Pure & Appl. Chem., Vol. 51, pp. 1875-1883.
Pergamon Press Ltd. 1979. Printed in Great Britain.

0033/4545/79/0901-1875 $02.00/0

SEVERAL COMPOUNDS FROM PALYTHOA TUBERCULOSA (COELENTERATA)

Yoshimasa Hirata, Daisuke Uemura, Katsuhiro Ueda, and Satoshi Takano

Department of Chemistry, Nagoya University, Nagoya, Japan

Abstract- The isolation and structural studies of palytoxin, $C_{145}H_{264}N_4O_{78}$, a potent coelenterate toxin, are reported. Although palytoxin has not been obtained up to now in crystalline form, the nature of the chromophoric portions giving rise to the λ_{max} at 233 nm and 263 nm has been elucidated by spectral and chemical studies of the fragments resulting from $NaIO_4$ oxidation. The structure of the ninhydrin-positive moiety is also discussed.
During the course of our studies on the toxins of Palythoa tuberculosa we have isolated four compounds, characterized by their absorption maxima at 310, 320, 332 and 360 nm, respectively. The structures of these compounds, together with that of a 334 nm absorbing compound isolated from Porphyra species, have been fully elucidated.

INTRODUCTION

We have been extensively involved in the isolation, structural determination and synthesis of active substances from marine animals: e.g. bromine-containing compounds from sea hare (Aplysia kurodai) (Ref. 1), bioluminescent substance (Cypridina luciferin) from Cypridina (Ref. 2), and tetrodotoxin from puffer fish (Ref. 3).

Palythoa tuberculosa (Coelenterata, Zoanthidae), a species of hexacorallia living on the coral reefs in tropical and subtropical regions, contains palytoxin, well known as the most powerful toxin among those obtained from marine animal sources. The structure and chemical properties of palytoxin are expected to be of great interest in view of the following facts: i) toxicity of palytoxin is ten times stronger than that of tetrodotoxin; ii) palytoxin induces powerful vasoconstriction; iii) in spite of the supposed molecular formula, $C_{145}H_{264}N_4O_{78}$, palytoxin does not seem to contain repeating units of sugars and amino acids. In view of these highly interesting properties, an extensive chemical study of palytoxin was undertaken.

In addition to palytoxin we have examined other constituents of Palythoa tuberculosa in order to clarify the symbiotic relation between coral and algae. We have isolated a series of compounds possessing characteristic UV absorption. Some of them were known to exist in species of red algae, corals, jellyfishes and planktons, and have attracted much attention with respect to their role in these living systems. However, no structural information on these compounds has been recorded. The results obtained in our laboratory on the isolation and structural elucidation of these compounds, together with their structural and biogenetic relationship, will be reported here.

PALYTOXIN

Scheuer (Ref. 4) found in 1971 that the coelenterate Palythoa toxica, called in Muolea Island "limu-make-o-Hana" (deadly seaweed of Hana) contains an unstable compound, palytoxin, having molecular weight 3300 and molecular formula $C_{145}H_{264}N_4O_{78}$. In a subsequent paper (Ref. 5) he reported that the intravenous lethality of palytoxin exceeds that of tetrodotoxin or saxitoxin by an order of magnitude, and that the biological action against animals is unique, as exemplified by powerful vasoconstriction and coronary spasms. In 1974 Scheuer and coworkers (Ref. 6) determined the structure of the 263 nm chromophore (Fig. 1) in palytoxin by spectral data, chemical degradation and syntheses of model compounds.

In 1973 Hashimoto, who had had a long interest in ciguatera, reported that the viscera of a filefish in Okinawa was lethal, and that the gut content of the toxic fish mainly consisted of a zoanthid, which was identified as Palythoa tuberculosa (Ref. 7). He also recognized this toxin as palytoxin itself or a closely related compound.

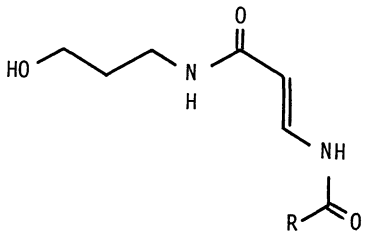


Fig. 1. The structure of the 263 nm chromophore according to Scheuer.

Materials

Palythoa tuberculosa was collected in Ishigaki Island, which is situated in the southern part of Japan. The toxicity of Palythoa tuberculosa is confined to the female polyps, being stronger in mature eggs, which are kept from May through September. In order to have Palythoa tuberculosa with high toxicity, we collected it during this period, and transported it by air, frozen with dry ice,to Nagoya.

Isolation

There are two methods of isolation by Scheuer (Ref. 4) and Hashimoto (Ref. 7), but an improved method for large-scale operations has been developed in our laboratory. This new method is described in the following.

The frozen animals were crushed in a blender in 75% aqueous ethanol. After removal of the solid residue by filtration, the orange-colored filtrate was concentrated carefully under reduced pressure at low temperature. The remaining aqueous solution was charged on a Toyo-Soda TSK G3000S polystyrene gel column. Inorganic salts were washed out with water and then the toxin fractions eluted with 75% ethanol. A DEAE Sephadex A-25 column equilibrated with phosphate buffer (pH 6.9) was charged with this fraction, and the toxin, which is weakly basic, eluted in the solvent front without absorption. The toxin fraction was freed from the buffer by passing through a TSK G3000S column, which was thoroughly wahed with water and then eluted with 75% ethanol; the toxin fractions were monitered by an UV detector. Further purification was achieved using a column of CM Sephadex C-25 buffered with pH 4.6 phosphate buffer. The toxin was eluted with the buffer solution and desalted by TSK G3000S column chromatography. The purity of palytoxin was checked by HPLC and HPTLC.

Properties

Spectral data of palytoxin are: UV(H_2O) 233 (ε 47000), 263 nm (ε 28000); IR(KBr) 3600,1655 cm^{-1}; PMR(DMSO-d_6, 100 MHz) δ 7.60 (1H, dd, J=10, 14 Hz), 7.82 (1H, br.s), 10.2 (1H, d, J=10 Hz); CMR (D_2O, 67.9 MHz) Fig.2; and LD_{50} for mice was 0.5 μg/Kg by intraperitoneal injection. Catalytic hydrogenation of this toxin with platinum oxide in aqueous ethanol gave a perhydropalytoxin, in which the 263 nm chromophore and some other double bonds had been reduced.Since palytoxin is a fairly large molecule having molecular weight 3300 and molecular formula $C_{145}H_{264}N_4O_{78}$, catalytic hydrogenation only slightly affected its molecular structure, but yet toxicity was completely lost:perhydropalytoxin showed no toxicity by 9.2 mg/Kg injection. Similar results were obtained by treatment with 0.1 N NaOH at room temperature for 50 min, or 0.1 N acetic acid at 80° for 24 hours, which afforded non-toxic products. Acetylation of palytoxin with p-nitrophenyl acetate in water containing trace amounts of pyridine gave N-acetylpalytoxin, which responded negatively to the ninhydrin test and showed a dramatic decrease of toxicity. Therefore, we believe that toxicity of palytoxin mostly depends on its ninhydrin-positive moiety.

Acetylation of palytoxin

As described above, under mild condition palytoxin afforded a monoacetate, the structure of which is closely related to that of palytoxin. On the other hand, palytoxin has many hydroxyl groups as shown by its molecular formula and spectral data. Practically, treatment of palytoxin with acetic anhydride-pyridine gave polyacetate(s), CMR spectrum of which indicated minor changes in the structure of palytoxin.

Oxidation of palytoxin with $NaIO_4$

Oxidation of palytoxin with $NaIO_4$ was carried out on a TSK G3000S polystyrene gel column.

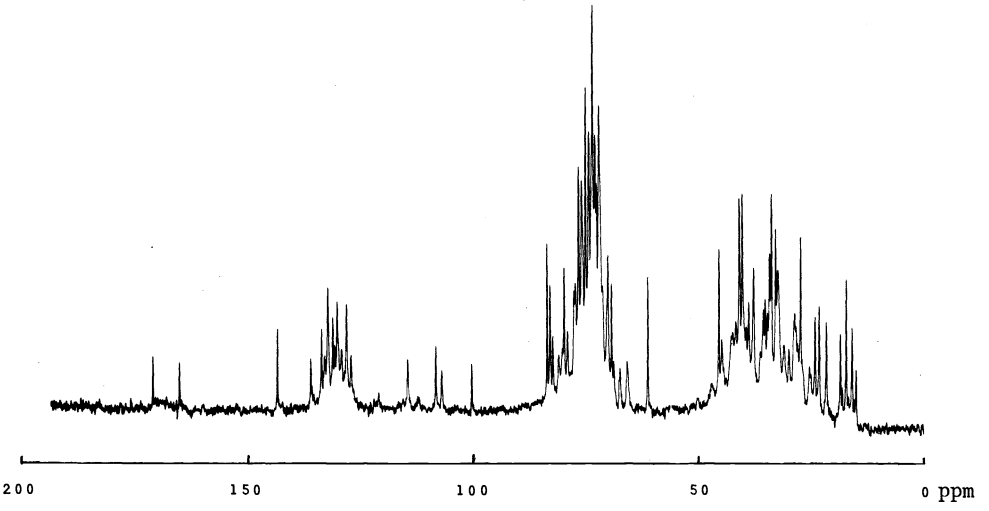
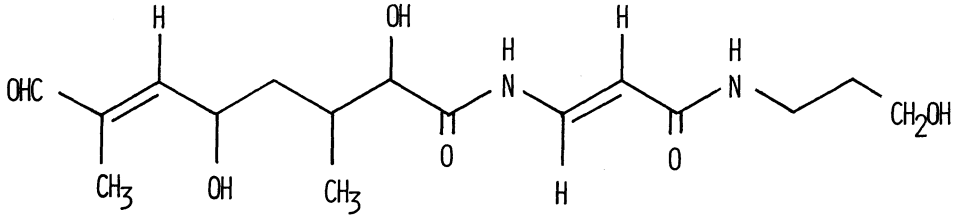
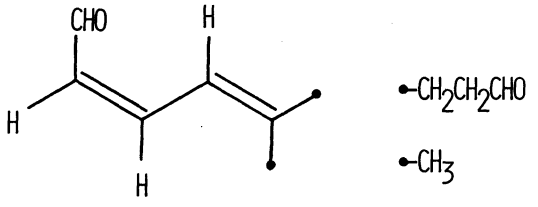


Fig. 2. The ^{1}H-noise-decoupled ^{13}C FT NMR spectrum of palytoxin at 67.9 MHz in D_2O.



(1)

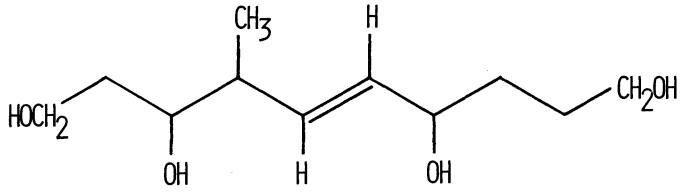


(2)

Palytoxin was charged on this column and treated with an aqueous solution of excess $NaIO_4$. After elution of periodate and formic acid, a ninhydrin-positive fraction was obtained by washing with water. The spectral data of this substance and its monoacetate obtained by treatment with p-nitrophenyl acetate show the presence of $CHO-CH_2-CHOH-$ and $-CH_2NH_2$ groups. It is suggested that high polarity and lability of palytoxin are due to this ninhydrin-positive moiety.

The polystyrene column was then eluted with increasing concentrations of ethanol in water. Two compounds were obtained from the 50% ethanol eluate, and their structure established as 1 and 2 by detailed analysis of the spectral data of themselves and their derivatives. Compound 1 has the same 263 nm chromophore present in palytoxin. This is consistent with the Scheuer's report concerning this chromophore. Compound 2 contains the 233 nm of palytoxin, as results from theoretical calculation of the value of the UV maximum. However, from the value of the extinction coefficient it can be deduced that palytoxin contains a second chromophore of the same type. Finally, sodium borohydride reduction of the pure ethanol eluate afforded a tetraol assigned the structure 3 and compound 4. The crystalline compound 4, mp 99.5-101°, $C_{30}H_{58}O_7$, which is derived from the lipophilic part in palytoxin, possesses an isopropyl group, a tertiary methyl and some ether functions.

At the present, the fragments obtained account for appoximately 74 carbon atoms of palytoxin. We believe that the structure elucidation of the ninhydrin-positive substance and of compound 4 will lead us to the determination of the full structure of palytoxin and its structure-toxicity relationship.



(3)

A SERIES OF COMPOUNDS POSSESSING CHARACTERISTIC UV ABSORPTION AT 310-360 nm

During the course of our study of palytoxin, we have isolated several water-soluble compounds with strong absorption maxima in the range of 310-360 nm. Although it is well-known that compounds having UV bands in this range are widespread in marine plants (Ref. 8) and animals (Ref. 9), there are only few reports dealing with their structures and roles in vivo. We have reported previously the structure of two compounds, mycosporine-Gly 5 (λ_{max} 310 nm) (Ref. 10) and palythine 6 (λ_{max} 320 nm) (Ref. 11) from Palythoa tuberculosa. Recently Tsujino et al. have isolated from the red alga Chondrus yendoi a compound with an absorption maximum at 320 nm which is identical to palythine but exhibits optical activity (Ref. 12). Further investigation on the UV absorbing compounds from Palythoa tuberculosa has yielded two new compounds, palythinol (λ_{max} 332) and palythene (λ_{max} 360), to which structures 7 and 8 are assigned.

A new compound 9 with a maximum at 334 nm, supposed to be biogenetically related to palythene, has been extracted from Porphyra tenera Kjellman and its structures determined.

Isolation

As shown in Fig. 3, an oily material containing mycosporine-Gly, palythine, and palythinol was obtained by repeated chromatography of extracts of Palythoa tuberculosa on Dowex 50W (H^+ form) and TSK G3000S. Then, purification of this material by preparative TLC on silica gel gave three compounds with characteristic UV absorption maxima. The 360 nm absorbing compound, palythene, which contrarily to the related compounds was slightly absorbed on the polystyrene column, was obtained by concentration of the 10% ethanol eluate.

Isolation of the 334 nm absorbing substance 9 from Porphyra tenera Kjellman is shown in Fig. 4.

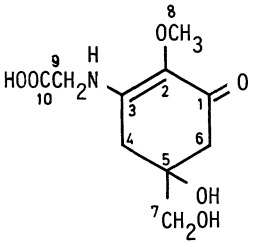
Structures of mycosporine-Gly, palythine, palythinol and palythene

The molecular formula of each compound was determined by elemental analysis. The CMR data,

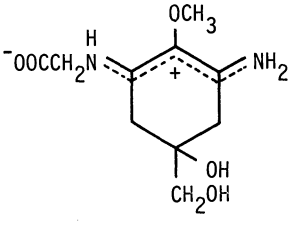
Palythoa tuberculosa
- 75% EtOH extract concentrated

TSK G3000S (polystyrene gel)
- eluted with 10% EtOH concentrated → An oily material
  - preparative TLC (SiO_2) → Palythene
- eluted with H_2O concentrated desalted → Dowex 50W (H^+ form)
  - eluted with 0.5 N HCl concentrated → TSK G3000S
    - eluted with H_2O → An oily material
      - preparative TLC (SiO_2) → Palythine and Palythinol
  - eluted with H_2O → Mycosporine-Gly

Fig. 3. Scheme for the isolation of a series of compounds possessing characteristic UV-absorption at 310-360 nm from Palythoa tuberculosa.



(5)
Mycosporine-Gly
310 nm



(6)
Palythine
m.p. 145-146°
320 nm (ε 36200)

Dried Porphyra tenera
- 80% MeOH extract concentrated
- MeOH filtrated concentrated

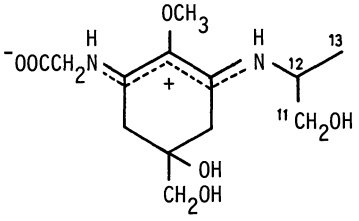
Sephadex G-10
- eluted with H_2O concentrated

DEAE Sephadex A-25
- eluted with pH 6.9 phosphate buffer concentrated

Sephadex G-10
- eluted with H_2O

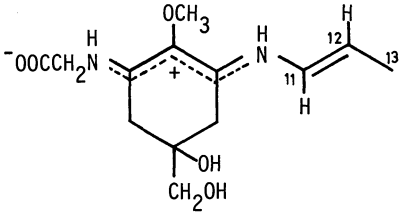
Norit A
- eluted with 50% EtOH concentrated

Compound 9

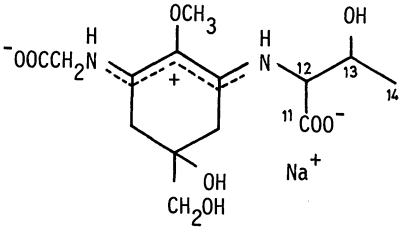


(7)
Palythinol
m.p. 155-156°
332 nm (ε 43500)

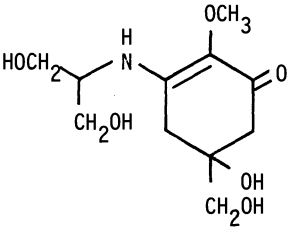
Fig. 4. Scheme for the isolation of the 334 nm UV-absorbing substance from Porphyra tenera Kjellman.



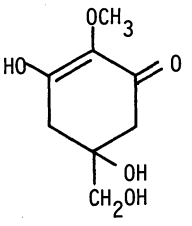
(8)
Palythene
m.p. 145-146°
360 nm (ε 50000)



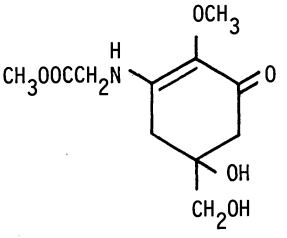
(9)
334 nm



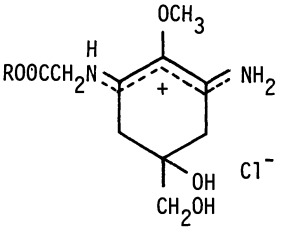
(10)



(11)

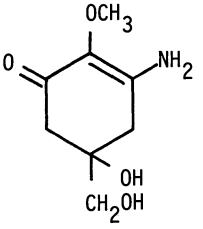


(12)

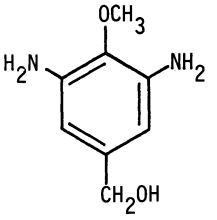


(13) R= CH_3

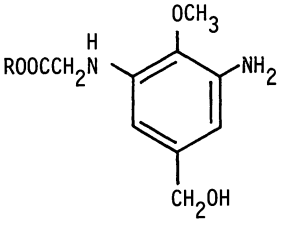
(18) R= H



(14)

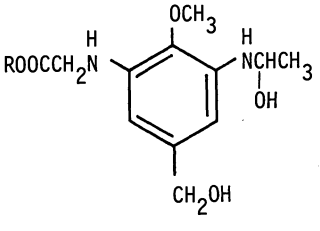


(15)



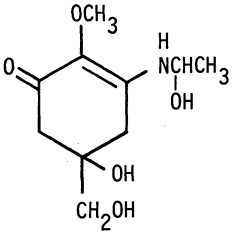
(16) R= H

(17) R= CH_3

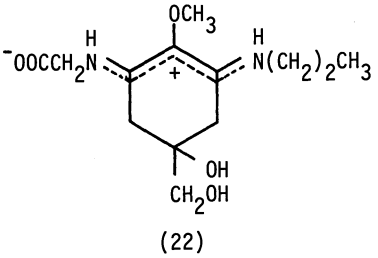


(19) R= CH_3

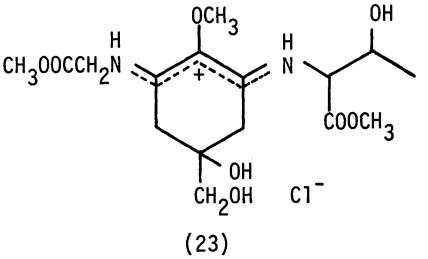
(21) R= H



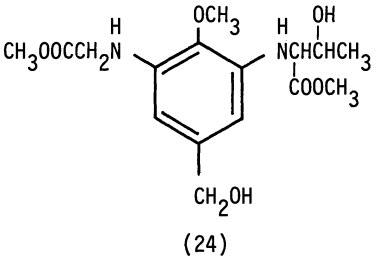
(20)



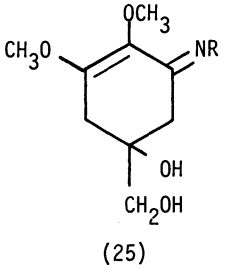
(22)



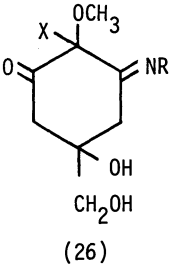
(23)



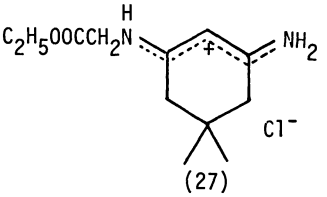
(24)



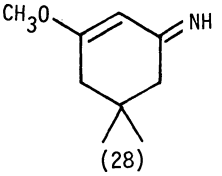
(25)



(26)



(27)



(28)

Table 1. ^{13}C Chemical shifts (δ in ppm) of mycosporine-Gly 5, palythine 6, palythinol 7, palythene 8, compound 9, and mycosporine 10.

Carbon Number	1	2	3	4	5	6	7	8	9	10	11	12	13	14
5	187.2	130.4	159.7	33.8	72.9	45.4	68.4	60.2	43.7	174.5				
6	162.5*	125.4	160.9*	34.2	72.0	36.6	68.2	59.7	47.5	177.5				
7	160.9*	126.1	160.4*	33.6	71.9	33.9	68.2	59.9	47.4	175.9	50.7 (t)	67.4 (d)	20.2 (q)	
8	161.5*	126.4	154.2*	33.8	71.8	33.8	68.4	60.3	47.6	175.4	124.5 (d)	117.9 (d)	15.2 (q)	
9	161.2*	126.4	157.7*	33.7	71.8	34.1	68.2	60.2	47.5	175.5	175.5 (s)	65.2 (d)	69.0 (d)	20.2 (q)
10	187.9	132.5	160.5	35.9	74.5	45.4	70.0	61.5						
Multiplicity	s	s	s	t	s	t	t	q	t	s				

* Assignments may be reversed.

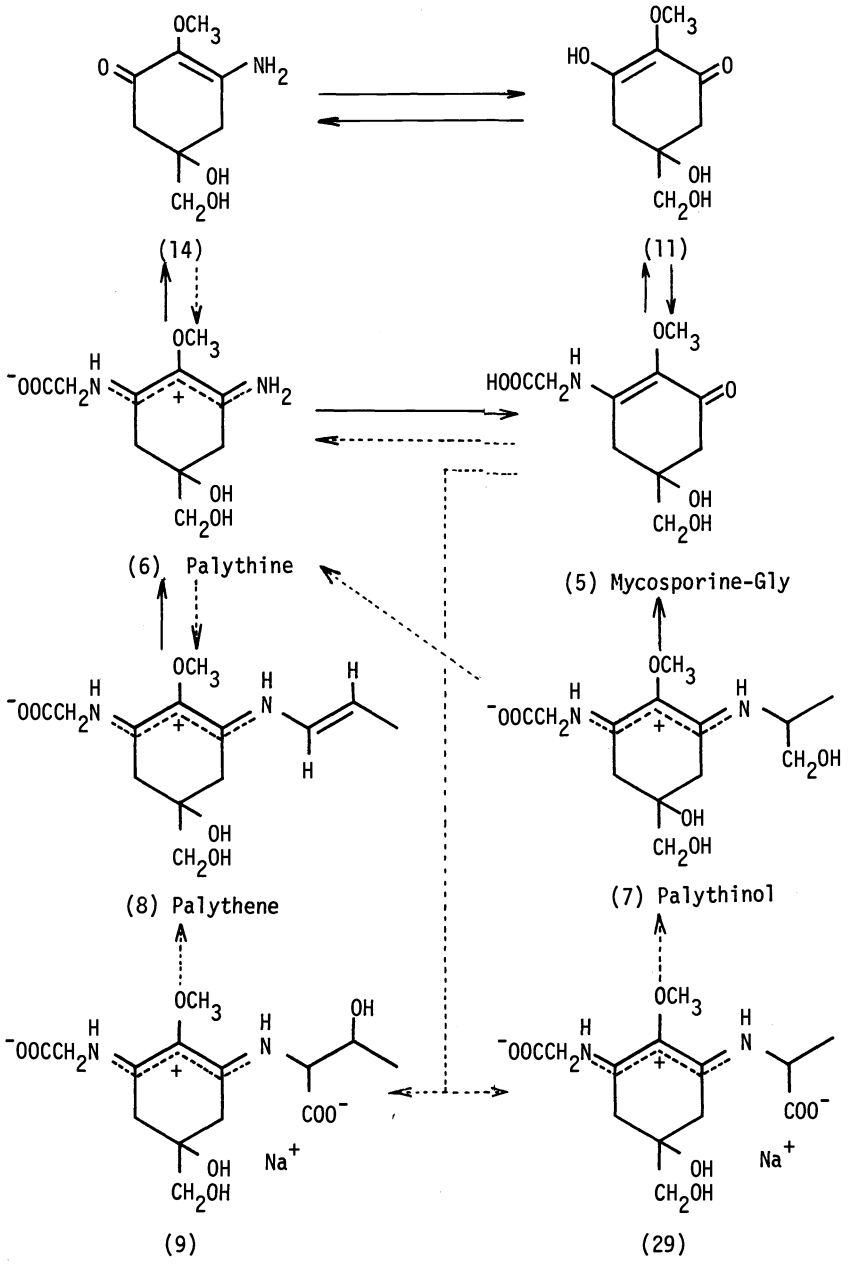


Fig. 5. The biogenetic interrelationships of a series of compounds possessing characteristic UV-absorption at 310-360 nm. Solid arrows indicate chemically feasible steps.

which were very useful to elucidate the structures of these compounds, are shown in Table 1. The structures of these compounds have been elucidated on the basis of spectral data and chemical evidence. Mycosporine 10 (Ref. 13) in Table 1 is the 310 nm absorbing substance isolated from fungi by the Favre-Bonvin group. Interestingly, near ultraviolet radiation in many fungi stimulates reproduction, which is accompanied by the formation of these compounds (Ref. 14).

Mycosporine-Gly was converted to β-diketone 11 and glycine when heated in water at 80° for 3 hrs. As mycosporine-Gly itself was rather unstable, it was converted by standard methylation with diazomethane into the stable methyl ester 12.

Palythine was stable in acidic media, and on treatment with HCl-MeOH gave the methyl ester 13. On the contrary, under basic conditions palythine was remarkably unstable, and easily underwent hydrolysis and dehydration. When palythine was treated with conc. NH_4OH at room temperature overnight, it gave glycine and compounds 14, 15, and 16. Formation of these compounds indicates that palythine has the same substituents on a cyclohexene ring as mycosporine except for the absence of the ketone group in palythine. Remarkably, treatment of palythine with diazomethane afforded the aromatized methyl ester 17 with only two methoxy groups. Probably, the methyl ester 17 is produced through proton abstraction induced by diazomethane, followed by dehydration. From its IR spectrum, palythine appears to be an innersalt, as shown in structure 6, and the actual structure is visualized as a resonance hybrid of the stable vinylogs of amidine. In addition, from CMR spectral data it can be deduced that delocalization of the positive charge on C-1 and C-3 is more effective in palythine hydrochloride 18 and its methyl ester 13 than in palythine itself.

Palythinol 7 on treatment with diazomethane in ether-methanol was easily aromatized by dehydration, similarly to palythine, to give methyl ester 19. Palythinol was converted by hydrolysis with NH_4OH into compounds 5, 20, 21, 2-aminopropanol, and glycine. Formation of these compounds indicates that palythinol has structure 7.

Finally, palythene 8 was treated with refluxing 2N HCl for 3 hours to afford palythine and propanal by hydrolysis of the enamine group. These results strongly support structure 8. Additionally, hydrogenation of palythene with Pd-C in water for 1 hr gave dihydropalythene 22, whose absorption maximum was shifted to 331 nm. The stereochemistry of the aminopropene moiety of palythene was readily determined by pmr measurement: the coupling constant between the olefinic protons (13 Hz) clearly indicated their trans-relationship (Ref. 15).

The structure of a compound possessing a 334 nm chromophore

Although the presence of a 334 nm absorbing substance had been reported by several groups, the structure has not yet been determined (Ref. 16). Comparison of chemical and spectral properties of this substance with those of palythine, palythinol and palythene led to structure 9.

Treatment of 9 with HCl-MeOH afforded dimethyl ester 23, which was easily dehydrated in pyridine to afford the aromatic compound 24. Furthermore, by treatment with HCl compound 9 gave glycine and threonine.

Biosynthetic pathway, biochemical correlation and physiological roles of UV absorbing compounds

These compounds can be considered to be derived from β-diketone 11, which in turn can be theoretically derived from shikimic acid as shown in Fig. 5. The information obtained from the correlation among those compounds led us to the following consideration concerning their biosynthetic pathways. Introduction of the second nitrogen-containing group into an enaminone-type compounds (i.e. 12 and 14) may proceed through activated intermediates such as 25 and 26. For example, compound 27 was obtained from dimedone in three steps through 28. However, it may occur in their biosynthetic pathways that direct introduction of the second nitrogen-containing group proceeds without the activation of enaminone and that compound 9 or a hypothetical compound 29 is converted to palythene, palythinol, palythine and mycosporine-Gly as depicted in Fig. 5.

These compounds are widely distributed in living organisms, but their biochemical roles are obscure. Shibata proposed two possible hypotheses (Ref. 17): (1) UV absorbing compounds may play a protective role against strong ultraviolet irradiation, and (2) they may be precursors of the various pigments in algae and corals. On the other hand, several authors have hypothesized that they may play an important role in the photosynthetic system or reproduction of many fungi. It is interesting to note that the production of mycosporine (10) occurs during the UV-stimulated reproduction stage of many fungi (Ref. 14).

REFERENCES

1. S. Yamamura and Y. Hirata, Tetrahedron 19, 1485 (1963).
   S. Yamamura and Y. Hirata, Bull. Chem. Soc. Jpn. 44, 2560 (1971).
2. O. Shimomura, T. Goto, and Y. Hirata, Bull. Chem. Soc. Jpn. 30, 929 (1957).
   Y. Kishi, T. Goto, Y. Hirata, O. Shimomura, and F.H. Johnson, Tetrahedron Lett. 3427(1966).

3. T. Goto, Y. Kishi, S. Takahashi, and Y. Hirata, Tetrahedron 21, 2059 (1965).
4. P.J. Scheuer and R.E. Moore, Science 172, 4951 (1971).
5. R.J. Seltzer, Chem. Eng. News Dec. 16 (1974) p20.
P.J. Scheuer, Acc. Chem. Res. 10, 33 (1977).
6. R.E. Moore, R.F. Dietrich, B. Hatton, T. Higa, and P.J. Scheuer, J. Org. Chem. 40, 540 (1974).
7. S. Kimura and Y. Hashimoto, Publ. Seto. Mar. Bio. Lab. 20, 713 (1973).
8. I. Tsujino and T. Sato, Bull. Faculty of Fisheries, Hokkaido Uni. 12, 49 (1961).
9. J.H. Price and H.S. Forrest, Comp. Biochem. Physiol. 30, 879 (1969).
10. S. Ito and Y. Hirata, Tetrahedron Lett. 2429 (1977).
11. S. Takano, D. Uemura, and Y. Hirata, Tetrahedron Lett. 2299 (1978).
12. I. Tsujino, K. Yabe, I. Sekikawa, and N. Hamanaka, Tetrahedron Lett. 1401 (1978).
13. J. Favre-Bonvin, N. Arpin, and C. Brevard, Can. J. Chem. 54, 1105 (1976).
14. R.E. Hite, Plant Dis. Reptr. 57, 760 (1973).
15. J. Sauer and H. Prahl, Tetrahedron Lett. 2863 (1966).
16. T. Yoshida and P.M. Sivalingam, Plant & Cell Physiol. 11, 427 (1970).
17. K. Shibata, Plant & Cell Physiol. 10, 325 (1969).