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Tetrahedron Letters
Tetrahedron Letters 46 (2005) 53–56

Bioluminescence activity of *Latia* luciferin analogues: replacement of the 2,6,6-trimethylcyclohexene ring onto the methyl-substituted phenyl groups

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Received 30 September 2004; revised 1 November 2004; accepted 5 November 2004

Abstract—A series of *Latia* luciferin analogues having methyl-substituted phenyl groups instead of the natural 2,6,6-trimethylhexene ring was synthesized and their bioluminescence activity were measured. The *Latia* luciferase was found to be able to moderately recognize the appropriately methyl-substituted phenyl analogues with the same light production kinetics as that of natural luciferin.

The limpet-like snail *Latia neritoides* is only found in the clear and shallow streams of North Island in New Zealand, and is the only luminous animal, which lives its whole life cycle in fresh water. Upon mechanical stimulation, the snails secrete mucus, from which greenish light (λ_{max} 536 nm) is emitted.[1] The bioluminescence system exhibits a luciferin–luciferase reaction.[1,2] Shimomura established the structure of the luciferin (**1**) having a characteristic, enol formate functionality, and proposed that the bioluminescence requires a luciferin, 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**) is oxidized into *Latia* oxyluciferin (**2**), CO_2 and formic acid with the emitting of the green light (Scheme 1). Shimomura suggested that light emission should arise from a flavin compound covalently bound to the luciferase because **1**, **2** and the 'purple protein' do not have green fluorescent properties.[2,3] However, the precise bio-oxidation mechanism of **1** and the detailed molecular bases for the light-emitting process are still unclear.

For understanding the *Latia* bioluminescence reaction, we studied the relationship between the structure and bioluminescence activity of the luciferin analogues (Scheme 2).[4] The luciferin possesses the characteristic structural profiles: the 2,6,6-trimethylcyclohexene ring

Scheme 1. Proposed bioluminescence reaction of *Latia neritoides*.

Keywords: *Latia neritoides*; Luciferin; Luciferase; Bioluminescence.
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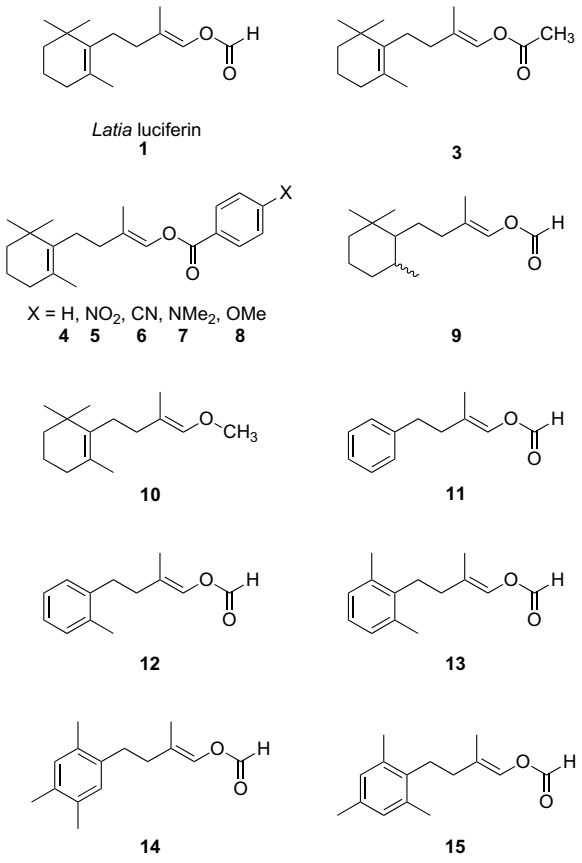
doi:10.1016/j.tetlet.2004.11.043



Scheme 2. The structures of *Latia* luciferin and its analogues.

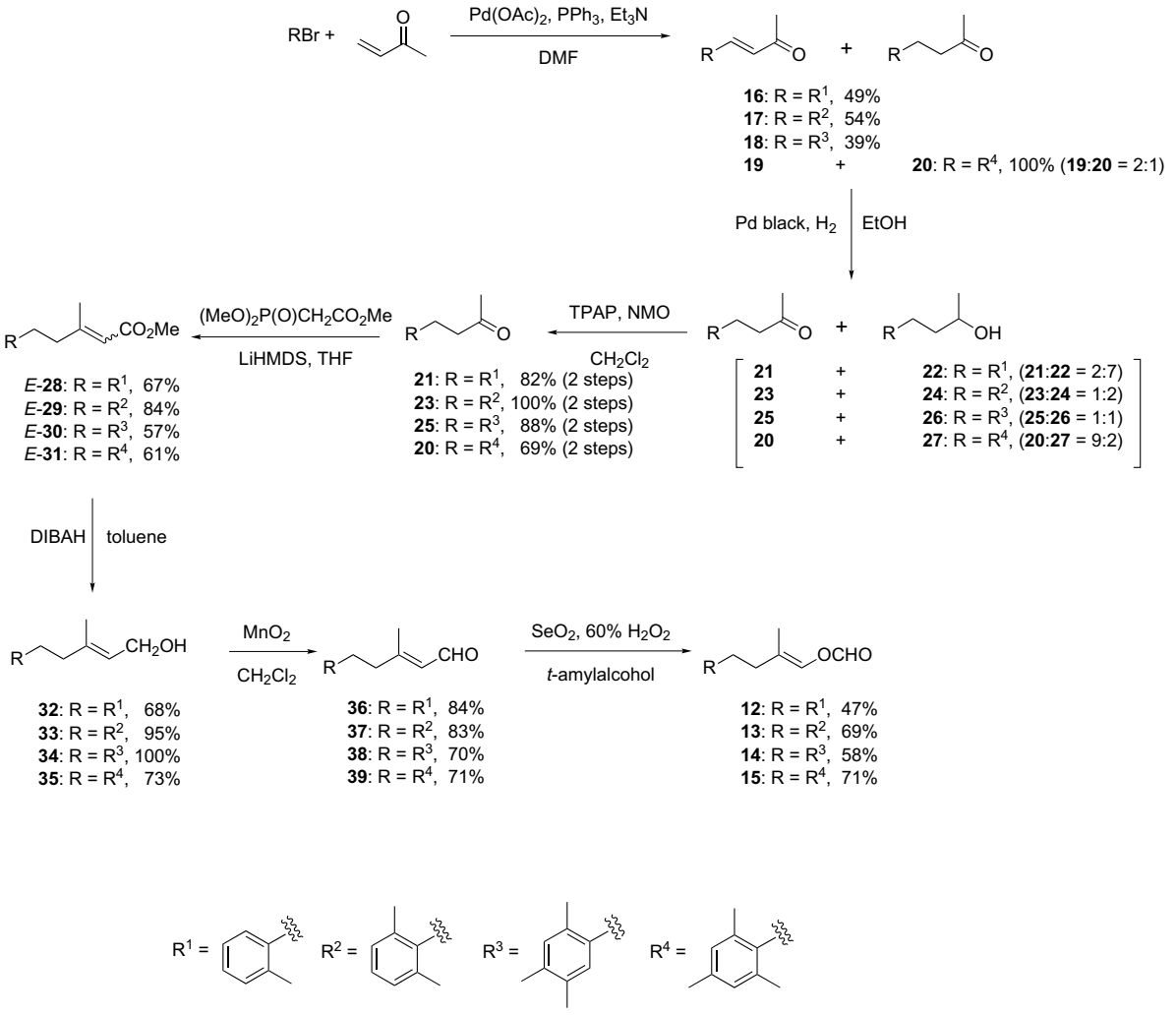
similar to the vision pigment retinal, and the enol formate functionality. Upon saturation of the cyclohexene ring into the corresponding cyclohexane (analogue **9**), or upon replacement of the ring system by the phenyl group (analogue **11**), the bioluminescence activity disappeared, implying that the 2,6,6-trimethylcyclohexene ring moiety is important for the substrate recognition with the luciferase. While the corresponding enol ether analogue (**10**) had no bioluminescence activity, the corresponding enol acetate **3** and enol benzoate analogues **4**–**8** exhibited substantial light production.[4] In addition, light production was delayed when the enol acetate and enol benzoate analogues (**3**–**8**) were used.

Herein we report the bioluminescence activity of the *Latia* luciferin analogues **12**–**15** having methyl-substituted phenyl groups instead of the natural 2,6,6-trimethylcyclohexane ring system.

The luciferin analogues **12**–**15** were prepared as follows (Scheme 3). The Heck reaction of 2-bromotoluene with methyl vinyl ketone in the presence of $Pd(OAc)_2$, PPh_3 and Et_3N, gave the α,β-unsaturated ketone **16**, which upon treatment with Pd black under a H_2 atmosphere, yielded a mixture of ketone **21** and alcohol **22**. Oxidation of the mixture of **21** and **22** with tetrapropylammonium perruthenate (TPAP) furnished the pure ketone **21** in 82% yield from **16**. The ketone **21** was subjected to the Horner–Emmons reaction to afford the α,β-unsaturated ester **28** as a mixture of geometrical isomers (*E*/*Z* = 3:1). This mixture was separated by silica gel column chromatography and the stereochemistry of each geometrical isomer was determined by NOE experiments. The reduction of *E*-**28** with DIBAH followed by the oxidation of the resulting allylic alcohol **32** with MnO_2 gave the aldehyde **36**. The Baeyer–Villiger oxidation of **36** was achieved using 60% hydrogen peroxide in *t*-amyl alcohol in the presence of selenium dioxide to give the *Latia* luciferin analogue **12**. Analogues **13**–**15** were synthesized from 2-bromo-*m*-xylene, 5-bromo-1,2,4-trimethylbenzene, or 2-bromomesitylene in a manner similar to that described.

The bioluminescence activity of **12**–**15** was measured by a previously described procedure.[4b] Thus, frozen *Latia* was homogenized in 50 mM 2-amino-2-hydroxymethyl-1,3-propanediol Tris–HCl buffer (pH 7.6) at 0 °C and the homogenized mixture was centrifuged at 7000 rpm for 20 min at 4 °C. The supernatant was diluted 10 times, and used for the bioluminescence measurement as a crude luciferase. The light production was measured by mixing 100 μL of the crude luciferase solution with 100 μL of *Latia* luciferin or the analogue (60 μM) in 25% ethanol solution.[4b] The photons were counted by a luminometer luminescencer-PSN AB-2200 (Atto, Tokyo, Japan) for 30 min. The wavelength of luciferin and its analogue was measured by a LumiFLSpectro-Capture AB-1850 (Atto). The inhibitory activity was measured by competition assay with natural luciferin. The inhibitory activity was measured by mixing 100 μL of the crude luciferase solution with 100 μL of 60 μM *Latia* luciferin and the analogue (various concentrations) in 25% ethanol solution. The photons were counted by a luminometer luminescencer-JNR II AB-2300 (Atto) for 30 min.

The bioluminescence activity of the synthesized analogues are summarized in Table 1. The analogues *E*-**13**, *E*-**14** and *E*-**15** showed luminescent activity, although these analogues were less potent than the authentic[4a] *Latia* luciferin (**1**). The bioluminescence activity of *E*-**12** exhibited no significant differences in the blank luminescent measurement similar to *E*-**11**,[4a] while, upon increasing the substrate and luciferase, *E*-**12** exhibited a small luminescent activity (data not shown). The bioluminescence spectra of *E*-**13**, *E*-**14** and *E*-**15** were identical with those of the authentic *Latia* luciferin (**1**) (λ_{max} 536 nm) (Table 1), indicating that the 2,6,6-trimethylcyclohexene moiety of the luciferin does not affect the bioluminescence spectra. The inhibitory activity of the inactive analogues *E*-**11** and *E*-**12** against the bioluminescence activity of the authentic *Latia* luciferin (**1**) was measured (Table 2). The *E*-**12** was found to exhibit the same inhibitory activity as oxyluciferin, while *E*-**11** exhibited approximately 10 times less potency. It is indicated that the recognition of *E*-**11** and oxyluciferin (**2**) for luciferase was the same, and supported the result that *E*-**11** had slightly bioluminescence and *E*-**10** did not have bioluminescence. These results indicated that the number and the position of the methyl group(s) on the phenyl ring are important for the substrate recognition in the *Latia* luciferase. In addition, the presence of the



Scheme 3. Synthesis of *Latia* luciferin analogues.

Table 1. Relative bioluminescence activity of *Latia* luciferin analogues

Substrate	Light intensity[a]	Total light[b]	Emission max. (nm)
E-**1**	100	100	536
E-**12**	—[c]	—[c]	536[d]
E-**13**	7.2	32	536
E-**14**	0.54	1.4	536
E-**15**	3.0	7.7	536

[a] The peak height was recorded as the light intensity.
[b] Total light was obtained by the integration of the photon counts for 30 min.
[c] The light intensity and total light of *E*-**12** did not significantly differ from that of the blank measurement.
[d] Upon increasing the substrate and luciferase, *E*-**12** had a slight luminescent activity.

O,*O*′-dimethyl group on the phenyl ring may be in a good position, like natural luciferin. Previously, we reported that the *Latia* luciferin benzoate analogues **4**–**8** delayed the emission for natural luciferin in bioluminescence.[4b] In contrast, no delay emission was observed for the luciferin analogues **13**–**15** in bioluminescence, though they exhibited a lower emission activity (Fig. 1). From these results, it is discussed that by modification of the enol ester moiety of luciferin, the luminescent rate is able to be controlled, and by modification of the 2,6,6-trimethylcyclohexene moiety, the luminescent quantity is able to be controlled.

In conclusion, the 2,6,6-trimethylcyclohexene moiety recognition site of *Latia* luciferase had some flexibility,

Table 2. Inhibitory activity of luciferin analogues against bioluminescence of natural luciferin

Substrate	Inhibitory activity IC_{50} (mM)
2 (oxyluciferin)	0.436
E-**11**	6.96
E-**12**	0.460

The inhibitory activity was measured by mixing 100 μL of the crude luciferase solution with 100 μL of 60 μM *Latia* luciferin and the analogue (various concentrations) in 25% ethanol solution. The inhibitory activity against bioluminescence of natural luciferin was shown with the effective concentrations that inhibit the concentration to 50% (IC_{50} values).

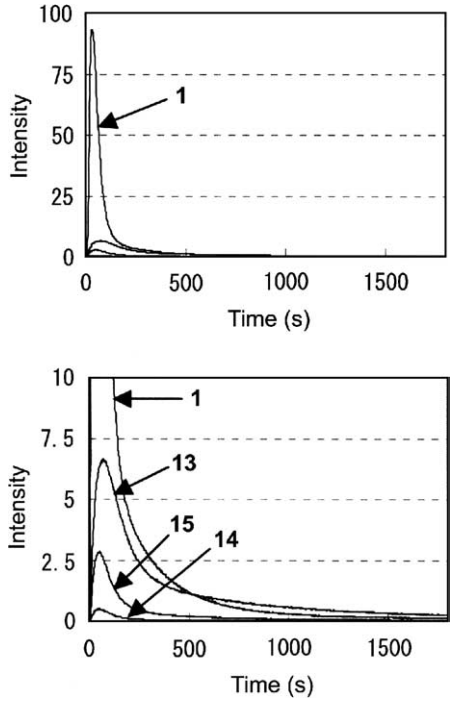


Figure 1. The bioluminescence activity of *Latia* luciferin and its analogues. Below is a magnified figure portion of the above plot.

and thereby the 2,6,6-trimethylcyclohexene moiety of the luciferin structure is able to be substituted into the *O*,*O*′-dimethyl-substituted phenyl ring system.

Acknowledgements

The authors thank Dr. Yoko Ohashi at the University of Electro-Communications and Mr. Yoshiyuki Itoh at Yokohama City University for their helpful discussions. This study was supported in part by a NEDO grant (Dynamic Biology Project) from the Ministry of Economy, Trade and Industry of Japan and by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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