds of these structure types have not been isolated from marine flora.



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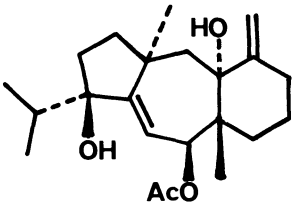

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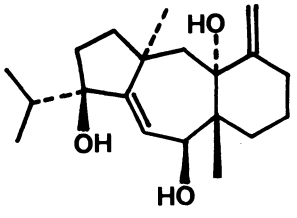

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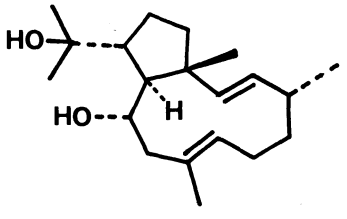
We have recently investigated the chemical components of the brown alga Glossophora galapagensis (Dictyotaceae) from the Galapagos Islands, and have isolated the dolabelladiene 8, as well as the related structures 11 and 12 (Ref. 23). Compounds 11 and 12 were also isolated from D. californica.



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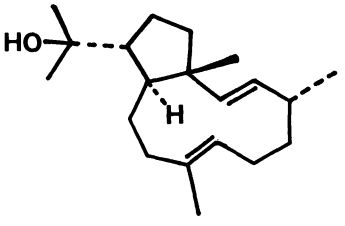

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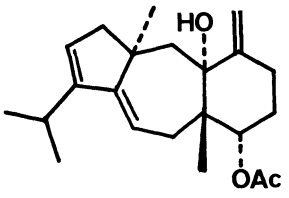
In addition, a recent study in this laboratory of the chemistry of the Caribbean brown alga Dictyota divaricata (Dictyotaceae) has led to the isolation of divarol acetate (13) and two related compounds 14 and 15 (Ref. 24). Compounds 14 and 15 were converted to divarol acetate which when hydrolysed yielded a crystalline diol. The structures of these compounds (relative stereochemistries only) were secured by an X-ray elucidation of



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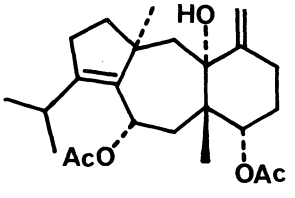

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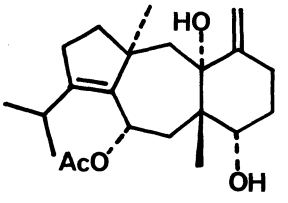

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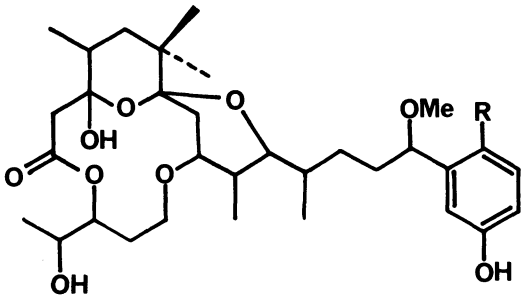
the diol. These tricyclic diterpenoids are, obviously, closely related to dolatriol 6-acetate. A series of compounds of the dolatriol ring system has recently been isolated from an Indopacific soft coral (Ref. 25). It is unlikely that the anaspideans obtain these compounds from corals, however, as they are well known to be exclusive herbivores. Hence, while circumstantial in nature, these studies clearly indicate that Dolabella californica and D. auricularia parallel Aplysia vaccaria and A. depilans in their feeding preferences, selecting from a closely related group of diterpenoid-producing brown algae of the family Dictyotaceae. These conclusions are summarized in Table 2.

TABLE 2. Anaspideans (Sea Hares) which concentrate brown algal metabolites

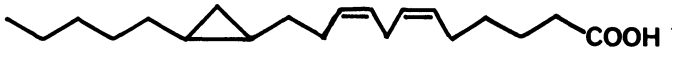
Anaspidean (location)	Compounds observed	Apparent dietary sources
Aplysia vaccaria (Calif.) (Ref. 16)	Pachydictyol A	Pachydictyon coriaceum or Dictyota spp.
A. depilans (Med.) (Refs. 18,19)	dictyols	Dictyota dichotoma
Dolabella californica (Gulf of Calif.) (Refs. 20,21)	dolabelladienes	Glossophora galapagensis or related Dictyotaceae
D. auricularia (Indian Ocean) (Ref. 22)	dolatriols	Dictyota spp. or related Dictyotaceae

The feeding preferences of the sea hares must, based upon recent observations, be expanded to include the blue-green algae. The Hawaiian sea hare Stylocheilus longicauda was found to contain the exceptional and toxic metabolite aplysiatoxin (16) (Refs. 26-28). Based on structural and biogenetic arguments, it seemed unlikely that 16 was of red or brown algal origin. Recent work by the Moore group has now shown that debromoaplysiatoxin (17) is a major component of several blue-green algae, particularly Lyngbya gracilis (Ref. 29).



16 R = Br aplysiatoxin
17 R = H debromoaplysiatoxin

A recent study in this laboratory of the digestive gland components of the sea hare Bursatella leachii, collected in marsh areas along the Texas coastline, seems to add confusion to the concepts of algal feeding preferences in the anaspideans. In this study, none of the aforementioned structure types were observed; instead, we isolated the C_{20} cyclopropane-containing fatty acid 18, which composed over 75% of the fatty acid content of the digestive gland (Ref. 30). Compound 18, which contains Δ^6 and Δ^9 olefins, is a unique fatty acid not yet found in marine algae. A possible source for polyunsaturated fatty acids, however, lies in the diatoms (Bacilliarophyceae) which produce large amounts of C_{18} - C_{22} trienyl through pentaenyl fatty acids including the C_{20} tetraenyl acid (Ref. 31), which are conceivable precursors to 18. The structure of 18 was secured by ozonation of the corresponding methyl ester to yield, after methylation, the cyclopropane-containing cleavage product 19. Reduction of 19 gave the primary alcohol 20, which by ^1H NMR analysis using incremental shift reagent, illustrated the cyclopropyl group to be at C-4 - C-5.

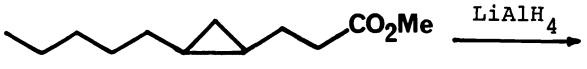


$C_{20}H_{34}O_2$

75% total fatty acids

18

1.) CH_2N_2
2.) O_3
3.) CH_2N_2



19 20

In summary, the majority of the anaspideans appears to have co-evolved with chemically noxious seaweeds as a strategy providing both a guaranteed food supply (by virtue of the general herbivore avoidance of these seaweeds) and an "accumulated" chemical defensive system. While not reviewed here, a similar situation involving food chain concentration of defensive compounds from both plant and animal prey is known for other opisthobranchs, particularly the dorid nudibranchs (Ref. 32), a sacoglossan (Ref. 33) and a notaspidean (Ref. 34).

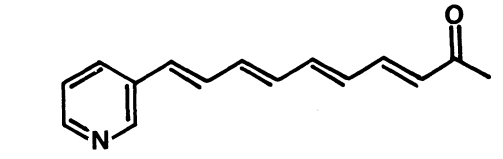
GLANDULAR PRODUCTION OF DEFENSIVE COMPOUNDS

The production of defensive compounds in specialized glandular tissues is a well known process in many opisthobranchs, particularly in the Orders Cephalispidea, Nudibranchia, and Onchidiaceae (Refs. 2,35).

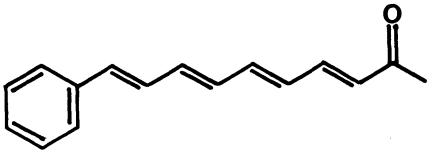
In recent studies in this laboratory , we observed that the ornately colored sea slug Navanax inermis (syn. Chelidonura inermis; Cephalaspidea) produces, when heavily molested, a bright yellow secretion from glandular tissue located near the anus of the animal. In their natural habitat, Navanax locate one another, for reproductive purposes, by following their own slime trails which are deposited as the animals crawl. We observed that when a trail-following Navanax encounters this yellow secretion, it expeditiously terminates trail-following behavior and turns at angles of greater than 90°, in avoidance of the secretion. We interpret this response as an intraspecific communication of danger (alarm pheromone) which diverts a migrating Navanax population away from areas of high predator populations.

An alternative explanation, which may require considerable experimentation to totally exclude, is that the yellow secretion is produced as a classical defensive strategy to discourage the predator. Perhaps the most compelling argument against this interpretation is the unfavorable location of the gland beneath the animal in a position which renders predator contact unlikely.

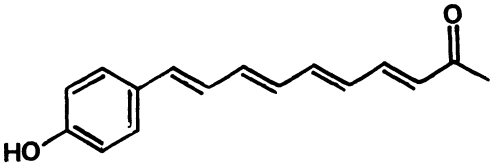
The composition of this interesting secretion was next investigated, and the three major compounds, described as the navenones A - C (21 - 23), were described in a preliminary communication based upon chemical transformations of navenone A and extensive ^{1}H and ^{13}C NMR studies (Ref. 36).



21 navenone A

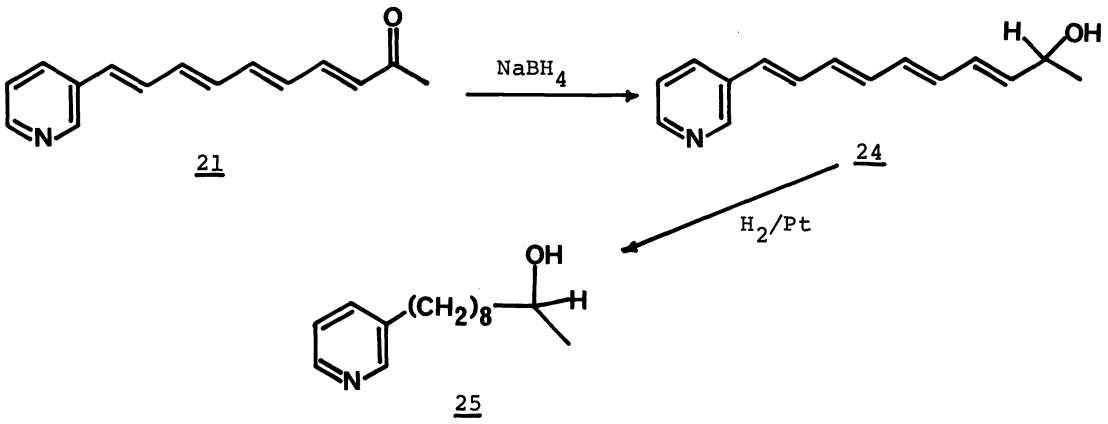


22 navenone B



23 navenone C

The major compound of the secretion, navenone A (21) was also reduced to the alcohol 24 with $NaBH_4$, and 24 was subsequently hydrogenated to yield the colorless pyridine derivative 25. Compound 25 showed UV absorptions superimposable with pyridine itself.



In the ^1H NMR spectra (220 MHz) of the navenones, the methyl groups and aromatic protons were nicely resolved. The olefinic protons of the tetraene system were, however, closely spaced which precluded coupling constant analysis and spin-decoupling experiments. Spectra were then recorded for both navenone A and B after sequential additions of $Eu(fod)_3$ reagent in $CDCl_3$. The results in Figure 1 illustrate the $\Delta\delta$ values obtained after least squares treatment of the shift data through a 1 to 1 molar ratio of compound to $Eu(fod)_3$. As protons shifted it became possible to measure vicinal coupling constants and to interrelate the protons of the central tetraene system by selective decoupling. As all eight olefin protons possess a 15 Hz coupling constant, the stereochemistry of the tetraene system was concluded as all trans. The data in Figure 1 clearly illustrate that both oxygen and nitrogen function as the complexing atoms for navenone A, while for navenone B the data obviously illustrate complexation only with oxygen.

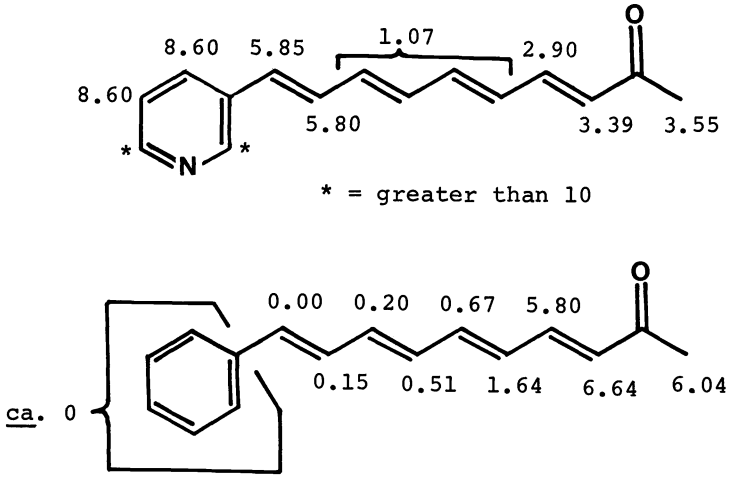
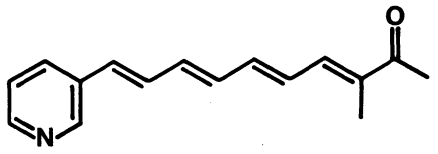


Fig. 1. Lanthanide induced shift (LIS) data for navenones A and B. $Eu(fod)_3$ was used in $CDCl_3$ solution and the spectra were recorded at 220 MHz. $\Delta\delta$ values were obtained by least squares refinement of the shift data obtained through a 1:1 $Eu(fod)_3$: substrate molar ratio.

Navenones A - C are the major compounds in the natural secretion (~90%), but several minor compounds have now been isolated which are also capable of inducing a trail-breaking alarm response. Further separation of the crude navenone A and navenone B fractions by HPLC on μ-porasil has now led to the isolation of the 3-methyl derivatives, 26 and 27, as well as the corresponding 3,5-di-cis analogs of navenones A and B. Several other minor compounds were isolated by HPLC, which are assumed to be unknown cis isomers of navenones A and B since they were rapidly transformed to all trans before NMR data could be obtained. The structures of the homologs 26 and 27 follow from spectral analysis, particularly the existence in their ^{1}H NMR spectra of the extra olefin methyl group, as well as the loss of the high field doublet (α proton of α,β-unsat. ketone) at C-3 clearly visible in the spectra for 21 and 22. Assignments of 28 and 29 as the 3,5-di-cis compounds followed from the analysis of the resolved ^{1}H NMR bands for protons at C-3, C-4, and C-5, all of which showed coupling constants with a maximum magnitude of 10 Hz.



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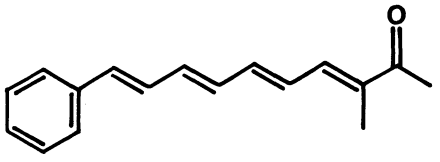

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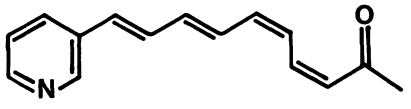

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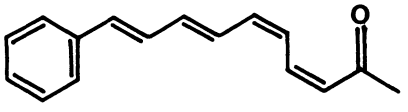

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Samples of HPLC purified navenone A and B, when irradiated with visible light, produced both 28 and 29 in an apparent photoequilibrium. After several hours irradiation (500 W projection bulb) a 9:1 ratio of 21:28 and an 8.5:1.5 ratio of 22:29 was observed.

In view of the glandular secretion of the navenones, it seemed unlikely that these compounds could be obtained by food-chain concentration. But, since this argument could not be totally excluded, the basic biosynthesis of the navenones was investigated. Large Navanax were kept in aquaria, in isolation, and fed both heavily and sparingly. The secretion was obtained from these animals, without damage, by irritating the sensitive anterior portion of the animal. With prosperous feeding of any accepted food source, the navenones were regenerated in about three days. However, the ratio of navenones A:B:C, which is 4:2:1 in natural populations, was heavily shifted toward navenone C with little, if any, navenone A being produced. If longer periods of regeneration time were given, the quantities of navenone A were found to slowly increase. This may indicate that the phenol ring in navenone C is the precursor to the benzene and pyridine rings in navenones A and B. Under stress conditions, the regeneration of the secretion was slower and the production of the 3-methyl homologs, 26 and 27, seemed to increase. This observation may illustrate increased levels of in vivo propionate production from carbohydrate catabolic processes.

To confirm the in vivo synthesis of the navenones, a single dose of ^{14}C-labelled sodium acetate was given to Navanax by injection into its food. After two days, the secretion was obtained, its radioactivity was measured, and the major navenones (C and B only) were isolated by thin-layer chromatography. Measurement of the radioactivities of the pure compounds

clearly illustrated they were produced, at least in part, from acetate. Table 3 illustrates the results of this experiment. It should again be pointed out that navenones B and C were the major compounds produced, and that navenone C had proportionately higher activity than B, in support of the phenol→benzene→pyridine biogenetic hypothesis.

TABLE 3. Incorporation of ^{14}C-sodium acetate into navenones B C (1μCi=2.22 x 10^6 dpm)

Dose	Start dpm*	Total secretion* dpm	Navenone B dpm*	Navenone C dpm*
0.7μCi	1.55 x 10^6	19,918 (1.28% incorporation)	(6mg) 746 (0.05% incorporation)	(2mg) 4326 (0.28% incorporation)

*adjusted to approximate background of 20 dpm.

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