Bioluminescence

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The Chemical Basis of Fungal Bioluminescence**

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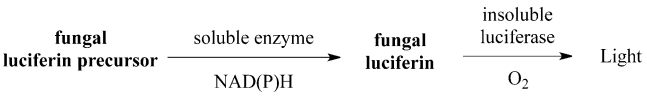
Abstract: *Many species of fungi naturally produce light, a phenomenon known as bioluminescence, however, the fungal substrates used in the chemical reactions that produce light have not been reported. We identified the fungal compound luciferin 3-hydroxyhispidin, which is biosynthesized by oxidation of the precursor hispidin, a known fungal and plant secondary metabolite. The fungal luciferin does not share structural similarity with the other eight known luciferins. Furthermore, it was shown that 3-hydroxyhispidin leads to bioluminescence in extracts from four diverse genera of luminous fungi, thus suggesting a common biochemical mechanism for fungal bioluminescence.*

Bioluminescence of higher fungi has been known at least since the time of the ancient Greeks.[1] Fruiting bodies of several species produce a constant bright glowing light, which can be seen easily with the naked eye. The luminescence of cell-free extracts from luminous fungi was demonstrated by Airth and McElroy in 1959,[2] with the emission of light achieved by adding nicotinamide adenine dinucleotide phosphate (NADPH) to a mixture of cold and hot aqueous extracts prepared from the mycelium of *Collybia velutipes* and *Armillaria mellea* fungi. The terms "cold and hot extracts" were introduced by Dubois in 1885.[3] He found that extraction of luminous beetle biomass with cold water preserved the enzymatic activity of luciferase, while the same extraction using hot water denatured the enzymes to leave intact the low-molecular-weight luminescence substrate—luciferin. The two extracts produced light when mixed together.

The bioluminescence of fungi is thought to be a two-step process. In the first step, a luciferin precursor is reduced by an NAD(P)H-dependent enzyme to a true luciferin. In the second step, luciferin is oxidized by air under luciferase catalysis to produce visible light[4,5] (Scheme 1).



Scheme 1. Two-stage fungal bioluminescence scheme, proposed by Airth and Foerster.[5]

Despite multiple attempts to isolate and structurally characterize fungal luciferins,[6–10] the structural and chemical basis of bioluminescence has remained obscure. The main obstacle impeding the study of fungal luciferins is the difficulty to obtain pure substances suitable for structural studies because of their likely instability and low content in the biomass. As a result, no reliable structural data has been reported concerning the chemical nature of fungal bioluminescence, and the two-stage process of the bioluminescence of fungi remains a hypothesis supported only by indirect data. Cross-reactions between cold and hot extracts from diverse fungal species suggests a uniform bioluminescence mechanism in higher fungi.[11,12]

We focused on assaying the fruiting bodies of several nonluminous fungal species collected in the forests around Krasnoyarsk (Russia) for the presence of a luciferin precursor. For this, the enzymatic bioluminescence assay[13] was carried out on cold extracts from the bioluminescent mycelium of *Neonothopanus nambi*. We found activity, corresponding to a fungal luciferin precursor, to be present in the hot aqueous extracts of five nonluminous forest species. Moreover, the luciferin precursor content in the fruiting bodies of these five nonluminous fungi appeared to be about 100 times higher than in the mycelium of well-known luminous species, such as *N. nambi* and *Mycena citricolor* (see Figure S1 in the Supporting Information). We focused on

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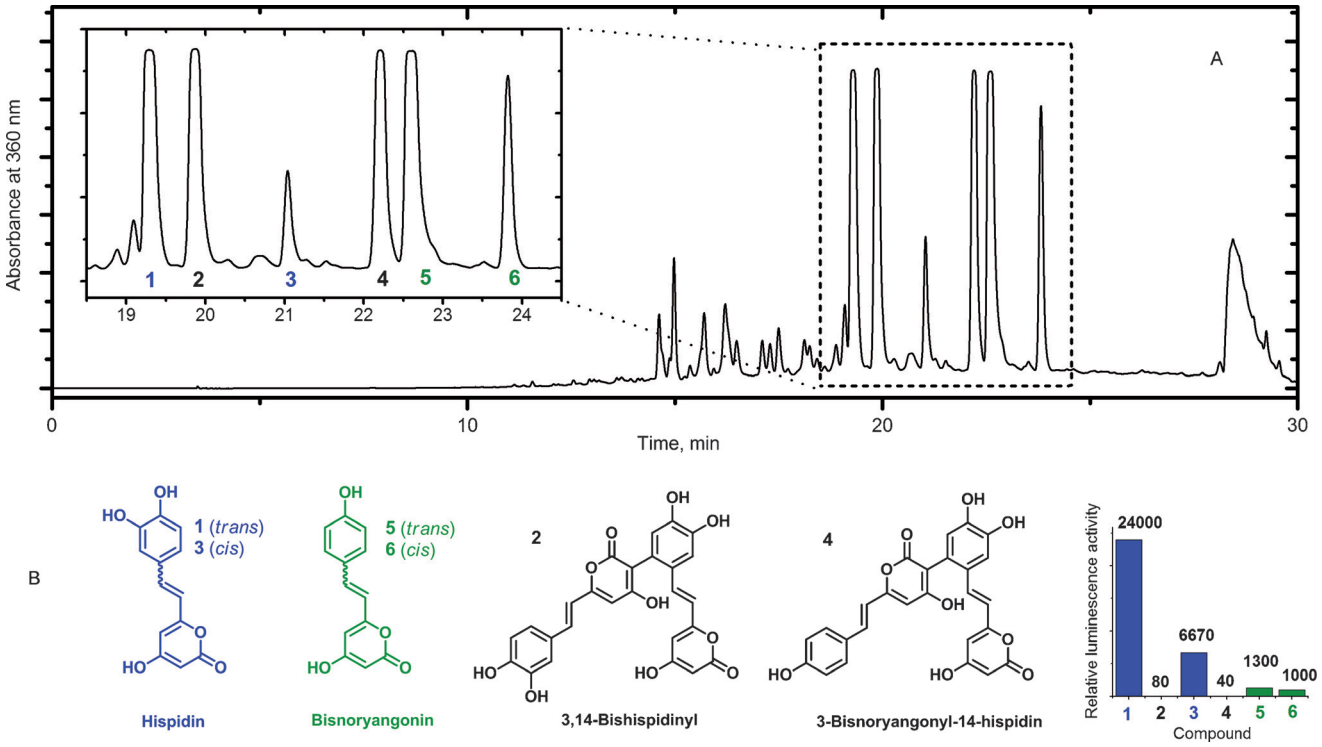


Figure 1. A) Chromatographic profile of the ethyl acetate extract from *Pholiota squarrosa*. B) Structures of the compounds corresponding to the chromatographic peaks. The luminescence activities of **1**–**6** are shown (signal/background ratio).

the fruiting bodies of *Pholiota squarrosa* in further isolation experiments.

The ethyl acetate extract from the fruiting bodies of *Ph. squarrosa* was concentrated and subjected to preparative reversed-phase HPLC to yield six different compounds **1**–**6** (Figure 1). Compounds **1**–**6** showed pronounced activities in the enzymatic bioluminescence assay developed by Oliveira and Stevani.[13] The luminescence of **1**–**6** was 24000, 80, 6670, 40, 1300, and 1000, respectively (signal/background ratio of the assay, see the Supporting Information for details). In addition, two pairs (1,3 and 5,6) were found to undergo tautomerization to produce equilibrium mixtures of tautomers during further chromatography of isolated pure fractions.

Chromatography was then carried out on all six compounds dissolved in either [D_6]DMSO or [D_6]acetone, and their NMR spectra (^{1}H, 2D DQF-COSY, 2D ^{1}H-^{13}C HSQC, and 2D ^{1}H-^{13}C HMBC) were recorded. These spectra in combination with the HRMS spectra allowed their chemical structures to be determined: the spectra of the pairs **1** and **3**, and **5** and **6** were completely identical and contained two sets of signals corresponding to the double-bond isomers of hispidin (**1**: *trans*, **3**: *cis*) and bisnoryangonin (**5**: *trans*, and **6**: *cis*). Compound **2** was identified as a homodimer of hispidin (3,3′-bishispidinyl) and compound **4** as a heterodimer of hispidin and bisnoryangonin (3-bisnoryangonyl-14-hispidin). The structural assignment of **1, 3, 5**, and **6** was also confirmed by comparing the chromatograms and spectra of these samples with those of commercially available hispidin (Sigma) and synthetic bisnoryangonin (for the NMR and HRMS spectra as well as the synthesis, see the Supporting Information).

Hispidin is a well-known representative of a styrylpyrone class of fungal and plant secondary metabolites.[14,15] To confirm its role as a luciferin precursor in luminous fungi, we isolated it from glowing mycelium of *N. nambi*. In these experiments, we took advantage of the observation that soaking the mycelium in distilled water overnight leads to a dramatic increase in the specific activities of the hot and cold extracts (up to 250- and 140-fold, respectively). Ethyl acetate extraction of the hot aqueous extract prepared from the soaked mycelium of *N. nambi* followed by HPLC led to the isolation of two bioluminescent compounds with retention times and UV spectra identical to those of hispidin (**1**) and its *cis* isomer (**3**; Figure 2). Moreover, these two compounds

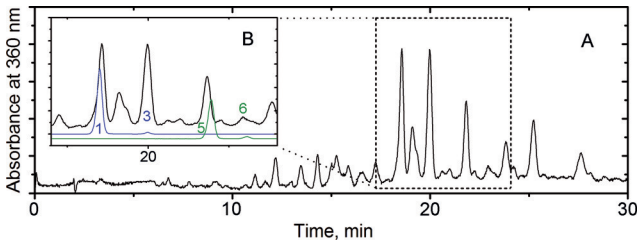


Figure 2. A) RP HPLC chromatogram of the ethyl acetate extract from the mycelium of *Neonothopanus nambi* soaked in distilled water. B) Comparison of (A) with HPLC profiles of synthetic hispidin (blue) and bisnoryangonin (green) under the same chromatographic conditions. Both hispidin and bisnoryangonyn occur as a mixture of *cis* and *trans* double-bond isomers.

showed tautomerization behavior, specific bioluminescence activities, and HRMS spectra (see Figure S6) identical to hispidin and its isomer, identified above as luciferin precursor(s). Overall 0.5 μg of a mixture of tautomers **1** and **3** was isolated from 10 g of the soaked mycelium.

The content of the luciferin precursor hispidin—even in soaked specimens of *N. nambi* and other bioluminescent species, such as *M. citricolor* and *Panellus stipticus*—was many times lower, than that in non-bioluminescent fungi, for example, *Ph. squarrosa*. This fact probably explains why previous attempts to identify hispidin as a substrate of fungal bioluminescence were unsuccessful.

We then tested the hypothesis of Stevani and co-workers that all luminous fungi share a common substrate.[11] We analyzed three other bioluminescent species by using chromatography coupled with an enzymatic assay for the presence and specific activity of hispidin. All the analyzed specimens (*M. citricolor*, *P. stipticus*, and *Armillaria borealis*) were found to contain hispidin and its *cis* isomer, which showed luminescence activity in the bioenzymatic assay with cold extracts from *N. nambi*.

We also tested commercial hispidin for activity with the cold extracts prepared from the mycelia of *N. nambi*, *M. citricolor*, *P. stipticus*, and *A. borealis* upon addition of NADPH. In all cases, we observed dose-dependent bioluminescence, strongly suggesting that hispidin is a common luciferin precursor of all higher luminous fungi. To confirm this conclusion, we measured the luminescence characteristics of the cold extract from the *Mycena chlorophos* fruiting body upon addition of hispidin and NADPH. The intensity of the light emission produced by the mixture of the cold extract from *M. chlorophos* with hispidin (6.1 μM) and NADPH (0.18 mM) was easily seen with the naked eye and comparable to that of the live fruiting body of the same fungus (Figure 3 A–C). The luminescence spectrum of an in vitro mixture was identical to the in vivo bioluminescence spectrum of the *M. chlorophos* fruiting body, and also with the fluorescence emission spectrum of hispidin (Figure 3 D).

In accordance with the previous studies of fungal bioluminescence, our results showed that two different enzymatic activities within the cold extract were required to produce light from hispidin and NADPH. The first enzyme utilized hispidin and NADPH as substrates and produced true luciferin, which is capable of luminescence when mixed with the second enzyme (luciferase). No additional cofactors except molecular oxygen were required for the reaction of luciferin with luciferase. The two enzymes present in the cold extract could be separated by two independent methods: ultracentrifugation or gel filtration using a Superdex 75 column (see the Supporting Information for experimental details).

Considering the likely role of hispidin as the fungal luciferin precursor, we converted it enzymatic into luciferin. For this purpose, commercial hispidin was incubated with a partially purified and soluble NADPH-dependent enzyme obtained by gel filtration of the cold extract from *N. nambi* mycelium (ca. 35 kDa fraction, Superdex 75 column) in the presence of NADPH. HPLC analysis of the reaction mixture showed gradual appearance of one major new component

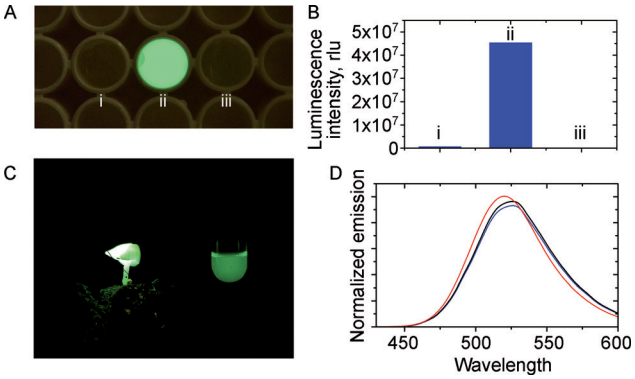


Figure 3. Comparison of the A) in vitro luminescence of the cold extract from the *M. chlorophos* fruiting body with the i) hot extract from the same fungus and NADPH, ii) hispidin and NADPH, iii) NADPH. Exposure 5 s, ISO 6400, F3.5, NIKON D5100. B) The same mixtures as in (A) measured with a Berthold Centro LB960 luminometer at 10 min accumulation. i) 650 000 rlu, ii) 45 000 000 rlu, iii) 43 000 rlu. C) In vivo luminescence of the *M. chlorophos* fruiting body compared to the in vitro luminescence of the cold extract from the *M. chlorophos* fruiting body with hispidin and NADPH. D) Fluorescence spectra of hispidin in methanol, with excitation at 379 nm (black line), in vivo bioluminescence spectrum of the *M. chlorophos* fruiting body (red line), and in vitro luminescence of the cold extract from *M. chlorophos* with hispidin and NADPH (blue line).

($t_r = 17.2$ min), reaching a maximum concentration 35 min after the onset of the reaction (Figure 4). At that point the reaction was stopped by acidifying to pH 2, and the compound eluting at 17.2 min was isolated by HPLC (19 μg was obtained from 32 μg of hispidin).

The new compound produced a bright NADPH-independent luminescence when mixed with the cold extract of *N. nambi* (Figure S8). We also found that the microsomal fraction of the cold extract was responsible of this luminescence, in full accordance with Airth's hypothesis[2,4,5] and findings by Stevani and co-workers[11–13] (Scheme 1), thus confirming the functional role of this compound as the fungal luciferin.

The UV/Vis spectra of luciferin were similar to those of hispidin (Figure 5). The ^{1}H NMR spectrum of fungal luciferin revealed the same pattern of protons as in hispidin. However, the disappearance of one signal corresponding to H-3 of hispidin was observed in luciferin (Figure 6). The HRMS spectrum of luciferin showed a molecular ion with m/z 263.0571, which corresponds to the formula $C_{13}H_{11}O_6^+$ (calcd m/z 263.0550). This formula shows that fungal luciferin

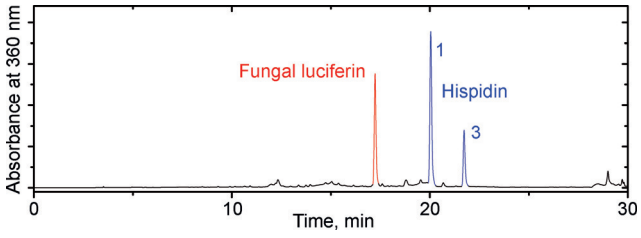


Figure 4. Enzymatic synthesis of fungal luciferin from hispidin catalyzed by a 35 kDa fraction of the cold extract from *N. nambi* mycelium in the presence of NADPH.

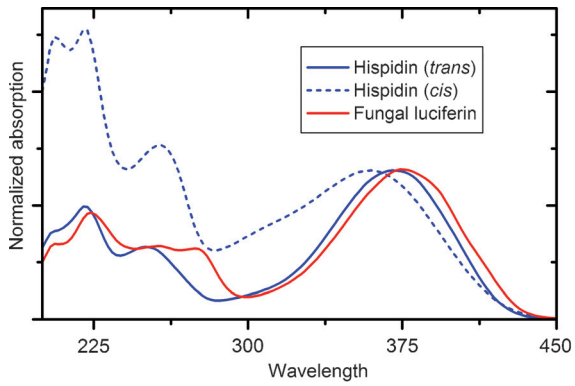
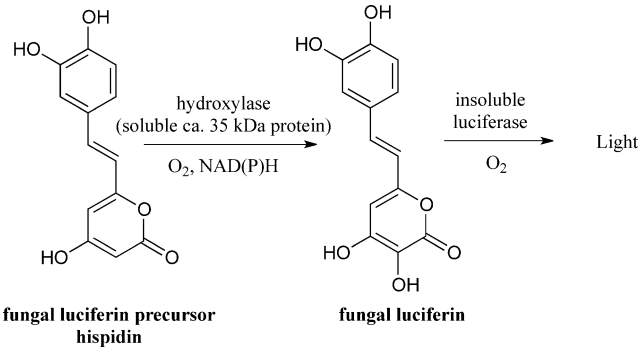


Figure 5. UV/Vis spectra of hispidin and fungal luciferin.



Scheme 2. Mechanism of fungal bioluminescence.

contains one additional oxygen atom compared to hispidin. All these data taken together allow unambiguous structural assignment of fungal luciferin as 3-hydroxylated hispidin or (*E*)-6-(3,4-dihydroxystyryl)-3,4-dihydroxy-2*H*-pyran-2-one (Figure 6).

The biochemical action of the soluble NADPH-dependent enzyme responsible for luciferin biosynthesis is hydroxylation, in contrast to the previous theory that it would reduce a luciferin precursor, a hypothesis that persisted for more than 50 years[2,16]. In fact, many oxygen-dependent hydroxylases utilize NAD(P)H as a cosubstrate, because full reduction of one oxygen molecule requires four electrons, while monohydroxylation of one substrate molecule provides only two electrons.[17] The data reported here suggest the mechanism of fungal bioluminescence shown in Scheme 2.

The moderate luminescence activities of hispidin analogues **2** and **4** might be explained by their ability to undergo analogous hydroxylation at the pyranone fragment followed by reaction with luciferase. Our examination of the ability of the cold extract of the fruiting bodies of *Ph. squarrosa* to catalyze 3-hydroxylation of hispidin in the presence of NADPH and to produce light upon addition of 3-hydroxy-hispidin proved the lack of both kinds of enzymatic activities in these extracts.

Our data indicate that the well-described fungal and plant secondary metabolite hispidin is a luciferin precursor in at least four evolutionary distant genera of luminous fungi. The reactivity of the identified luciferin, 3-hydroxyhispidin, with luciferases from the same four luminous fungi coupled with the evolutionary breadth of the considered fungi suggests that most, if not all, luminous fungi utilize hispidin as the luciferin precursor and 3-hydroxyhispidin as luciferin. As evident from the occurrence of hispidin in both luminous and nonluminous fungi, the fungal bioluminescence is defined by the presence of hispidin-3-hydroxylase and luciferase rather than by the ability to biosynthesize the luciferin precursor hispidin.

Keywords: bioluminescence · bioorganic chemistry · biosynthesis · luciferin · natural products

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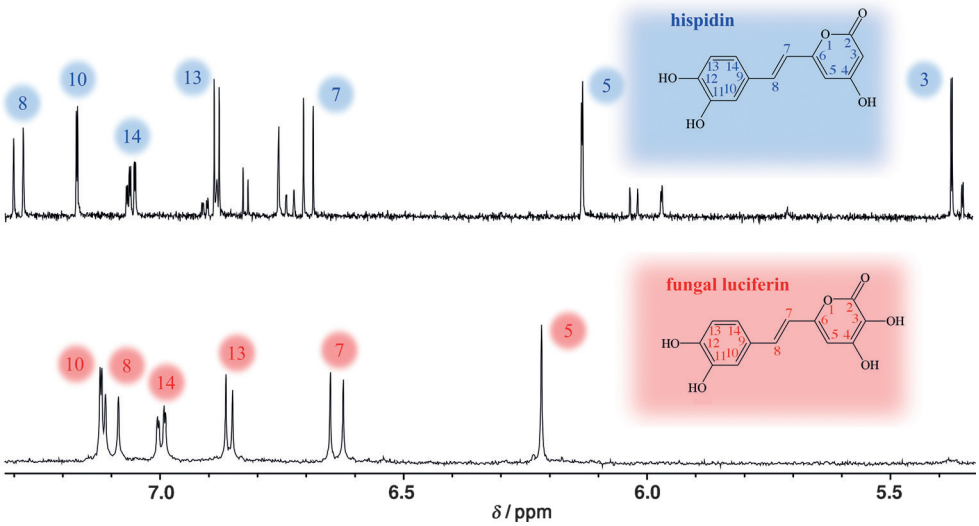

Figure 6. Comparison of the ^{1}H NMR spectra of hispidin (unlabeled peaks correspond to an admixture of the *cis* isomer of hispidin) and enzymatically synthesized fungal luciferin.

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