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SQUID BIOLUMINESCENCE III. ISOLATION AND STRUCTURE OF WATASENIA LUCIFERIN

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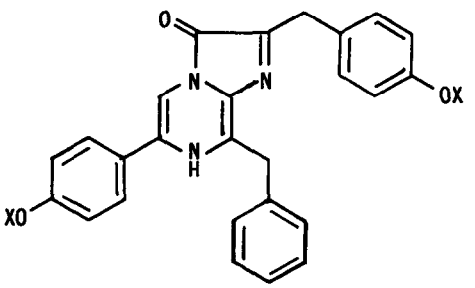
In Toyama Bay, Japan, is found the luminous squid, Watasenia scintillans (Japanese name: hotaru-ika) which gives brilliant blue light from the light organs located at the tip of each of the ventral pair of arms. Many attempts to isolate bioluminescent materials from the squid have been done for a long time without success. Recently we isolated from the light organs Watasenia oxyluciferin (III), a possible light emitter of bioluminescence of the squid.[1] Structure similarity between Watasenia oxyluciferin (III) and Cypridina oxyluciferin (V) coupled with considerations of mechanisms proposed for Cypridina bioluminescence[2] (IV $\longrightarrow$ V + hν) suggests that the luminescent compound supposed to be present in the squid could have structure II, although until now a complete luminescence system of Watasenia, either a luciferin-luciferase system or a photoprotein, has not been isolated in vitro.

During the search for such a compound in the squid we found that livers of the squid contain fairly large amounts of a strongly chemiluminescent compound, whose structure was determined as I.[3] From the structure of this compound it can be considered as a precursor of II. Thus, we name I Watasenia preluciferin.[4] Attempted isolation of II from the livers failed, but we finally succeeded in isolating it from the arm photophores.

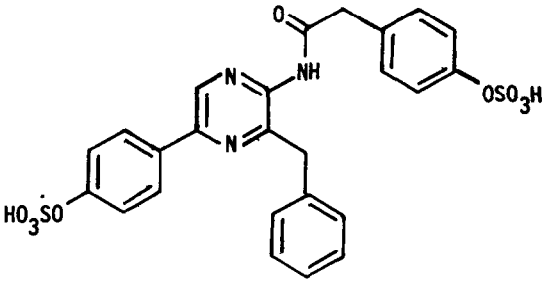
Lyophylized arm photophores (2.0 g, ca 2500 indiv.) were washed thoroughly with ether in a mortar, and the residue was extracted with oxygen-free methanol. The extracts contain Watasenia oxyluciferin (III) as reported previously.[1] The residue was then stirred with methanol containing 3% sodium methoxide (15 ml) at room temp. under complete exclusion of oxygen. After filtration, the filtrate was carefully neutralized with acetic acid and evaporated in vacuo. The residue was chromatographed on a silica gel column using MeOH-CH_2Cl_2 (1:3) as eluant, to give a yellow fraction, which was further purified by successive tlc separations under N_2 atm.

giving a yellow compound (ca 40 μg). The structure of this compound was determined as II by comparison of the Rf values on tlc [Merck Kieselgel $60F_{254}$ 0.2 mm, MeOH-CH_2Cl_2 (1:3): Rf 0.32; n-BuOH-AcOH-H_2O (4:1:2): Rf 0.11],[5] electrophoretic data [0.1M acetate buffer pH 3.9, 900 V, 1.4 mA, 20 min, moving distance 2.7 cm toward the anode],[5] and uv spectra (Fig. 1) with the synthetic sample (vide infra). This compound gave strong chemiluminescence in aqueous cellosolve without addition of a base.

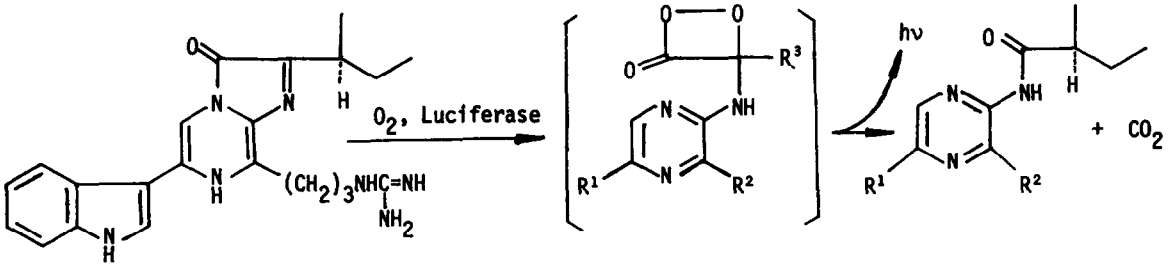


Watasenia preluciferin (I): X = H

Watasenia luciferin (II): X = SO_3H



Watasenia oxyluciferin (III)



Cypridina luciferin (IV) Dioxetane intermediate Cypridina oxyluciferin (V)

The mechanism of Watasenia bioluminescence may be as follows: Watasenia preluciferin (I) is sulfurized to II in the liver or the photophores and stored in a bound form (with a protein ?), as II cannot be extracted from the photophores with methanol or pyridine. Light is emitted from the oxyluciferin (III) in a singlet excited state which is produced by oxidation of II in the bound state or after liberation. Although this bioluminescence system may not be a luciferin-luciferase type, we prefer to use the name "Watasenia luciferin" for this compound

(II) by structural and mechanistic analogies to Cypridina luciferin (IV), as we think[6] that a low molecular compound which can act as the energy source by oxidation on a given bioluminescence should be generally called "luciferin" apart from the initial definition given by DuBois.[7]

Synthesis of Watasenia luciferin (II) —— Watasenia preluciferin (I)[3] (50 mg) was heated with sulfur trioxide-pyridine (200 mg) in pyridine (0.5 ml) at 100° for 1 hr. The reaction mixture was diluted with ether and the solid precipitated was collected and washed thoroughly with ether. It was chromatographed under N_2 atm. on a silica gel column and then thin-layer plates (twice) using MeOH-CH_2Cl_2 (1:3) as eluant to give a yellow solid. It was dissolved in methanol and treated with a methanol solution of sodium methoxide. After neutralization with Amberlite IRC-50, the solution was evaporated to dryness and the residue triturated with ether to give a crystalline solid (54 mg), which was further purified by tlc [MeOH-CH_2Cl_2 (1:5)] giving pure II as disodium salt.[8] Uv (Fig. 1): λ_{max} nm (ε) (MeOH) 426 (7700), 350 (6600), 258 (24200); (MeOH-HCl) 335 (8600), 266 (25700); (MeOH-NaOH) 400 (7100), 348 (9700), 266 (29600); nmr (CD_3OD, ppm from int TMS) 4.18 (2H,s), 4.43 (2H,s), 7.2-7.6 (11H,m), 7.66 (2H, $\underline{A}'_2\underline{B}'_2$), 7.74 (1H,s); ir (KBr) 3450br, 1590br, 1508m, 1250vs, 1050s, 860 cm^{-1}. Addition of II (60 mg) in portion into DMSO (5 ml) under O_2 bubbling at room temp. gave III (55 mg, 96%) with chemiluminescence, thus confirming the disulfate structure (II).

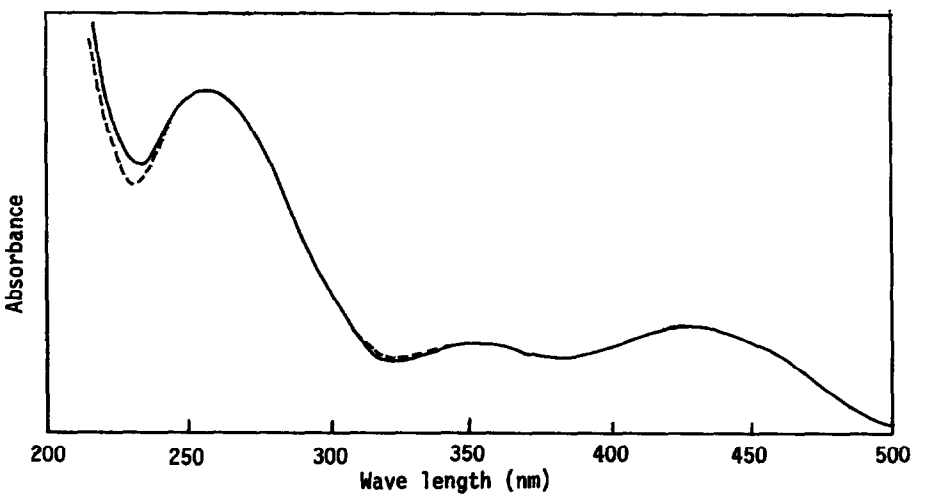


Fig. 1. Uv Spectra of Watasenia Luciferin (II)

—— natural ----- synthetic (in MeOH)

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REFERENCES AND FOOTNOTES

1) T. Goto, H. Iio, S. Inoue and H. Kakoi, Tetrahedron Letters, 2321 (1974).

2) T. Goto, Pure Appl. Chem., 17, 421 (1968); To Goto and Y. Kishi, Angew. Chem. Int. Ed., 7, 407 (1968).

3) S. Inoue, S. Sugiura, H. Kakoi, K. Hashizume, T. Goto and H. Iio, Chemistry Letters (Japan), 141 (1975).

4) Aequorin, a photoprotein extracted from Aequorea aequorea, is supposed to contain I as a modified form. Although I has not been isolated as such from aequorin, it was named coelenterazin [S. Shimomura and F. H. Johnson, Proc. Nat. Acad. Sci. USA, 72, 1546 (1975)]. It was also suggested, but not identified, that Renilla luciferin and Cavenurularia luciferin have the structure same as I.

5) Watasenia luciferin (II) is easily oxidized in aqueous as well as in organic solvents so that the tlc and the electrophoretic separations of II are always accompanied by a trace of oxyluciferin (III).

6) Cf. M. J. Cormier, J. Lee and E. Wampler, Ann. Rev. Biochem., 44, 255 (1975).

7) R. DuBois, Compt. Rend. Soc. Biol., 37, 564 (1887); cf. F. H. Johnson in M. Florkin and E. H. Stotz Ed., Comprehensive Biochemistry, 27, Elsevier Bubl. Co., Amsterdam, 1967, p 79.

8) One of the confirmation of the disulfate structure (II) was obtained from the comparison of nmr spectrum of II with that of I. Thus, both of the $\underline{A}_2'\underline{B}_2'$ signals appeared in the spectrum of I [nmr (CD_3OD, ppm from int. TMS) 4.08 (2H,s), 4.41 (2H,s), 6.69 (2H, $\underline{A}_2'\underline{B}_2'$), 6.88 (2H, $\underline{A}_2'\underline{B}_2'$), 7.0-7.8 (10H,m)], which are assigned to the four hydrogens ortho to the phenolic hydroxyl groups, are completely absent in the spectrum of II. The following elementary analysis of II also supports the structure although reliability may not be high due to the presence of ash which probably consists of Na_2SO_4. Found (dried over P_2O_5 at room temp.): C, 43.1; H, 3.7; N, 5.7; ash 22.8%. $C_{26}H_{19}O_9N_3S_2Na_2 \cdot 4H_2O$ requires: C, 44.6; H, 3.9; N, 6.0; ash (as Na_2SO_4) 20.3%.