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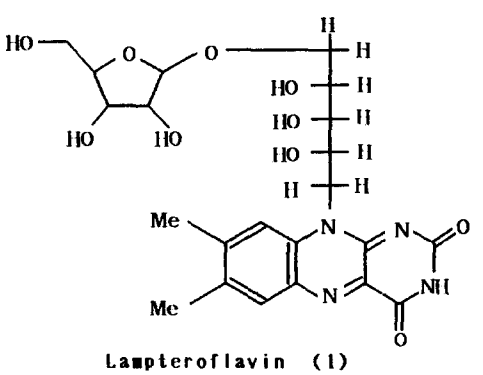
LAMPTEROMYCES BIOLUMINESCENCE – 2 LAMPTEROFLAVIN, A LIGHT EMITTER IN THE LUMINOUS MUSHROOM, *L. japonicus*

Minoru Isobe,* Duangchan Uyakul and Toshio Goto
Laboratory of Organic Chemistry, Faculty of Agriculture,
Nagoya University, Chikusa, Nagoya 464, Japan

A new fluorescent compound, lampteroflavin, was isolated from a luminous mushroom (Lampteromyces japonicus). Its properties suggest that it has a function as the light emitter in the mushroom bioluminescence. Its structure was assigned to be pentofuranosyl riboflavin, 1.

The emission of light from fungal species has been intermittently studied for a considerable period of time. The luminosity of fungi generally occurs in the mycelia *(Omphalia flavida)*, occasionally in the fruiting body or in part of it *(Pleurotus japonicus)*, or sometimes it is present in both *(Pleurotus olearius)*.[1] Moon night mushroom (月夜茸, *Pleurotus japonicus, Lampteromyces japonicus)* is known as a luminous mushroom as well as a poisonous mushroom in Japan. It occurs only for two weeks in October in a year. Information concerning the luminescence of *L. japonicus* has been insufficient to start this study. The first study on light intensity and temperature was done by Kawamura in 1915.[2]

The luminescence is observed in the gill (inner side of the fruiting body). The bioluminescence from wild mushroom is continuous but not so bright enough to record its spectrum. Since the spectrum should be identical with the fluorescent substance to emit light, several fluorescent compounds have been isolated from *L. japonicus* by Endo et al. in 1970 (from methanol extracts).[3] They concluded that illudin S (or lampterol, isolated in 4.4g from 80kg mushroom) and ergosta-4,6,8(14),22-tetraen-3-one (13.5 mg/52 kg) were involved in the bioluminescence of the mushroom, since their fluorescence emission maxima were reported to be 550 nm and 530 nm, respectively; the latter being identical with the bioluminescence peak of luminous fungi, for example, *Armillaria mellea, Collybia velutipes,* and *Omphalia flavida.*



Lampteroflavin (1)

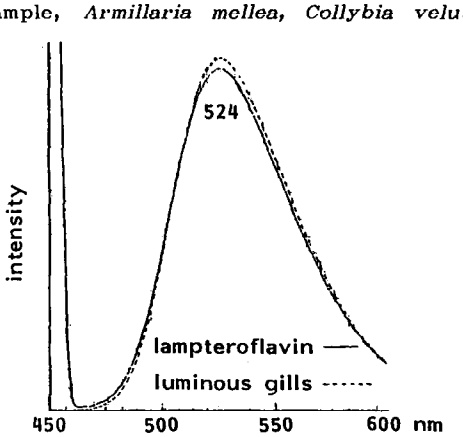


Fig.1

Recently, we have succeeded in measuring the bioluminescence spectrum of the mushroom, which showed the maximum at 524 nm. An aqueous extract of the mushroom gave a major fluorescent compound which was identified as riboflavin, (Rf), (*3*). It was isolated in less than 1 mg from 5 kg (whole body). Riboflavin showed its fluorescence maximum at 524 nm, which was identical with the bioluminescence spectrum of the mushroom. Thus, riboflavin was concluded to be the light emitter of the mushroom.[4] In our further studies has been found another new fluorescent compound named "**lampteroflavin**", (Lf), (*1*), which is closely related to riboflavin, but very unstable in the mushroom extracts. It is difficult to separate this minor component, lampteroflavin, from frozen mushrooms (whole body) because it usually decomposes during extraction with water. But it sometimes appeared in the HPLC analysis right after the riboflavin peak. When separated with a preparative ODS column, it was eluted with riboflavin as a shoulder, the ratio of Lf:Rf being ca. 1:>12.

We have developed a new method by using alive mushrooms instead of the frozen mushrooms and using only the luminous part (gills) instead of the whole body of the mushroom. Six kilograms of gills were separated from fruiting body and well washed with water. It was then immersed in distilled water (15 L) and air was introduced into this aqueous suspension for 10 hr at 19-21°C. The suspension was filtered through Celite, and the filtrate (20 L) containing lampteroflavin was concentrated by first adsorbing on an ODS column, and eluting with methanol. The methanol eluate, showing green fluorescence, was evaporated to nearly dryness. The residual oil was purified twice by two ODS columns (20x250 mm and 4.0x250 mm, at 5.0 and 0.8 mL/min, respectively, with MeOH:H_2O/ 25:75)[10] to obtain lampteroflavin (ca. 100 μg). The progress in acquiring better Lf:Rf ratio (1:0.3) was obtained by using dil. hydrochloric acid at pH 3[5] instead of distilled water at pH 6 (Lf:Rf=1:1.24). By this way about 4 times of lampteroflavin was obtained. Its UV and fluorescence spectra were identical to the riboflavin spectra, showing UV max (in H_2O) 265, 368 and 445 nm, fluorescence emission maximum at 524 nm, and excitation maximum at 445 nm. The fluorescence spectrum of lampteroflavin was identical to the bioluminescence spectrum of the mushroom as indicated in ***Fig. 1.***

Lampteroflavin was apparently soluble in water though riboflavin was sparingly soluble in water (less than 1 mg in 15ml).[6] Both flavins were analyzed with 500 MHz ^1H nmr to show two Me's signals at around δ 2.5 ppm and two aromatic H's around δ7.9 ppm characteristic to flavins. Lampteroflavin showed signals of a ribityl group same as riboflavin. The proton nmr data are summarized in Table 1. Lampteroflavin exhibited 6 additional signals. One of them (δ5.17 ppm) was assigned to be an anomeric proton (H-1"), since its chemical shift did not change a lot after acetylation. The appropriate double resonance experiments with the ^1H nmr of riboflavin acetate, lampteroflavin, and its acetate led us consider that the 6 protons should be a pentofuranosyl group which is connected to riboflavin with an acetal linkage. There were two methylene groups at C-1' and C-5' which could be assigned interchangeably even in riboflavin itself judging from the value of 0.5 ppm difference between free form and acetate form for primary alcohol. However, this problem was solved by comparing the chemical shifts with those of lampteroflavin and its acetate. We found that the chemical shifts of one of methylene groups of lampteroflavin (δ=4.01 and 3.84 ppm for the free form, δ=3.95 and 3.82 ppm for the acetate form) did not change significantly.[7] This fact suggested that this methylene group should be at C-5' position which further connected to the pentofuranosyl group. Connectivity between the flavin chromophore, the ribityl group and the pentofuranosyl group was concluded by nuclear Overhauser effect by

TABLE 1 ^{1}H NMR DATA of Lampteroflavin & Analogs

assignment	lampteroflavin[a] 1	lampteroflavin acetate[b] 2	riboflavin[a] 3	riboflavin acetate[b]
1'	5.08(m, 12.0,3.0)	5.68(m)	5.18(brd, *)	5.68(m)
1'	4.88(m *)	5.51(m)	*	5.46(m)
2'	4.38(m, 6.7,3.0)	5.68(m)	4.45(m)	5.68(m)
3'	4.01(dd, *)	5.51(m)	3.99(dd, *)	5.46(m)
4'	4.15(m, 11.7, 6.6,3.3)	5.37(m, 4.2,3.1)	3.95(m)	5.42(m)
5'	4.01(brd, 11.7)	3.95(dd, 11.6,4.2)	3.90(brd, 12.5)	4.43(dd, 12.5,2.5)
5'	3.84(brd, 11.7)	3.82(dd, 11.6,3.1)	3.75(dd, 12.5, 6.0)	4.25(dd, 12.5,*)
1"	5.17(d)	5.30(d, 4.2)		
2"	4.15(m)	4.94(m, 7.1,4.2)		
3"	4.07(m)	5.16(dd, 7.1,3.9)		
4"	4.15(m 5.0, 3.3,3.3)	4.26(m, 3.9,3.6, 2.9)		
5"	3.75(brd 13.0)	4.18(dd, 12.0,3.6)		
5"	3.69(brd 13.0)	4.34(dd, 12.0,2.9)		
7-Me	2.57(s)	2.57(s)	2.60(s)	2.57(s)
8-Me	2.45(s)	2.45(s)	2.51(s)	2.45(s)
6	7.84(s)	7.57(s)	7.99(s)	7.57(s)
9	7.89(s)	8.05(s)	8.00(s)	8.04(s)
3	*	8.36(s)	8.47(s)	8.34(s)
5'-Ac				2.29(s)
4'-Ac		2.22(s)		2.22(s)
3'-Ac		2.10(s)		2.08(s)
2'-Ac		1.71(s)		1.75(s)
2"-Ac		2.14(s)		
3"-Ac		2.13(s)		
5"-Ac		2.32(s)		

a) measured in D_2O, t-BuOH (δ 1.27ppm) as standard
b) measured in $CDCl_3$, TMS (δ 0.0ppm) as standard
* not readable

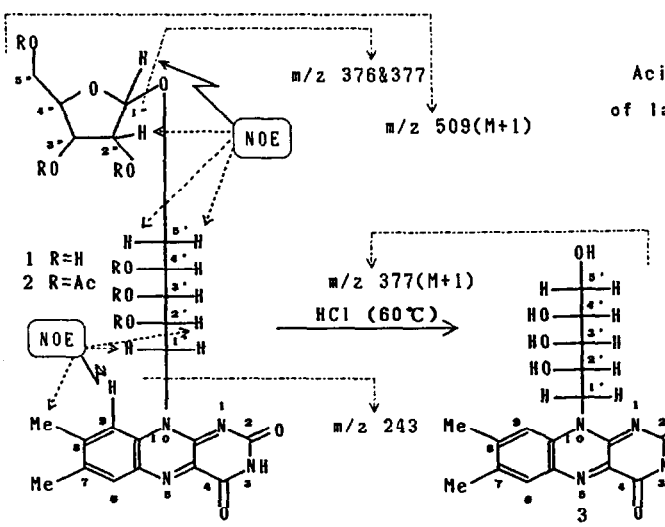


Fig.2 NOE and FAB-ms/ms of Lampteroflavin

Fig.3 Acid hydrolysis of lampteroflavin

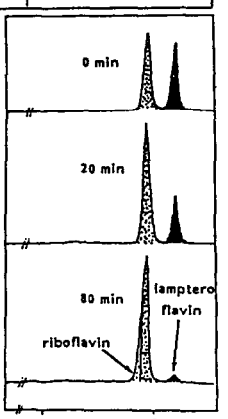
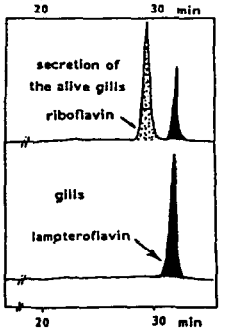


Fig.4 Occurrence of lampteroflavin in gills



irradiating H-1" or H-9, the results being summarized in ***Fig. 2.*** The data above allowed the assignment of lampteroflavin structure as shown in Table 1. The table also includes the first proton assignment of riboflavin itself. High resolution FAB-mass spectrum of lampteroflavin showed a peak at 509.1913 (M+1, calcd for $C_{22}H_{29}N_4O_{10}$ 509.1882) which confirmed the molecular formular. Tandem FAB-MS/MS of the He-collision of m/z 509 ion gave rise to three signals at m/z 377, 376 and 243, suggesting the pentofuranosyl, ribityl, and flavin moieties.[8] The presence of acetal linkage was chemically demonstrated by acid hydrolysis (0.2N HCl, 60°C) of lampteroflavin into riboflavin which was monitored by HPLC analysis.[10] Almost complete hydrolysis was observed in 80 min as shown in ***Fig. 3.***

Furthermore following analytical experiments demonstrated the occurrence of lampteroflavin as the precursor of riboflavin. The gills (600 g) were suspended in distilled water (3 L), aerated for 3 hours at 20°C, and drained. The green fluorescent substances were extracted from the residual gills and from the suspended water to which detectable amount of fluorescent compounds were secreted. HPLC analysis showed that the Lf:Rf ratio in the former was >25:1, while the latter was 2:3 as shown in ***Fig. 4.*** Thus, lampteroflavin was proposed to be the primary green fluorescent compound considered to be the light emitter of the mushroom. Riboflavin was concluded as an artifact, which was produced on acid hydrolysis of lampteroflavin or on degradation with enzymatic system in the mushroom. The occurrence of lampteroflavin is of interest because the structure is the first demonstration of pentofuranosyl riboflavin besides a glucosyl riboflavin.[9] Lampteroflavin may be a very important intermediate in the riboflavin (vitamin B_2) biosynthesis. It could lead us to a new field of biochemical function as a coenzyme.

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References and Notes

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2. S. Kawamura, *J. Coll. Sci. Tokyo,* 35, art. 3, 29 (1915).
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4. M. Isobe, D. Uyakul and T. Goto, *J. of Bioluminescence and Chemiluminescence,* 1, 181-188 (1987).
5. In acidic media, it luminesces with higher intensity than in neutral medium. See ref. #4. No effect on the luminescence intensity was observed with different counter anions, Cl^-, Br^-, I^-, HSO_4^- etc.
6. There was, so far, no report on ^1H nmr analysis of riboflavin in D_2O.
7. For comparison, were prepared 5'-trityl-riboflavin and its per-acetate to show H-5' at δ 5.14 and 4.82 ppm and at δ 5.65 and 5.58 ppm, respectively. The acetylation of the neighbouring hydroxy groups affected the chemical shifts of the 5'-methylene OH as much as 0.5-0.7 ppm without its own acetylation.
8. Reduction of the flavin nucleus in riboflavin and lampteroflavin into dihydro body was observed during FAB-MS measurements, which showed $M+1^+$ into $M+3^+$ in the positive FAB spectrum. Such reduction was only observed when glycerin was used as matrix, but no reduction was observed when measured in dithiothreitol matrix.
9. a) L.G. Whitby, *Biochem.,* 50, 433-438 (1952). b) L.G. Whitby, *Biochemical Journal,* 57, 390-396 (1954). c) L.G. Whitby, *Method in Enzymology,* 18b, 404-413 (1971). d) Y. Suzuki and K. Uchida, *ibid,* 66, 327-333 (1980). e) K. Uchida and Y. Suzuki, *Agric. Biol. Chem.,* 38, (1) 195-206 (1974). f) H. Ohkawa, N. Ohishi and K. Yagi, *J. Nutr. Sci. Vitaminol.,* 29, 515-522 (1983). g) S. Kasai, S. Isemura, M. Masuoka and K. Matsui, *The Journal of Vitaminology,* 18, 17-23 (1972).
10. HPLC was monitored both with UV at 254 nm and fluorescence Em_{max} 524 nm and Ex_{max} 445 nm.

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