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SQUID BIOLUMINESCENCE I. STRUCTURE OF WATASENIA OXYLUCIFERIN, A POSSIBLE LIGHT-EMITTER IN THE BIOLUMINESCENCE OF WATASENIA SCINTILLANS

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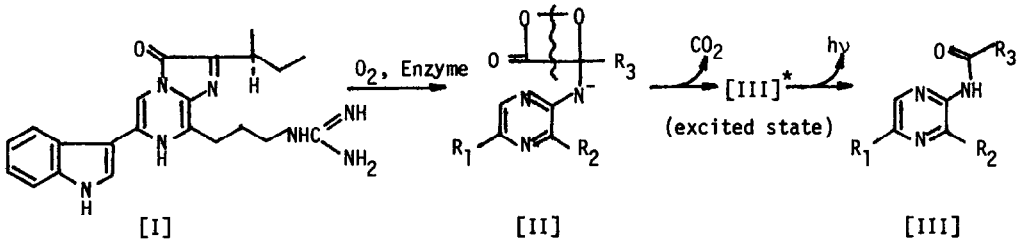
A luminous squid, Watasenia scintillans Berry (Japanese name: hotaru-ika), belongs to Oegopsida and is caught in large numbers in Toyama bay, Japan, in a certain period of the year. It has three tiny black spots, which are luminous organs, located at the tip of each of the ventral pair of arms.[1] Shima[2] first proposed that the luminescence of Watasenia is due to the symbiosis of luminous bacteria as had been observed in the light organs of many luminous Myopsid squids.[1] But, soon later Kishitani[3] and then Okada et al.[4] reported that the source of luminescence of Watasenia is not luminous bacteria. Since then extraction of the luminescent substance from the squid has been attempted by many workers without success. The squid is very liable to die and no luminescence could be restored after death.

Recent studies[5] on the mechanism of bioluminescence indicate that a luminescent substance such as Cypridina luciferin (I) is usually oxidized to give a fluorescent compound (light emitter) such as Cypridina oxyluciferin (III) through a dioxetane intermediate (II). The



[I] Cypridina luciferin

[II]

[III] Cypridina oxyluciferin

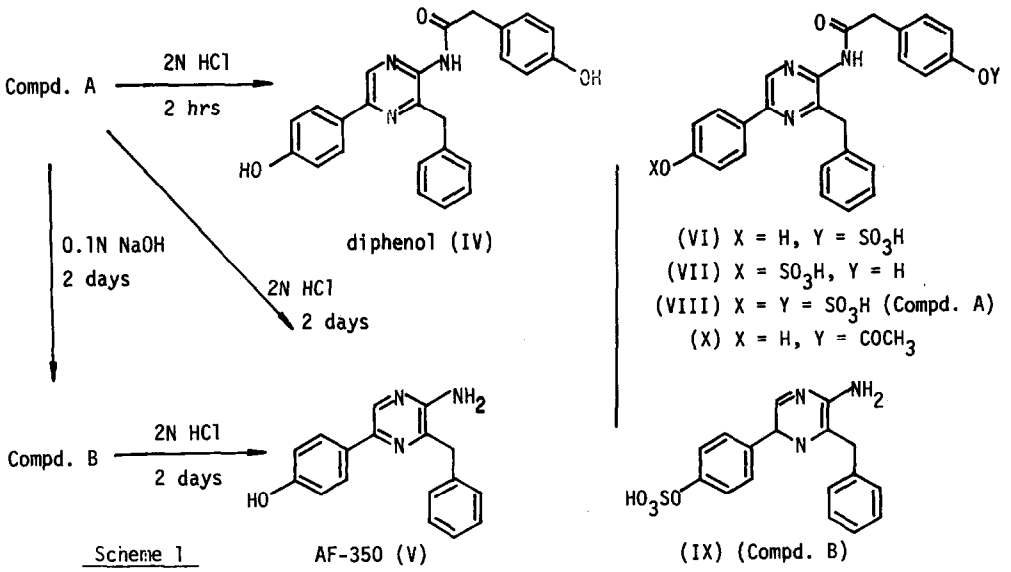
emitter such as III is first produced as an excited state from which light is emitted. In the case of aequorin, a photoprotein obtained from Aequorea by Shimomura and Johnson, essentially the same mechanism was suggested.[6] Since luminescence of the squid lasts until death, emitter of the luminescence might be accumulated in the light organs of the dead animal. Therefore, we extract fluorescent substance(s) from the dead squid in expectation of obtaining the light emitter.

The light organs (163 g) of Watasenia (ca 10,000 indiv.) were ground with silica sand, defatted with ether and extracted with methanol. The extracts were dried up and the residue was subjected to Avicel tlc using once n-BuOH:AcOH:H_2O (3:1:2) and twice with n-BuOH:AcOH:H_2O (4:2:1) to give three fluorescent compounds, A, B, and C, as shown in Table 1.

Table 1

compd.	yield	Rf on Avicel tlc		fluorescence
		n-BuOH:AcOH:H_2O (3:1:2)	n-BuOH:AcOH:H_2O (4:2:1)	color
A	4 mg	0.4	0.3	blue
B	—	0.8	0.75	white-blue
C	8 mg	0.3	0.4	green

Hydrolysis of compound A under various conditions as shown in Scheme 1 gave compound B, a diphenol (IV), and AF-350 (V), the latter two compounds had been isolated from aequorin.[6]



Scheme 1

NMR spectrum of compound A [δ ppm in CD_3OD: 3.71 (2H,s), 4.23 (2H,s), 7.06-7.44 (4H,m), 7.35 (5H,s), 7.49 (2H,d), 8.11 (2H,d), 8.87 (1H,s)] shows similar signals to that of IV [δ ppm in CD_3OD: 3.58 (2H,s), 4.11 (2H,s), 6.78 (2H,d), 7.20 (2H,d), 7.00-7.30 (5H,m), 6.89 (2H,d), 7.92 (2H,d), 8.71 (1H,s)] and number of hydrogens that are non-exchangeable with deuterium are same in the both NMR spectra. Electrophoretic data indicate that strongly acidic groups must be in the molecule of compound A since A moves toward the anode at pH 3.9, while IV does not. Coupled with the NMR and UV (Fig. 1) data it is suggested that the phenolic hydroxyl group(s) could be esterified with sulfuric acid. Two monosulfates (VI and VII) and a disulfate (VIII) of IV were synthesized (see below) and compared with A. The data shown in Table 2 and Fig. 1 indicate that compound A is identical with the synthetic disulfate (VIII).[7] Similarly compound B is identified as monosulfate of V (IX)[7] which was prepared from AF-350.

Table 2

compound	Rf on Avicel tlc BuOH:AcOH:H_2O 4 : 2 : 1	electrophoresis pH 3.9 3 mA, 45 min	fluorescence (H_2O) λ max nm	relative intensity	NMR
compd. A and synth. VIII	0.3	+ 5.2 cm	400	500	in text
synthetic VI	0.8	+ 3.1			
synthetic VII	0.8	+ 2.6			
diphenol IV	1.0	0	400	1	in text
compd. B and synth. IX	0.75	+ 3.2			

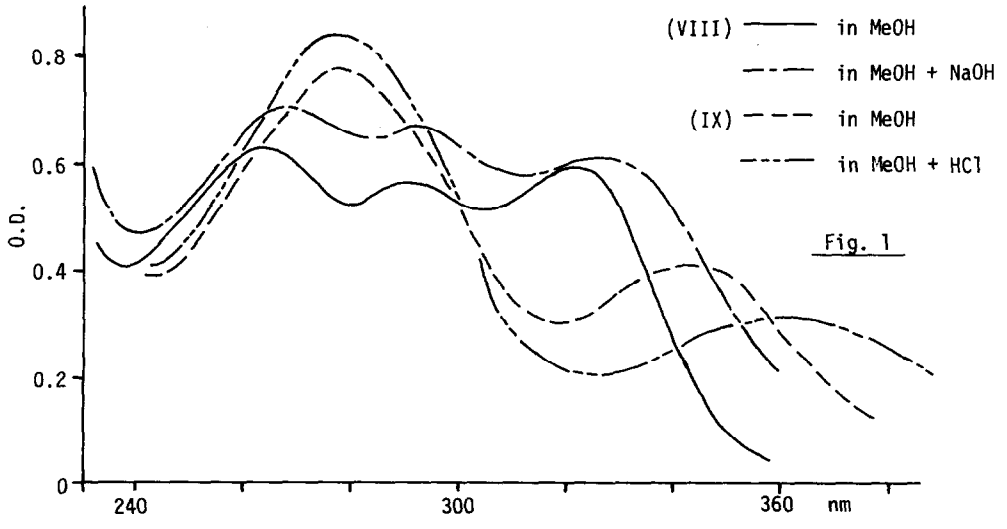


Fig. 1

Similarity of the structure of compound A (VIII) to that of Cypridina oxyluciferin (III) and the diphenol (IV) strongly suggests that compound A is the direct oxidation product of hitherto unknown Watasenia luciferin or a chromophore part of Watasenia photoprotein and hence we name compound A (VIII) Watasenia oxyluciferin. Fluorescence intensity of VIII at 400 nm in water is 500 times stronger than that of IV suggesting that higher luminescence efficiency of VIII. The green fluorescent compound (C) might act as an energy acceptor from the excited A to produce a longer wavelength luminescence than 400 nm as has been observed in the case of Aequorea and Renilla.[8] Structure determination of compound C is in progress.

Synthesis of the sulfates. —— The diphenol (IV)[6] was treated with chlorosulfonic acid and N,N-dimethylaniline to give a mixture of sulfates from which monosulfate VI and disulfate VIII were isolated by basification with Na_2CO_3, extraction of dimethylaniline with CH_2Cl_2 and then of the sulfates with n-BuOH which was subjected to tlc separations. Monosulfate VII was synthesized as follows: AF-350 (V)[6] was condensed with p-acetoxyphenylacetic acid by heating under vacuum to give the diphenol monoacetate (X), which was esterified with chlorosulfonic acid and dimethylaniline followed by hydrolysis with alkali affording VII. AF-350 sulfate (IX) was also synthesized by direct sulfurization of AF-350 (V).

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