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STRUCTURE OF CYPRIDINA BILUCIFERYL, A DIMER OF CYPRIDINA LUCIFERYL RADICAL HAVING BIOLUMINESCENT ACTIVITY [1]

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Structure of Cypridina biluciferyl (luciferyl radical dimer), which is produced by chemical oxidation of C. luciferin with such as ferricyanide, was determined to be the symmetric 5,5'-dimer of C. luciferin. It gives light in the presence of C. luciferase, although the bioluminescent rate is very low. We suggest that the biluciferyl is an intermediate in the oxidation of the luciferin to C. luciferinol.

Cypridina luciferin gives brilliant light with C. luciferase in the presence of oxygen.[2] The luciferin is very susceptible toward oxygen even in the absence of the enzyme. Thus, orange colored aqueous solution of the luciferin turns red in air, and then becomes colorless owing to further autooxidation.[3] The initial red shift indicates the formation of a red substance (we named it luciferin-R[4]). The similar red substance is formed by chemical oxidation of luciferin (1) with ferricyanide,[3] lead dioxide, or diphenylpicrylhydrazyl radical (DPPH).[4] It emits light only very slowly in the presence of the enzyme, but the luminescent activity similar to the luciferin can be restored by chemical reduction with sodium hydrosulfite.[5] One of us had suggested that a one-electron oxidation of (1) gave the luciferyl radical, which dimerized to its dimer.[4]

Recently we isolated Cypridina luciferinol (3),[6] which is produced without light production by oxidation of luciferin (1) with lead dioxide or DPPH and which can be reduced with sodium hydrosulfite to the luciferin (1). However, luciferinol (3) is a two electron oxidation product and contrary to the previous observation[4] it has no bioluminescent activity with the luciferase.[6] We found by means of HPLC analysis that besides luciferinol (2) another oxidation product was also formed, particularly when ferricyanide was used as the oxidant. Although the product was extremely labile, we could determine its structure as described below.

3,7-Dihydro-6-(3-indolyl)-2-methylimidazo[1,2-a]pyrazin-3-one (4)[7] was first used as a model compound for the luciferin (1) to elucidate structure of the oxidation product, since the corresponding oxidation product of the luciferin model (4) is somewhat more stable and easier to handle than that of the luciferin (1) and since its structure would be easier to be analyzed. The luciferin model (4) was dissolved in water and to this solution 1.1 eq of aq $K_3[Fe(CN)_6]$ was added. The mixture was immediately extracted with dichloromethane and the organic layer was dried over

anhydrous sodium sulfate and then concentrated in vacuo at room temperature. The residue was refined by silica gel TLC (Merck Art. 5715 plate) using 5% acetone-AcOEt as a developing solvent. The purified product was washed with AcOEt and centrifuged, yielding a dark violet powder (5), which is unstable in water or methanol, but tolerably stable in aprotic solvents such as dimethylsulfoxide (DMSO), acetone, and dichloromethane (in the latter two solvents the purified product was soluble, but the powder was not).

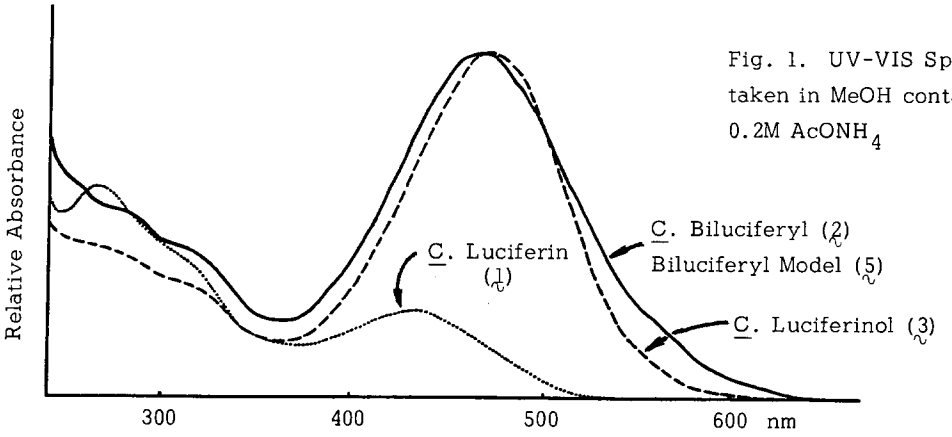
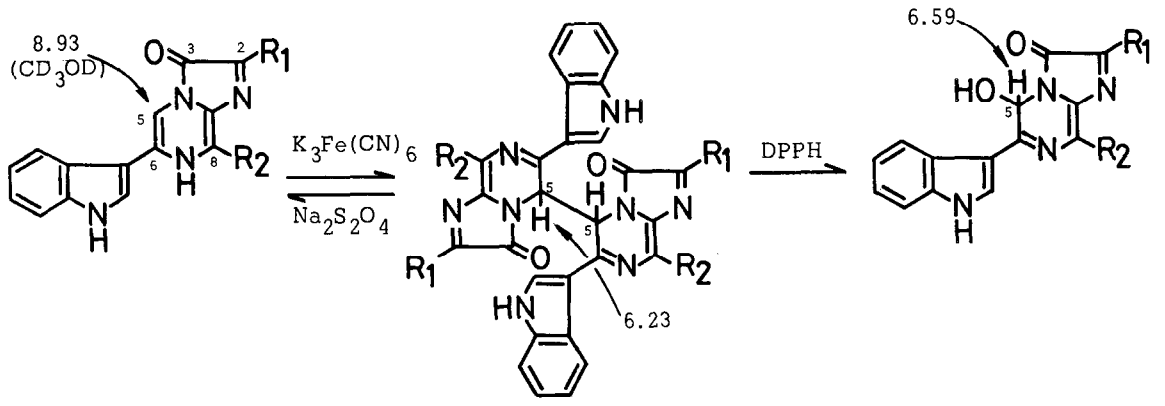


Fig. 1. UV-VIS Spectra taken in MeOH containing 0.2M $AcONH_4$

The product (5)[8] [FAB-MS m/z 527 (M+1)] showed very similar electronic (Fig. 1) and PMR[8] spectra to those of the corresponding luciferinol model (6),[9] which was synthesized by oxidation of (4) with DPPH (2.9 eq).[6] The FAB-MS spectrum indicated a dimeric structure for (5). Since only one-half signals of the total protons were detected, (5) must be a symmetric dimer. The position of dimerization was determined as follows. For solubility problem, we used N-methylindolyl derivatives for further experiments. When a dichloromethane solution of the analogous symmetric dimer (7) having a methyl group at C-2 was mixed with a dichloromethane solution of another symmetric dimer (8) having an ethyl group at C-2, the asymmetric dimer (9) was produced and the three dimers, (7), (8) and (9), soon became in equilibrium. FAB-MS of the equilibrium mixture gave peaks at m/z 555, 569, and 583, corresponding to (7), (9), and (8), respectively. The equilibration was so fast that (9) could not be isolated by means of HPLC of TLC unless at low temperatures below -20 °C. PMR spectrum (Fig. 2) of the mixture clearly showed, besides two singlet signals at 6.07 and 6.08 ppm assignable to H-5 of the symmetric dimers, a pair of doublets (J = 1.8 Hz) at 6.06 and 6.09 assignable to H-5 of the asymmetric dimer (9). Incidentally, H-8 signal could be distinguished from H-5 signal by a long-range coupling between the methyl on C-2 and H-8.

Cypridina biluciferyl (2) was similarly obtained from C. luciferin (1) by oxidation with ferricyanide in DMSO followed by chromatography on an alumina column [Woelm N Act I gel packed with AcOEt, developing solvent:upper layer of AcOEt:i-PrOH:H_2O = 5:2:3]. Although the product could not be completely purified, its precise electronic spectrum (Fig. 1) obtained by a three-dimensional detector (Shimadzu SPD-M1A) attached to a column [Develosil ODS-5 HPLC at -50 °C with MeOH containing 0.2M $AcONH_4$] was completely superimposable to that of the model dimer (5). FAB-MS [m/z 809 (M+1)] and PMR spectra[10] also indicated the structure of biluci-

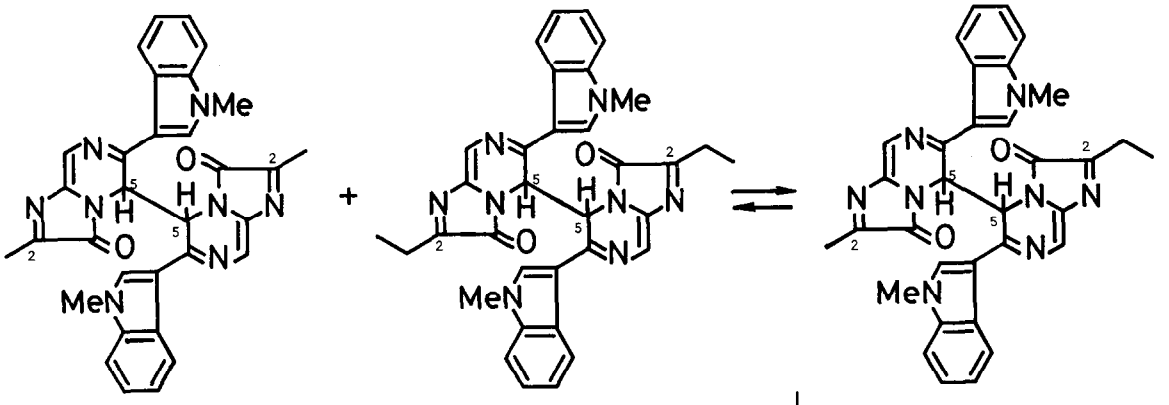


C. Luciferin (1)
Luciferin Model (4)

C. Biluciferyl (2)
Dimer of Luciferin Model (5)

C. Luciferinol (3)
Luciferinol Model (6)

C. luciferin series: $R_1 = -CH(CH_3)CH_2CH_3$, $R_2 = -CH_2CH_2CH_2NHC(=NH)NH_2$
Luciferin model series: $R_1 = CH_3$, $R_2 = H$
PMR chemical shifts (ppm in CD_3COCD_3) are for the model compounds



(7) (8) (9)

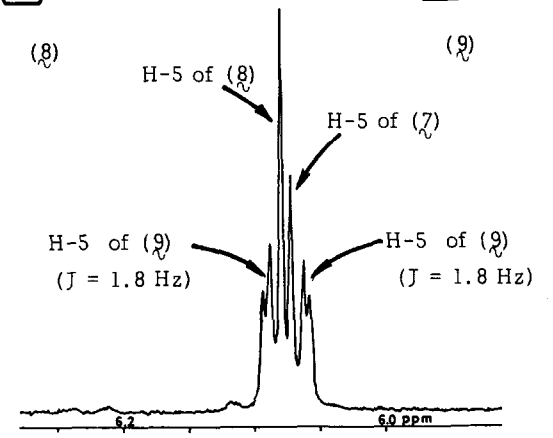


Fig. 2. PMR spectrum of an equilibrium mixture of (7), (8), and (9) (400 MHz, in CD_2Cl_2)

feryl to be (2). C. biluciferyl (2) was oxidized with DPPH to C. luciferinol (3) and reduced with sodium hydrosulfite or acid[11] to C. luciferin (1). Whereas luciferinol (3) gave no light with C. luciferase, bioluminescence was observed on biluciferyl (2) in presence of the enzyme, although the reaction was very slow. The above results indicate that (2) is an intermediate in the oxidation of (1) to (3) and the slow luminescent reaction of "reversibly oxidized luciferin" that Anderson had observed[5] could be due to biluciferyl (2). Because almost the same rate of light production was observed on biluciferyl (2) in presence of different amounts of C. luciferase, we consider that biluciferyl (2) is slowly disproportioned to luciferin (1) and probably luciferinol (3) (unconfirmed),[11] and the former gives light with the enzyme.

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8. The biluciferyl model (5): PMR (200 MHz, in acetone-d_6) δppm 10.06 (1H, br.s, ind-NH), 8.35 (1H, br.d, J = 7.5 Hz, ind-4), 8.24 (1H, m, ind-2), 7.55 (1H, br.d, J = 7.5 Hz, ind-7), 7.24 (2H, m, ind-5 & 6), 6.53 (1H, br.s, H-8), 6.23 (1H, s, H-5), 2.28 (3H, br.s, CH_3 at C-2); protons are doubled in this molecule.
9. The luciferinol model (6): PMR (200 MHz, in acetone-d_6) δppm 11.08 (1H, br.s, disappeared by addition of D_2O, ind-NH), 8.59 (1H, m, ind-4), 8.33 (1H, br.t, s by addition of D_2O, ind-2), 7.52 (1H, m, ind-7), 7.24 (2H, m, ind-5 & 6), 7.07 (1H, br.s, H-8), 6.59 (1H, m, s by addition of D_2O, H-5), 2.28 (3H, br.s, CH_3 at C-2).
10. C. biluciferyl (2): PMR (200 MHz, in DMSO-d_6) since this sample was not completely pure and since it is a mixture of two diastereomers, the spectrum of (2) could not be analyzed completely; 6.22 ppm (1H, s, H-5).
11. In acidic solution, biluciferyl (2) is disproportioned to luciferin (1) and some unknown compound(s). Luciferinol (3) is also decomposed by acid treatment.

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