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STRUCTURE OF *CYPRIDINA* LUCIFERINOL, "REVERSIBLY OXIDIZED *CYPRIDINA* LUCIFERIN"[1]

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Structure of *Cypridina* luciferinol (reversibly oxidized luciferin), which is produced by autooxidation or PbO_2 oxidation of *Cypridina* luciferin without light production and can be reduced to *C.* luciferin with $Na_2S_2O_4$ or $NaBH_4$, was determined to be 2.

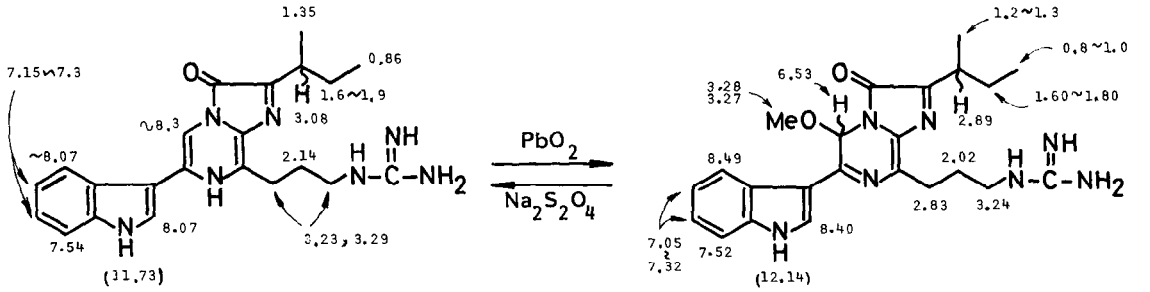
The chemical reactions occurring in bioluminescence and chemiluminescence of *Cypridina* luciferin (1) have been well established.[2] About 60 years ago Harvey[3] observed an interesting phenomenon that a test tube of clear solution of crude *Cypridina* luciferase (which initially contained some of *C.* luciferin), although it may give off no light when shaken with air (because the luciferin is consumed), after standing a day or so emits quite a bright light if disturbed, and later found that reducing agents such as sodium hydrosulfite are effective in restoration of the luminescence by reduction of an oxidized product of luciferin present in the luciferase solution. In 1936, Anderson[4] established that the oxidation in presence of the luciferase with light production gives a product (*C.* oxyluciferin)[2] which cannot be reduced, but oxidation without light production, taking place spontaneously or with oxidants like ferricyanide, is reversible. He named the product in the latter case "reversibly oxidized luciferin".

During the spontaneous oxidation of luciferin in aq. solution[5] the VIS maximum changes from 435 nm to 465 nm and finally becomes colorless. The first oxidation product having λ_{max} at 465 nm is the "reversibly oxidized luciferin", which by addition of hydrosulfite and then *C.* luciferase gave a flash of light. Lead dioxide and diphenylpicrylhydrazyl radical (DPPH) were also effective for this oxidation. Although the proudct is fairly unstable and difficult to handle, we were albe to isolate it and determine its structure to be 2.

Synthetic (±)-*Cypridina* luciferin (1)[6] was dissolved in methanol and treated with PbO_2[7] at room temp for 20 min. After filtration, the filtrate was concentrated *in vacuo* and the residue was chromatographed on a silica gel column twice using at first a mixed solvent composed of AcOEt:MeOH:H_2O = 5:1.1:1 and secondly 30% i-PrOH/CH_2Cl_2. The purified product was dissolved in acetone and precipitated by addition of benzene, yielding a dark-red powder [*Cypridina* luciferinyl methyl ether (3); FAB-MS m/z 436 (M+H); UV (i-PrOH) nm (ε) 472 (16,000), 310 (7,700), 260 (9,200), 254 (9,600), 248 (9,300); PMR in Fig. 1], which was un-

stable in aqueous solutions, but fairly stable in methanol and even more stable in i-PrOH or DMSO.

The PMR spectrum of 3 taken in DMSO-d_6 (Fig. 1) indicated the presence of 3-indolyl moiety and two side chains unchanged. The signal of the proton on the dihydropyrazine ring of the luciferin (1) was markedly shifted in the spectrum of 3 toward a higher field (8.33 ppm ⟶ 6.45 ppm in $CDCl_3$:CD_3OD = 7:3) and a new signal corresponding to a methoxy group appeared at 3.28 ppm (the product 3 was actually a mixture of two diastereoisomeric luciferyl methyl ethers and hence two methoxy peaks appeared at 3.28 and 3.27 ppm). The PMR assignments as well as the molecular weight 435 disclosed structure 3 for the luciferyl methyl ether.



Cypridina luciferin (1) C. luciferyl methyl ether (3)

The signals in parentheses disappeared after addition of D_2O

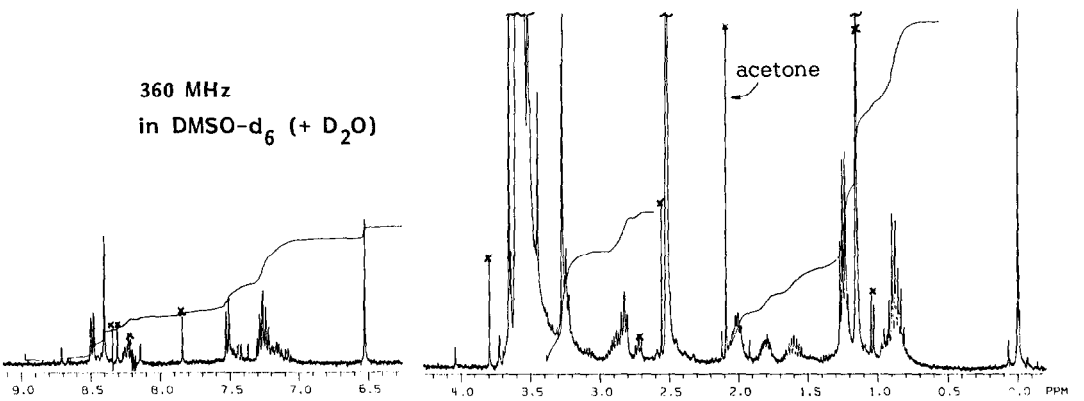


Fig. 1. PMR spectrum of C. luciferyl methyl ether (3)

When isopropanol and n-butanol were used instead of methanol as the solvent, luciferyl i-Pr ether (4) and n-Bu ether (5), respectively, were obtained (Fig. 2). Parent luciferinol (2) was obtained by oxidation of luciferin (1) with DPPH in DMF, and much more unstable than the ether derivatives, 3 ~ 5. C. luciferinol (2) was characterized by FAB-MS [m/z 422 (M+H)] and PMR spectrum; the proton signal on the pyrazine ring of 2 was a broad singlet which became a sharp singlet by addition of D_2O, indicating a hydroxyl group on the position of the pyrazine ring.[8]

(±)-Luciferinol (2)[8] has two asymmetric carbon atoms and hence exists in two diastereoisomeric forms; HPL chromatogram (Fig. 2) showed two peaks in case of 2, but other alkyl ethers showed only one peak and no separations were observed in this condition.

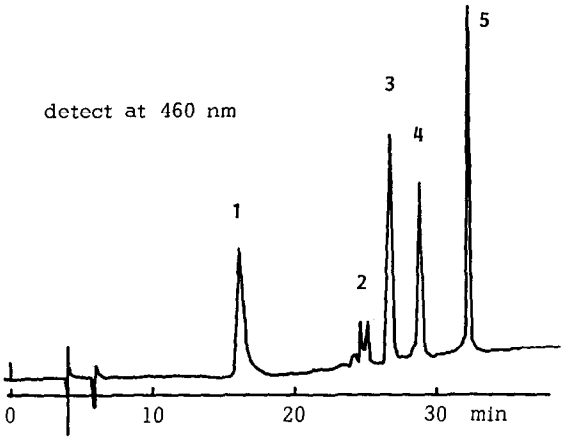
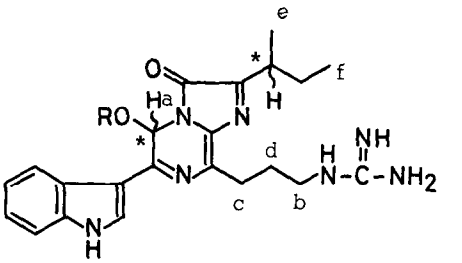


Fig. 2. HPLC of C. luciferin and its oxidized products, 2 to 5.

Develosil ODS-5; MeOH:H_2O(containing 0.2M NH_4OAc) 0-10 min 50:50; 10-40 min linear glad. 50:50 → 95:5; flow rate 1.0 ml/min; temp. 35°C



Cypridina luciferinol (2) R= H
Luciferyl methyl ether (3) R= Me
Luciferyl i-propyl ether (4) R= i-Pr
Luciferyl n-butyl ether (5) R= n-Bu

Optical- and diastereo-isomers of the luciferyl methyl ether (3) could be separated by HPLC using a column containing an optically active solid phase [Chiralpak OT(+)] (Fig. 3); three isomers, 3a, 3b, and 3c, could be separated in a ratio of ca 2:1:1. These isomers showed

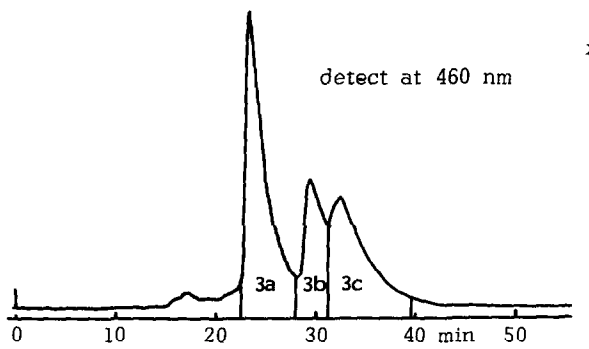


Fig. 3. HPLC resolution of optical isomers of luciferyl methyl ether (3). Chiralpak OT(+) 4.6x250 mm; MeOH:H_2O (8:2) containing 0.2M NH_4OAc; flow rate 0.5 ml/min; temp. 20 °C

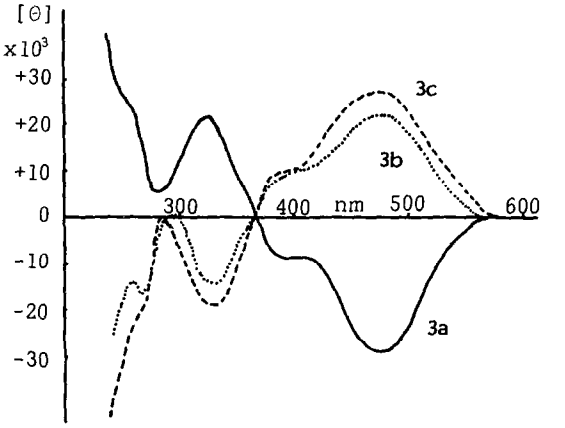


Fig. 4. CD spectra of 3a, 3b, and 3c in MeOH:H_2O (8:2) containing 0.2M NH_4OAc

very large CD (Fig. 4); the molecular ellipticities being around 30,000. 3a showed a negative Cotton, whereas a positive Cotton was observed with 3b and 3c. The CD curves of 3b and 3c were almost mirror image of that of 3a. Since only a small CD is expected from the asymmetric center of 2-butyl side chain,[9] the observed large CD must originate from the chiral center having the methoxy group. Therefore, 3b and 3c have the same methoxy configuration and different configuration of the 2-butyl group, whereas 3a may be a mixture of diastereoisomers which have the opposite configuration of the methoxy group to that of 3b and 3c.

Luciferinol (2) and its methyl ether (3) gave no light with C. luciferase, but after reduction with $Na_2S_2O_4$ or $NaBH_4$ they gave light. Formation of the luciferin (1) on this reduction was proved by HPLC; the luciferin fraction was obtained by HPLC separation of the reaction mixture and characterized by measurement of the rate of bioluminescence with C. luciferase. Yields of the luciferin (1) by reduction with $Na_2S_2O_4$ from 2 and 3 were 32% and 50%, respectively. Reduction with $NaBH_4$ gave the luciferin in poorer yields.

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4. R. S. Anderson, J. Cell. Comp. Physiol., 8, 261 (1936).
5. O. Shimomura, T. Goto, and Y. Hirata, Bull. Chem. Soc. Jpn., 30, 929 (1957).
6. S. Inoue, S. Sugiura, H. Kakoi, and T. Goto, Tetrahedron Lett., 1609 (1969).
7. Lead dioxide was prepared as follows: lead tetraacetate was decomposed with methanol and the black solid formed was washed with methanol, acetone, and then ethyl ether, dried and stored at -20 °C.
8. C. luciferinol (2) (a mixture of two diastereoisomeric DL-pairs): dark-red powder; PMR (200 MHz, in acetone-d_6) ppm 11.14 (1H, Br.s. → disappeared by addition of D_2O, ind-NH), 8.63 (1H, dd, J = 6 & 2.5 Hz, ind-H-4), 8.35 (1H, br.s. → s by addition of D_2O, ind-H-2), 7.55 (1H, dd, J = 6 & 3 Hz, ind-H-7), 7.26 (2H, m, ind-H-5 & 6), 6.53 (1H, br.s → s by addition of D_2O, Ha), 3.47 (2H, br.q → t, J = 7 Hz by addition of D_2O, Hb), 2.96 (2H, t, J = 7 Hz, Hc), 2.22 (2H, qi, J = 7 Hz, Hd), 1.30 & 1.28 (3H, d, J = 7 Hz, He), 0.94 & 0.92 (3H, t, J = 7 Hz, Hf).
9. Natural C. luciferin showed only very small CD.

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