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Bioluminescence activity of *Latia* luciferin analogs

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Abstract

Latia luciferin analogs were synthesized and their bioluminescence activities were measured. The *Latia* luciferase was found to recognize strictly the 2,6,6-trimethylcyclohexene ring moiety in the luciferin structure. While the enol ether analogs exhibited no bioluminescence activity, the corresponding enol acetate analog possessed 60% activity compared to natural luciferin having an enol formate structure, implying that the initial step of the light producing reaction is an enzymatic hydrolysis to yield the corresponding enolate anion.

Keywords: *Latia neritoides*; luciferin; luciferase; bioluminescence; enol formate; enol acetate.

The limpet-like snail *Latia neritoides* is only found in clear and shallow streams of North Island in New Zealand, and is the only luminous animal which lives in fresh water. On mechanical stimulation, the snails secrete mucus, from which greenish light (536 nm) is emitted.[1] The *Latia* bioluminescence system exhibits a typical luciferin–luciferase reaction.[2,3] Shimomura established the structure of *Latia* luciferin (**1**) having a characteristic, enol formate functionality, and proposed the *Latia* bioluminescence reaction as shown in Scheme 1.[2–4] Thus, the *Latia* bioluminescence requires four components: the luciferin (**1**), the luciferase (178 000 Da), a cofactor so-called 'purple protein (38 000 Da)' (red-fluorescent protein), and molecular oxygen. During the bioluminescence reaction the *Latia* luciferin (**1**) was oxidized into *Latia* oxyluciferin (**2**), CO_2, and formic acid with an emitting the green light. Shimomura suggested that light emission should arise from a flavin compound covalently bound to the luciferase because none of **1**, **2** and 'purple protein' have green fluorescent properties.[3] However, the precise bio-oxidation mechanism of **1** and the detailed molecular bases for the light-emitting process are still unclear. In this paper, we wish to report the bioluminescence activity of the *Latia* luciferin analogs and the substrate specificity of *Latia* luciferase.

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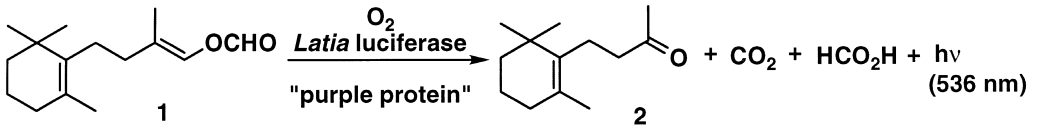
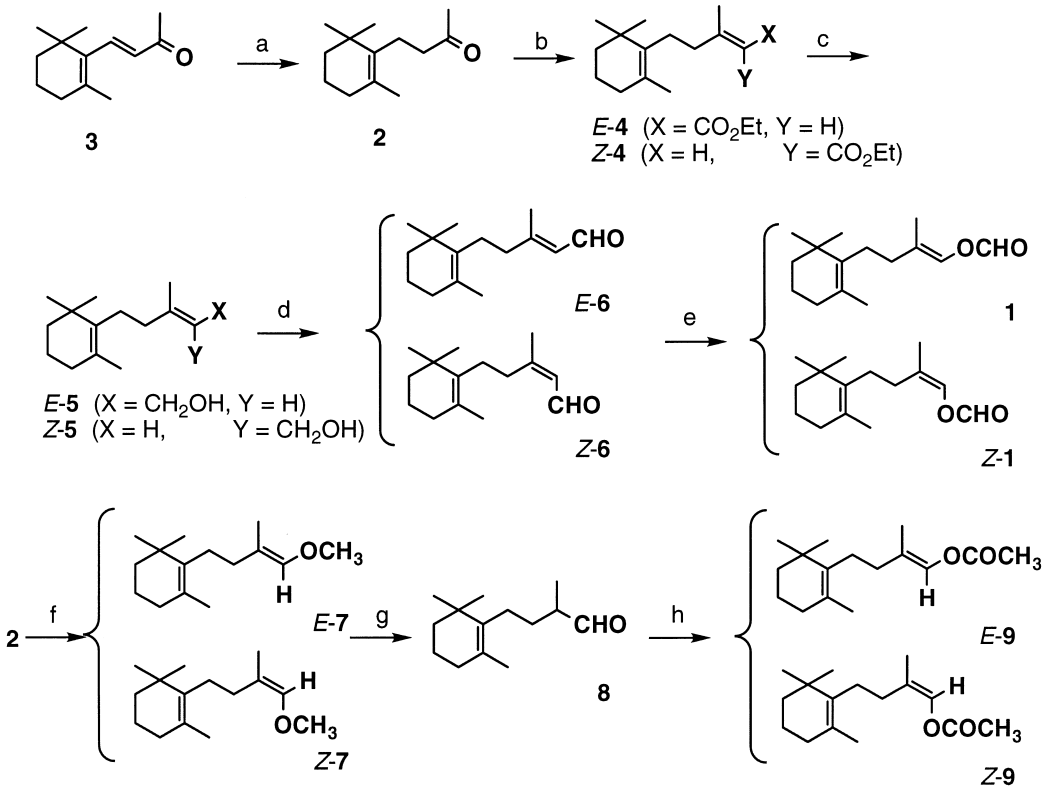


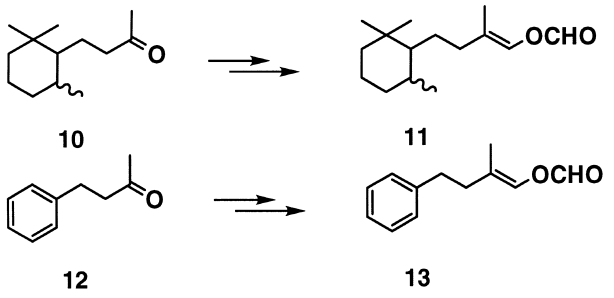
Scheme 1. Bioluminescence reaction of *Latia neritoides*

Scheme 2 shows the preparation of *Latia* luciferin (**1**) and the analogs **6**–**9**, **12** and **13** studied.[8] *Latia* luciferin (**1**) was prepared by the slightly modified procedure reported by Kishi.[5] Thus, selective hydrogenation of β-ionone (**3**) with $(PPh_3)_3RhCl$[6] followed by Horner–Emmons olefination of the resulting ketone **2** (*Latia* oxy-luciferin) afforded α,β-unsaturated esters *E*-**4** (overall 76%) and *Z*-**4** (overall 9%) after separation. Reduction of *E*-**4** with DIBAH followed by the oxidation of the resulting allylic alcohol with MnO_2 gave the desired α,β-unsaturated aldehyde *E*-**6** (overall 95%). Baeyer–Villiger oxidation of *E*-**6** was achieved by using 60% hydrogen peroxide in *t*-amylalcohol in the presence of selenium dioxide to give *Latia* luciferin (**1**) (45%). The corresponding geometrical isomer *Z*-**1** was prepared via *Z*-**4** by a similar procedure (overall 24%).



Scheme 2. Preparation of *Latia* luciferin (**1**) and its analogs. *Reagents and conditions*: (a) $(PPh_3)_3RhCl$, H_2, benzene, rt, 14 h, 100%; (b) $(EtO)_2P(O)CH_2CO_2Et$, LiHMDS, THF, reflux, 5 h, 76% for *E*-**4**, 9% for *Z*-**4** after separation; (c) DIBAH, toluene, 0°C, 100% for *E*-**5**, 100% for *Z*-**5**; (d) MnO_2, CH_2Cl_2, rt, 2 h, 95% for *E*-**6**, 95% for *Z*-**6**; (e) SeO_2, 60% H_2O_2, *t*-amylalcohol, 0°C, 45% for **1**, 25% for *Z*-**1**; (f) $MeOCH_2P^+(Ph_3)Cl^-$, LiHMDS, THF, 0°C, 4 h, 95% (*E*:*Z* = 1:1); (g) *p*-TsOH, 3:1 acetone:H_2O, reflux, 4 h, 100%; (h) *p*-TsOH, isopropenylacetate, reflux, 12 h, 42% for *E*-**9**, 21% for *Z*-**9**

Analogs **7**, **8** and **9** were synthesized from ketone **2** (Scheme 2). Wittig reaction of **2** with methoxymethylidenetriphenylphosphorane gave enol ether **7** as a 1:1 separable mixture of *E*:*Z*-isomers, which, upon acidic hydrolysis, afforded the corresponding aldehyde **8** (100%). Treatment of **8** with isopropenyl acetate-*p*-toluenesulfonic acid[7] gave the desired enol acetates *E*-**9** (45%) and *Z*-**9** (21%). By using quite similar sequences, analogs **11** (a racemic, 1:1 mixture of *cis* and *trans* ring-isomers) and **13** were prepared starting with **10** (a racemic, 1:1 mixture of *cis* and *trans* ring-isomers) obtained by hydrogenation of **3** and **12**, respectively (Scheme 3).



Scheme 3.

A crude *Latia* luciferase solution used for the bioluminescence measurement was prepared as follows: Specimens of frozen *Latia* were homogenized in 50 mM Tris–HCl buffer (pH 7.2) at 0°C and the homogenized mixture was centrifuged at 7000 rpm for 20 min at 4°C. The supernatant was subjected to fractional ammonium sulfate precipitation. The active precipitates obtained by 33–60% saturation were collected, resuspended in a 50 mM Tris–HCl buffer (pH 7.2), and subjected to gel filtration using Superdex 200 with the same buffer, yielding a crude luciferase solution containing 'purple protein'. The protein concentration of the luciferase solution was determined by Protein Assay Kit® (Bio Rad) to be 0.14 mg/ml.

The bioluminescent activity was measured by mixing 100 μl of the luciferase solution with 300 μl of *Latia* luciferin (**1**) or the analog solution in water (an 8×10^{-3} M EtOH solution was diluted with deionized H_2O to the final concentration of 8×10^{-6} M). The photons generated were counted by a luminometer Lumi Counter 1000 (Niti-on) for 10 min in each measurement. The results are summarized in Table 1. The cyclohexane analog **11** and the phenyl analog **13** had essentially no bioluminescence activity. These results suggest that the 2,6,6-trimethylcyclohexene ring moiety may be recognized strictly by the luciferase, and essential for binding to the active site.

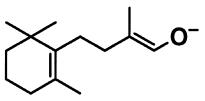
The *Z*-**1** regioisomer of *Latia* luciferin (**1**) had 60% activity of **1**, as reported by Kishi.[5] This result indicates that the recognition site of the enol ester moiety in the luciferase has a certain flexibility.

α,β-Unsaturated aldehyde *E*-**6** is supposed to be a biosynthetic precursor of *Latia* luciferin (**1**) because a certain flavin-containing oxygenase is known to catalyze Baeyer–Villiger-type oxidation of ketones and aldehydes.[9] However, both *E*-**6** and *Z*-**6** had no bioluminescence activity, indicating that *Latia* luciferase has no such activity. We expected to find that if the bioluminescence reaction starts with an electron transfer reaction from the substrate to a flavin component such as flavin (hydro)peroxide, enol methyl ether analogs *E*-**7** and *Z*-**7**, having a more electron donating double bond, may act as better substrates. However, both analogs were not active substrates. As mentioned by Shimomura, aldehyde **8**, the hydrolyzed product of **1** also has no bioluminescence activity.

Table 1
Relative bioluminescence activity of *Latia* luciferin analogs

Substrate	Relative Activity (%)
1	100
***Z*-1**	60
11	1
13	0
***E*-6**	0
***Z*-6**	0
***E*-7**	0
***Z*-7**	0
8	0
***E*-9**	66
***Z*-9**	44

Surprisingly, enol acetate ***E*-9** showed 66% activity compared to that of natural *Latia* luciferin (**1**) having the enol formate functionality. Furthermore, the regioisomer ***Z*-9** also had 44% activity compared to **1**. These results indicate that the formyl (-CHO) group is not essential functionality. Thus, the *Latia* bioluminescence system is completely different from that of bacteria. In addition, the *Latia* luciferase has an esterase activity and generation of the enolate anion **14** from the enol esters in the luciferase environment, but not the enol **15**, may be an important step for the bioluminescence reaction. Further study using substrate analogs is now in progress.



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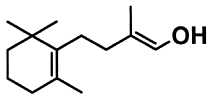

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In summary, *Latia* luciferase was found to recognize the 2,6,6-trimethylcyclohexene moiety strictly. The enol acetate functionality is also susceptible to the bioluminescence reaction.

Acknowledgements

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