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COMPLETE STRUCTURE OF *RENILLA* LUCIFERIN AND LUCIFERYL SULFATE[1]

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Extensive studies by Cormier et al. on the luminescence of the sea pansy *Renilla* have disclosed that light is emitted when *Renilla* luciferase[2] catalyzes the oxidation of *Renilla* luciferin[3] which is formed from *Renilla* luciferyl sulfate[4] in the presence of 3',5'-diphosphoadenosine and luciferin sulfokinase.[5] They have suggested for the luciferin the structure Ia containing an unknown group mainly from UV and mass spectral comparisons with those of synthetic analogs;[3] molecular weight of the luciferin being reported to be 513 as determined by mass spectrometry.[3]

Renilla luciferyl sulfate + 3',5'-Diphosphoadenosine $\xrightarrow{\text{Luciferin sulfokinase}}$ *Renilla* luciferin + 3'-Phosphoadenosine-5'-phosphosulfate

Renilla luciferin + O_2 $\xrightarrow{\text{Luciferase}}$ Light + CO_2 + Product

They also suggested structure IIa for the luciferyl sulfate[6] and claimed to have synthesized it from the luciferin by treatment with sulfamic acid in pyridine.[4] The method reported, however, had been devised without knowledge of the existence of phenolic hydroxyls in the luciferin molecule (*vide infra*), and insufficient data of the product were reported; thus the results being very ambiguous.

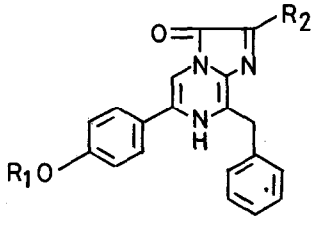
Shimomura and Johnson[7] later identified the product of bioluminescence of *Renilla* to be coelenteramide (III), implying that luciferin present in *Renilla* could probably be coelenterazine (Ib), although it conflicts with the molecular weight 513 reported by Hori and Cormier.[3]

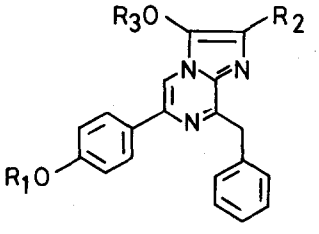
We have now established by examining of extracts of *Renilla*, that natural *Renilla* luciferin is indeed coelenterazine (Ib) (mol. wt. 423). We have also determined that natural luciferyl sulfate has the structure IIb by comparison of natural luciferyl sulfate with an authentic

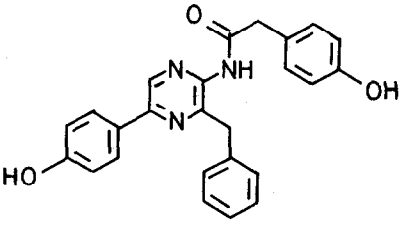
sample synthesized by the unambiguous route described below.

Natural Renilla luciferyl sulfate and luciferin Renilla mülleri (200 g, ca 40 animals, Gulf Specimen Company, Panacea, Florida) frozen in dry ice was homogenized (Servall Omni-mixer) with methanol (700 ml) and filtered, throughout at -5° to -10°. The residue was further extracted with methanol (600 ml). The extracts were combined, evaporated to 70 ml, and washed 3 times each with pentane and benzene. The aqueous layer was then extracted 4 times with ethyl acetate and the combined extracts were evaporated to dryness. The luciferyl sulfate in the residue was further purified on a column of Sephadex LH-20 (0.6 x 24 cm) as described.[4] The purified luciferyl sulfate (yield ca 15 μg) was proven to be identical with the synthetic sample (IIb) (vide infra) in UV absorption ($\lambda_{max}^{H_2O}$ 271 nm at pH 7.5, 297 nm at pH 12),in tlc behavior [silica gel, Rf values of the blue fluorescent spot: MeOH:AcOEt (1:13) 0.65; EtOH:AcOEt (1:8) 0.40; MeOH:CH_2Cl_2 (1:5) 0.20; MeOH:C_6H_6 (1:3) 0.20], and also in the elution volume from the Sephadex LH-20 column.

Free luciferin was obtained by hydrolyzing the sulfate with 0.1N HCl at 90° for 50 sec.[8] followed by extraction with ethyl acetate. The product was proven to be identical with an authentic sample of synthetic Ib[9] in tlc [Rf: MeOH:CH_2Cl_2 (1:10) 0.37; acetone:CH_2Cl_2 (1:10) 0.09; MeOH:C_6H_6 (1:5) 0.29]; in the rate of luminescence reaction catalyzed by Renilla luciferase, and in its mass spectrum which showed the molecular peak at m/e 423 without any signal at m/e 513.







(Ia) R_1=H, R_2=unknown

(Ib) R_1=H, R_2=$CH_2C_6H_4OH$(p)

(V) R_1=Ac, R_2=$CH_2C_6H_4OAc$(p)

(VII) R_1=SO_3^-, R_2=$CH_2C_6H_4OSO_3^-$(p)

(IIa) R_1=H, R_2= unknown, R_3=SO_3^-

(IIb) R_1=H, R_2=$CH_2C_6H_4OH$(p), R_3=SO_3^-

(IV) R_1=R_3=Ac, R_2=$CH_2C_6H_4OAc$(p)

(VI) R_1=Ac, R_2=$CH_2C_6H_4OAc$(p), R_3=SO_3^-

(VIII) R_1=R_3=SO_3^-, R_2=$CH_2C_6H_4OSO_3^-$(p)

(III)

Synthetic Renilla luciferyl sulfate (IIb) Treatment of synthetic Ib (100 mg)[9] at room temp. with acetic anhydride (4 ml) containing one drop of conc. H_2SO_4 afforded triacetate IV

(86% yield) [$\delta_{ppm}^{CDCl_3}$ 2.18 (3H,s), 2.28 (3H,s), 2.30 (3H,s), 4.40(2H,s), 4.68 (2H,s), 8.00 (1H,s)]. The triacetate (20 mg) dissolved in CH_2Cl_2 (2 ml) was carefully treated with 1% methanolic NH_3 (2 ml) at 0° to hydrolyze only the enol acetate moiety. After a few minutes of reaction, the mixture was evaporated to dryness. The residue was practically pure diacetate V [mass spec. m/e 479 (M^+-28),437 (M^+-28-42)]. To this residue was added a solution of pyridine-sulfur trioxide complex (60 mg) in pyridine (1 ml). After stirring the mixture at room temp. for 30 min, 1% methanolic NH_3 (2 ml) was added and the resulting precipitate was filtered off. The filtrate was dried under vacuum, and the residue was extracted with methanol. Tlc of the extract on silica gel (Merck 60F-254 plates) with MeOH:CH_2Cl_2 (1:10) yielded the diacetate-sulfate VI as Na salt[10] (12.3 mg) [$\delta_{ppm}^{DMSO-d_6}$ 2.25 (3H,s), 2.29 (3H,s), 4.12 (2H,s), 4.41 (2H,s), 6.98 (2H,$\underline{A}_2'B_2'$), 7.1-7.5 (9H,m), 7.93 (2H,$C_2'\underline{D}_2'$), 8.38 (1H,s)], which is more polar than V. Starting Ib (6.1 mg) was recovered from the tlc. Hydrolysis of two acetoxy groups of VI was achieved by treating it (12 mg) with 0.5% methanolic NaOH (1 ml) at room temp. for several minutes. The product was neutralized with acetic acid, concentrated to one third volume, and then chromatographed on a plate of silica gel (Merck 60F-254) with MeOH:CH_2Cl_2 (1:5) affording the luciferyl sulfate IIb as Na salt[11] (11 mg), in a pale yellow crystalline solid from methanol-ether [λ_{max}^{MeOH} nm (ε) 276 (35800), $\lambda_{max}^{MeOH-NaOH}$ 303 (31700); IR in Fig. 1; $\delta_{ppm}^{DMSO-d_6}$ 3.98 (2H,s), 4.38 (2H,s), 6,62 (2H,$\underline{A}_2'B_2'$), 6.83 (2H,$\underline{C}_2'D_2'$), 7.08 (2H, $A_2'\underline{B}_2'$), 7.1-7.5 (5H,m), 7.69 (2H,$C_2'\underline{D}_2'$), 8.21 (1H,s)]. Paper electrophoresis with 0.1M acetate buffer (pH 4.6): 3.6 cm toward anode after 3 hr at 600V/25cm. This compound (IIb) is readily converted to the luciferin (Ib) by heating in 0.1N HCl,[8] or by treatment with methanolic 0.1N HCl at room temp. for 2 min.

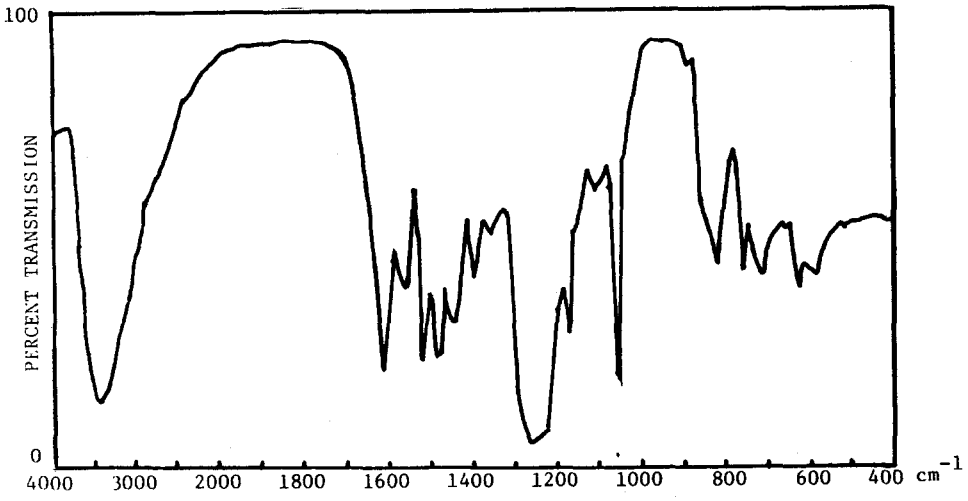


Fig. 1. Infrared spectrum of synthetic Renilla luciferyl sulfate (IIb) (Na salt)

Coelenterazine (Ib) has been found in some luminescent sea animals as their luciferin or precursor of luciferin: Watasenia preluciferin[9] from luminous squid Watasenia scintillans, Oplophorus luciferin from decapod shrimps,[12] and from luminous Myctophina fish.[13] It is now believed to be widely distributed as their luciferin among various luminescent coelenterates such as Cavernularia and Ptilosarcus[14] in addition to Renilla.

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10) VI: Found: C, 57.66; H, 3.95; N, 6.57; Na, 4.0(atomic abs.). $C_{30}H_{25}O_8N_3SNa\cdot H_2O$ requires: C, 57.41; H, 4.18; N, 6.70; Na 3.7%. During the tlc VI converted from its pyridinium salt to Na salt.

11) IIb: Found: C, 52.05; H, 4.54; N, 6.77; Na, 4.2 (atomic abs.). $C_{26}H_{20}O_6N_3SNa\cdot 4H_2O$ requires: C, 52.25; H, 4.72; N, 7.03; Na, 3.9%.

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