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Identification of enzyme produced firefly oxyluciferin by reverse phase HPLC

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Abstract—Firefly oxyluciferin (2-(6′-hydroxybenzothiazolyl)-4-hydroxythiazole) was chemically synthesized and characterized by means of ^{13}C and ^{1}H NMR, UV–vis spectrometry and RP-HPLC using different pH elution conditions. One of the chromatographic peaks observed in luciferase-catalyzed reaction mixtures was identified as corresponding to oxyluciferin. © 2001 Elsevier Science Ltd. All rights reserved.

2-(6′-Hydroxybenzothiazolyl)-4-hydroxythiazole (firefly oxyluciferin) was chemically synthesized by Suzuki et al. in the late 1960s and proposed to be the light emitter product of the bioluminescent reaction catalyzed by firefly luciferases.[1] The enzyme reaction involves the formation, from firefly luciferin (LH_2) and ATP, of an enzyme bound adenylyl intermediate and its subsequent oxidation with release of AMP, CO_2 and oxyluciferin.[2]

$$E+LH_2+ATP \rightarrow E^{\bullet}LH_2\text{-}AMP+PPi \quad (1)$$

$$E^{\bullet}LH_2\text{-}AMP+O_2 \rightarrow E+AMP+CO_2+\text{oxyluciferin}+\text{photon} \quad (2)$$

Firefly luciferase can also catalyze the synthesis of adenine containing dinucleoside polyphosphates (Ap_nN) and the oxidation of luciferyl-adenylate (LH_2-AMP) to dehydroluciferyl-adenylate (L-AMP). In an attempt to clarify the enzymic mechanism of synthesis of dinucleoside polyphosphates, it has been found that L-AMP is the enzyme intermediate in this enzyme activity.[3]

$$E^{\bullet}\text{L-AMP}+p_nN \rightarrow L+Ap_{(n+1)}N+E \quad (3)$$

Fontes et al.[3] successfully used RP-HPLC for the separation and identification of some compounds involved in the enzyme reaction, namely LH_2, dehydroluciferin (L) and L-AMP. Moreover, other chromatographic peaks presumably related to light production have also been detected.[3]

With the objective of identifying the enzyme-synthesized oxyluciferin, the previously used RP-HPLC technique was modified by changing the pH of the eluent mixture to three different values: 5.5, 7.0 and 7.7. This pH selection was based on the acid–base properties of chemically synthesized firefly oxyluciferin in the used eluent. Indeed, at pH 5.4 the absorbance maximum of firefly oxyluciferin was at about 371 nm, increasing to about 414 nm when the pH was raised; the point of inflexion was at about pH 7.0 (Fig. 1). It can be

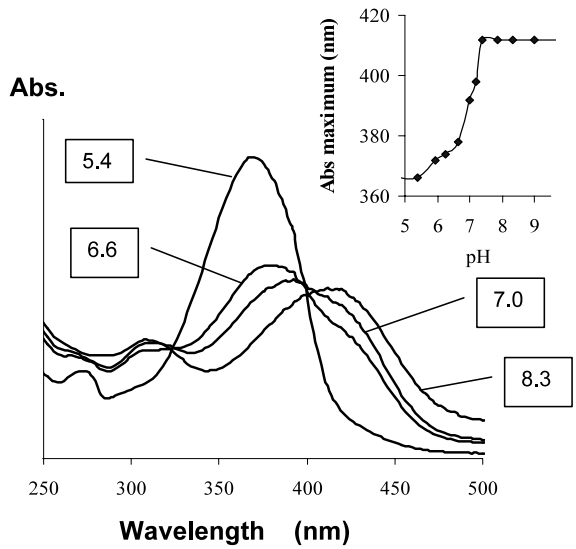

Figure 1. UV–vis spectra of chemically synthesized firefly oxyluciferin dissolved in a mixture containing 32% methanol (v/v) and 2.9 mM sodium phosphate buffer, where the pH was adjusted to the indicated values with NaOH.[5]

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inferred that in pH 5.5 oxyluciferin is protonated, at the highest eluent pH under research (7.7) it is almost all deprotonated (a higher pH cannot be used due to column limitation) and at the intermediate pH value (7.0) a mixture of the protonated and deprotonated oxyluciferin forms exists. Based on the studies of White and Rosewell on oxyluciferin methylated analogs,[4] it is most likely that the group corresponding to this deprotonation is the 4-hydroxyl of the hydroxythiazol moiety of the molecule.

Besides spectral variation induced by the changing pH, marked differences in the chromatographic retention time were expected (and actually observed) due to the increased polarity that occurs upon oxyluciferin deprotonation.

Chemically synthesized oxyluciferin was obtained from 2-cyano-6-hydroxybenzothiazole and ethyl thioglycolate, as described by Suzuki et al.[6] The parent compound 2-cyano-6-hydroxybenzothiazole was obtained from 2-cyano-6-methoxybenzotiazole (Aldrich, Steinheim, Germany), as described by Seto et al.[7] The purity of the product and of the intermediates was being checked by RP-HPLC. Additional purification of the synthesized compounds was not performed, besides that described by Seto et al.[7] and Suzuki et al.,[6] because minor components showed good chromatographic separation from the main product and, for the particular case of oxyluciferin, because purification attempts, according to Suzuki et al.,[6] degrades it.

^{1}H (Fig. 2), ^{13}C NMR (Fig. 3) and elemental analysis (46.6% C, 2.5% H, 10.4% N and 23.3% S)[8] showed that oxyluciferin was obtained about 85% pure relative to a

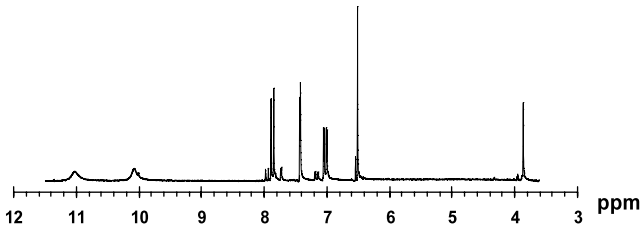

Figure 2. ^{1}H NMR spectrum for the chemically synthesized oxyluciferin (200 MHz: in $(CD_3)_2SO$ with minimum isotopic purity 99.96 atom% D (Aldrich, Milwaukee, USA); NMR equipment: Bruker AC200.

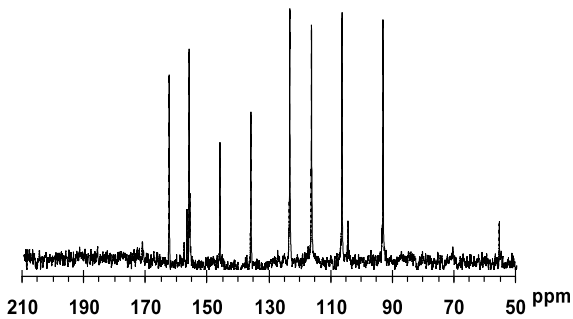

Figure 3. ^{13}C NMR spectrum for the chemically synthesized oxyluciferin (50 MHz: see Fig. 2 caption for other details).

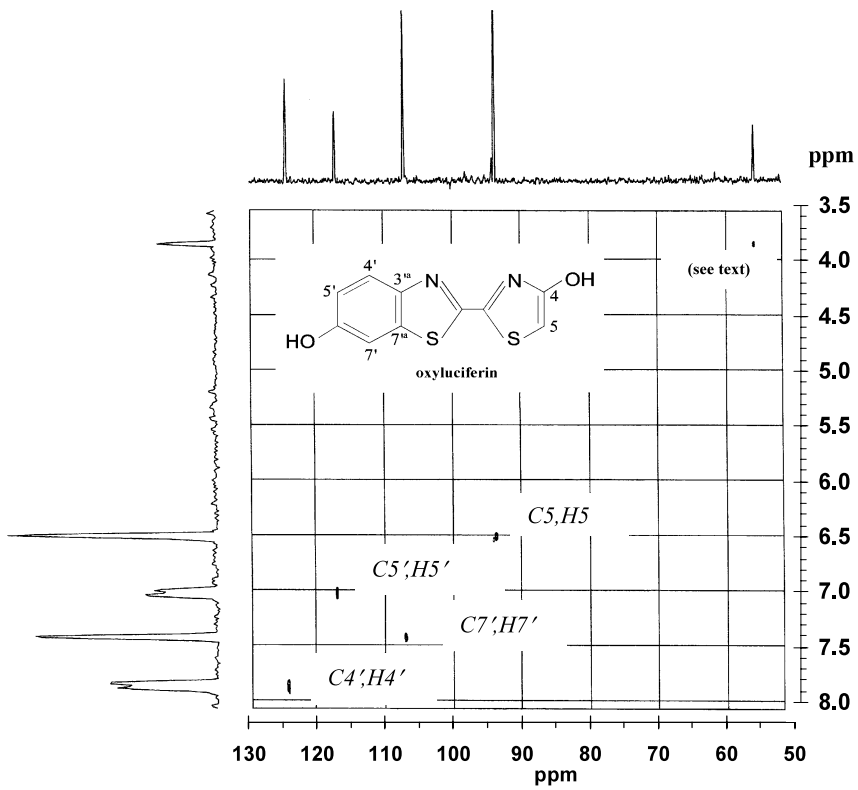

Figure 4. Heteronuclear C–H correlation spectrum for the chemically synthesized oxyluciferin (see Fig. 2 caption for other details).

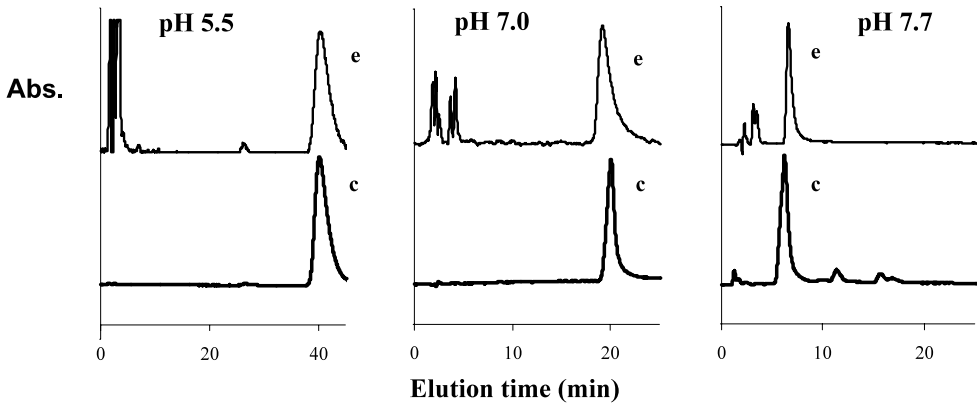

Figure 5. RP-HPLC analysis of luciferase-catalyzed reaction mixtures (above) and chemically synthesized oxyluciferin (below). Peaks labelled with letters e and c correspond to enzymatically and chemically synthesized oxyluciferin, respectively.[9,10]

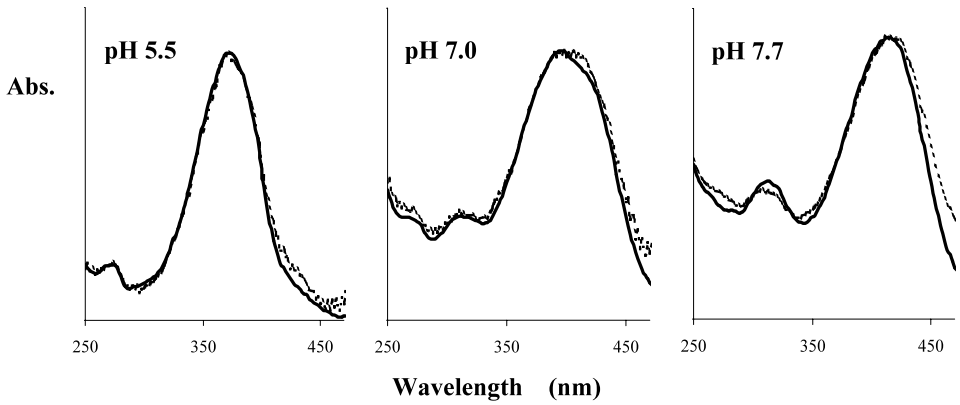

Figure 6. UV–vis spectra of chemically (continuous line) and enzymatically (discontinuous line) synthesized oxyluciferin obtained from chromatographic data.

main contaminant, identified as its 6′-methoxy derivative. A heteronuclear C–H correlation NMR spectrum is shown in Fig. 4. The lack of purity was also observed by RP-HPLC (Fig. 5, pH 7.7). The synthesized compound was kept under nitrogen in the freezer at about −20°C.

The analysis of ^{1}H NMR (Fig. 2) shows the following protons in the oxyluciferin molecule (Fig. 4): H-5, $\delta = 6.5$ ppm; H-5′, $\delta = 7.1$ ppm; H-7′, $\delta = 7.4$ ppm; H-4′, $\delta = 7.8$ ppm; phenolic protons, $\delta = 10.1$ and 11.0 ppm. The analysis of ^{13}C NMR (Fig. 3) shows the following carbons in the oxyluciferin molecule (Fig. 4): C-5, $\delta = 93.5$ ppm; C-5′, $\delta = 116.7$ ppm; C-7′, $\delta = 106.8$ ppm; C-4′, $\delta = 123.8$ ppm; C-3′^{a}, $\delta = 136.4$ ppm; C-7′^{a}, $\delta =$ 146.4 ppm; peaks due to C-2, C-2′ and C-6′ are in the range of δ between 156 and 158 ppm; C-4, $\delta = 163.0$ ppm. An important characteristic of the ^{13}C NMR (Fig. 3) is the evidence about the non-existence of carbonyl groups excluding the presence of dioxyluciferin (2-(6′-hydroxybenzothiazolyl)-5-hydroxythiazolin-4-one), an oxidized product of oxyluciferin detected by Suzuki et al. in the course of their attempts of synthesis of this compound.[6] This lack of carbonyl groups was also confirmed by infrared spectroscopy (not shown). The analysis of the heteronuclear C–H correlation NMR spectrum (Fig. 4) showed correlations compatible with the oxyluciferin molecule. The correlation C–H of the methoxy group in the 6′-methoxy derivative is also detected at C, $\delta = 55.7$; H, $\delta = 3.9$ ppm.

The observed chromatographic retention times for both chemically (c) and enzyme (e) synthesized oxyluciferin coincide and were: 40 min (pH 5.5); 20 min (pH 7); and 6 min (pH 7.7) (Fig. 5). This decrease in the retention time is compatible with the increase of polarity as a consequence of deprotonation. The absorbance spectra at the maximum retention time for the three eluent pH values under investigation are shown in Fig. 6 and were also coincident.

Under the used enzyme essay conditions, LH_2 was almost all consumed at 30 min of incubation and besides the peaks corresponding to oxyluciferin (peak e), we have, as expected from previous observations,[3,11] detected the formation of dehydroluciferin. The spectrum of this compound has a maximum at 348 nm at all the three pH values under study and eluted between minutes 3 and 4, 20 s after luciferin. The amount of dehydroluciferin formed was relatively high. Indeed, the ratio between the areas of the peaks (e) at maximum absorbance wavelengths (taken from the chromatograms in Fig. 5) and the area of the peaks corresponding to dehydroluciferin in the corresponding

chromatograms taken at 348 nm was about 1.5 when the pH was 7.7 and 7.0 and about 2 when the pH was 5.5.

The set of experimental results presented in this letter clearly show that 2-(6′-hydroxybenzothiazolyl)-4-hydroxythiazole is one of the main products of the bioluminescent firefly luciferase-catalyzed reaction. These observations are also in line with previous results of Suzuki and Goto who reported identification of oxyluciferin as the in vivo bioluminescent product by isolating it as the diacetate derivative.[12] This letter also supports the fact that separation techniques are useful in the detection and identification of the products of firefly luciferase catalyzed reactions because apart from the synthesis of oxyluciferin other pathways can take place in the reaction mixture.

Acknowledgements

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5. The adjusted pH aqueous solvents (0.68 mL) were deoxygenated by bubbling with ultra-pure nitrogen and kept in a nitrogen atmosphere for at least 1 day; before UV–vis spectra measurements a solution of oxyluciferin was prepared in pure methanol deoxygenated in the same way and a fixed quantity (0.32 mL) was added to a previously adjusted pH solution under nitrogen.
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8. The elemental analysis was performed in the Elemental and Thermal Analysis Service, Department of Chemistry, University of Manchester.
9. Enzyme reaction: The reaction mixtures contained in a final volume of 50 μl, 100 mM Hepes/NaOH (pH 7.5), 2 mM $MgCl_2$, 0.1 mM ATP (Sigma, Steinheim, Germany), 30 μM luciferin (Sigma, Steinheim, Germany), and luciferase (Fluka, Buchs, Switzerland; Cod. 62647) 0.95 mg of protein/ml. At 30 min of incubation (27°C) the enzyme reaction was stopped by the addition of 100 μl of ice cold 10 mM EDTA in 66% methanol, centrifuged for 2 min at 13,000 rpm and the supernatant stored at −20°C.
10. Chromatographic system: Aliquots of the supernatants obtained from the enzyme reaction mixtures or of the orange powder obtained from the chemical condensation of 2-cyano-6-hydroxybenzothiazole and ethyl thioglycolate dissolved in 32% methanol were injected into a Hypersil ODS column (4.6×100 mm) and eluted at a constant flow rate (0.5 ml/min) with mixtures containing 32% methanol (v/v) and 2.9 mM sodium phosphate buffer, where the pH was adjusted to 5.5, 7.0 and 7.7. The chromatograms shown in Fig. 3 represent the absorbance at 371 (pH 5.5), 395 (pH 7.0) and 414 nm of wavelength (pH 7.7).
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