Membrane-permeable luciferin esters for assay of firefly luciferase in live intact cells

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Five esters of luciferin were synthesized and compared with native luciferin as substrates for firefly luciferase expressed in live intact mammalian cells. The esters themselves were not substrates for purified luciferase, but four were substrates for a purified esterase and all appeared to be hydrolysed to luciferin within mammalian cells. At a substrate concentration of 0.01 mM, the peak luminescence from the *cos* cells expressing luciferase was up to 6-fold greater with the esters than with unmodified luciferin. At 0.1 mM, the difference between luciferin and the esters was decreased. The kinetics of the luminescent signal with the different luciferin esters varied significantly, indicating possible differences in the rates of uptake, breakdown and enzyme inhibition. The esters did not support luminescence from *Escherichia coli* cells expressing firefly luciferase, suggesting a lack of appropriate esterase activity in this particular strain. The esters could be useful for the assay of luciferase expression in intact mammalian cells when luciferin levels are limiting, for example in tissues, and in plants. Alternative luciferin derivatives may allow further improvements in sensitivity.

INTRODUCTION

Firefly luciferase has become an extensively used reporter enzyme for studies of gene expression in mammalian cells (DeWet *et al.*, 1987; Williams *et al.*, 1989). It has been used to monitor viral dissemination in infected cells (Rodriguez *et al.*, 1988) and, using viral-induced transactivation of luciferase under the control of the human immunodeficiency virus (HIV) long-terminal repeat, as a means of detecting and quantifying HIV infection (Schwartz *et al.*, 1990). The gene has also been expressed in plant cells (Ow *et al.*, 1986), yeast (Tatsumi *et al.*, 1988) and bacteria (Palomares *et al.*, 1989).

For maximum sensitivity, luciferase expression in mammalian cells is typically measured by luminometer analysis of cell lysates on addition of the substrates, ATP and luciferin (DeWet *et al.*, 1987). This method has the major disadvantage of destroying the biological sample, thus preventing further analysis and isolation of luciferase-expressing cells. Recently, charge-coupled device (CCD) cameras have been used for the direct imaging of the bioluminescence from intact mammalian cells expressing luciferase (Hooper *et al.*, 1990; White *et al.*, 1990). These cameras integrate the light signal over time, and the digital images can then be further used for spatial and quantitative analysis. However, the luciferase-directed light output from intact cells has been shown to be decreased when compared with that from cell lysates (DeWet *et al.*, 1987; White *et al.*, 1990). A possible reason for this is that the firefly luciferase substrate luciferin is an amphipathic molecule which, due to its carboxyl group (Fig. 1), is charged at physiological pH, thus preventing easy passage across cell membranes. In agreement with this, at lower pH when the luciferin molecule is protonated, light output from *Escherichia coli* colonies expressing luciferase appeared to increase (Wood & DeLuca, 1987).

In mammalian cells, it has been suggested that uncharged luciferin derivatives might be used to increase substrate uptake (Gould & Subramani, 1988). Previously, esterification of the carboxyl groups of fluorescent Ca^{2+} indicators such as quin-2 significantly increased their uptake by intact mammalian cells, where the esters were subsequently hydrolysed by intracellular esterases to yield native indicator (Tsien, 1981; Tsien *et al.*, 1982). The objective of the current work was to apply this approach to increase the sensitivity of detection of luciferase expression in live, intact cells using CCD camera analysis. We describe the synthesis and analysis of five esters of luciferin and compare them with unmodified luciferin in luminescent assays of firefly luciferase expression in eukaryotic and prokaryotic cells.

MATERIALS AND METHODS

Synthesis of luciferin esters

Fig. 1 shows the structures of D-luciferin and its derivatives synthesized for this study.

D-Luciferin. This was prepared by a procedure similar to that of the 'method B' described by White *et al.* (1965), except that 2-amino-6-methoxybenzothiazole was conveniently converted to 2-chloro-6-methoxybenzothiazole by addition of the reactant over 20 min to a well-stirred mixture of anhydrous $CuCl_2$ and *t*-butyl nitrite in acetonitrile at 60–65 °C. General details of this method of substitutive deamination are given elsewhere (Doyle *et al.*, 1977).

D-Luciferin 2-hydroxyethyl ester. D-Luciferin (0.25 g) in 3 ml of dry ethane-1,2-diol (Aldrich 'gold label', Gillingham, Dorset, U.K.), saturated with dry HCl, was stirred on a vacuum line at approx. 55 °C and 6.67 Pa (0.05 mmHg) so that it gently refluxed for 4 h. The solvent was occasionally resaturated with HCl, Ethane-1,2-diol was allowed to slowly distill off, leaving a residue of pale yellow powder which recrystallized from aqueous methanol at 3 °C. Fourier transform (FT) ^1H n.m.r. {[$^2H_6$]dimethyl sulphoxide (DMSO)}: $\delta = 3.70$ (distorted) (t), 4.21 (distorted) (t), 4.87 (t) (^2H_2O exchanged), 5.51 (t), 7.0–8.0 (aromatic H), 10.2 (s) (^2H_2O exchanged). M.s. [positive fast-atom bombardment (f.a.b.)]: 325 ($M+1$), 235 ($M-CO_2C_2H_4OH$). Microanalysis: found: C, 47.01; H, 3.66; N, 8.84% (calculated for $C_{13}H_{11}N_2O_4S_2$: C, 48.15; H, 3.70; N, 8.64%).

Abbreviations used: CCD, charge-coupled device; f.a.b., fast-atom bombardment; FT, Fourier transform; DMSO, dimethyl sulphoxide; HIV, human immunodeficiency virus; PBS, phosphate-buffered saline.

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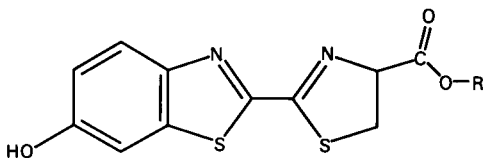

D-Luciferin	R = H
2-Hydroxyethyl ester	R = CH_2—CH_2OH
3-Hydroxypropyl ester	R = $(CH_2)_2$—CH_2OH
Ethoxyvinyl ester	R = —C(=CH_2)—O—CH_2—CH_3
Ethyl ester	R = CH_2—CH_3
Decyl ester	R = $(CH_2)_9$—CH_3

Fig. 1. Structure of the luciferin derivatives synthesized

D-Luciferin 3-hydroxypropyl ester. A procedure similar to that used for the 2-hydroxyethyl ester preparation was used, except that stronger warming was required to drive off all the propane-1,3-diol (approx. 75 °C). The residue recrystallized from aq. methanol to give 0.058 g of product from 0.1 g of D-luciferin. FT ^{1}H n.m.r. ($^2H_6$ DMSO): δ = 1.8 (p), 3.7 (m), 4.2 (t), 4.5 (t) (^2H_2O exchanged), 5.5 (t), 7.0–8.1 (aromatic H), 10.2 (s) (^2H_2O exchanged). M.s. (positive f.a.b.): 334 (M+1), 235 (M–$CO_2C_3H_6OH$). Microanalysis: found: C, 49.18; H, 4.12, N, 8.17% (calculated for $C_{14}H_{14}N_2O_4S_2$: C, 49.70; H, 4.14; N, 8.28%).

D-Luciferin 1-ethoxyvinyl ester. This compound was synthesized as described (Razavi, 1976), which was found to be preferable to the method of White *et al.* (1980). Ethoxyacetylene was either prepared from chloroacetaldehyde diethyl acetal (Jones *et al.*, 1963) or was fractionally distilled from a commercial solution in hexane.

D-Luciferin ethyl ester. Dry ethyl acetate (20 ml) was saturated with HCl, and 0.2 g of D-luciferin (White *et al.*, 1965; Doyle *et al.*, 1977) was added with stirring. The mixture was refluxed for 3 h with occasional resaturation by HCl. After a brief purge with nitrogen, the ethyl acetate solution was washed with sat. aq. $NaHCO_3$ and NaCl solutions and dried ($MgSO_4$). Solvent removal left a pale yellow residue which recrystallized from a mixture of acetone and pentane at −20 °C to give 0.045 g of fluffy white crystals. FT i.r. (nujol mull) 1738 cm^{-1}. FT ^{1}H n.m.r. (C^2HCl_3): δ = 1.35 (t), 3.75 (p), 4.31 (q), 5.37 (t), 7.05 (dd), 7.31 (d), 7.97 (d), 10.2 (s). M.s. (positive f.a.b.): 309 (M+1), 235 (M–CO_2Et). Microanalysis: C, 50.25; H, 3.77; N, 8.59 (calculated for $C_{13}H_{11}N_2O_3S_2$: C, 50.65; H, 3.90; N, 9.09).

D-Luciferin decyl ester. D-Luciferin (0.05 g), dissolved in 10 ml of dry tetrahydrofuran with 0.3 ml of di-isopropylethylamine and 0.4 ml of iododecane, was refluxed under argon for 6 h. After cooling and the addition of 0.5 ml of acetic acid, solvent was removed *in vacuo*. The residue was taken up in chloroform, washed with sat. aq. NaCl and then dried ($CaCl_2$). Removal of solvent *in vacuo* left a yellow oil which was dissolved in dry ether, filtered and precipitated with pentane. The precipitate, collected by centrifugation, was dried under vacuum to leave a pale yellow waxy solid of near single-spot purity by t.l.c. [silica gel; eluant chloroform/ethyl acetate (5:2, v/v), R_F = 0.69]. FT ^{1}H n.m.r. (C^2HCl_3/[$^2H_6$] DMSO): δ = 0.90 (t), 1.28 (broad) (s), 3.75 (dd), 4.24 (t), 5.37 (t), 7.05–7.95 (aromatic H), 10.2 (s).

Cell-free assays with purified luciferase

General assay conditions. All of the luminometer assays were performed at 25 °C in a Lumac Biocounter M 2010 luminometer (Lumac/3 m, Schaesberg, The Netherlands), and the peak luminescent signal (over 2 s integration periods) was measured immediately after initiation of the reaction. Purified luciferase enzyme (Boehringer Corp., Lewes, East Sussex, U.K.) was diluted in lysis buffer [25 mM-Tris/phosphate, pH 7.75, 1% (v/v) Triton X-100, 1% (w/v) bovine serum albumin (Sigma Chemical Co., Poole, Dorset, U.K.), 1 mM-dithiothreitol, 0.1 mM-Na_2EDTA, and 8 mM-$MgCl_2$ (Nguyen *et al.*, 1988)]. Stock solutions of luciferin or luciferin esters were made to 10 mM in DMSO and then diluted to 0.1 mM in assay buffer (25 mM-Tris/glycyl glycine, pH 7.75, and 150 mM NaCl). ATP (Sigma) was prepared in 25 mM-Tris/phosphate buffer (pH 7.75). The standard reaction mix contained 250 pg of luciferase, 0.45 mM-ATP and 29 μM-luciferin in a final volume of 350 μl per assay tube.

Activity of luciferin esters as luciferase substrates. To monitor the ability of the esters to act as luciferase substrates, luciferase and ATP were pre-incubated for 3 min in the luminometer. Either luciferin or luciferin ester was then added to a final concentration of 29 μM and the luminescence was measured.

Ester activity as esterase substrates. To analyse the ability of the luciferin esters to act as substrates for a purified esterase (rabbit liver; Sigma), each ester was pre-incubated with 50 μg of esterase (5 units; 1 unit hydrolyses 1 μmol of substrate/min at pH 7.5, 25 °C) for 15 min at 37 °C in assay buffer. The release of free luciferin was determined by addition of luciferase and ATP.

Luciferase activity in the presence of the esters. The luciferin esters were assayed for any inhibitory activity with the luciferase enzyme. This involved monitoring of the light output from the standard luciferase/luciferin reaction mixture after pre-incubation of the enzyme with ATP and various concentrations of luciferin ester (2.9–29 μM) for 3 min before injection of luciferin.

Live mammalian cell assay

Cos7 cells were transfected with plasmid pMW41 by lipofection as described previously (White *et al.*, 1990), except that, for increased standardization, cells were pooled from replicate tranfections, diluted 5-fold, and replated on to 60 mm-diam. Petri dishes 16–24 h before assay. Prior to analysis, *cos* cells were rinsed in assay buffer and a further 3 ml of assay buffer containing luciferin or esters was then added, keeping the DMSO concentration to 1% (v/v). Replicate Petri dishes of cells were assayed with a liquid-nitrogen-cooled CCD camera (Wright Instruments, Enfield, Middlesex, U.K.). The luminescent signal was measured for six consecutive 5 min integration periods and analysed by calculating the mean light output per pixel in a 65 × 65 pixel box around the centre of the image (an area approx. 45% of the dish).

Live bacterial cell assay

E. coli W3110 cells, transformed with plasmid pRSV-L (DeWet *et al.*, 1987), were grown to exponential phase with shaking at 37 °C, centrifuged and resuspended in either 0.1 mM-sodium citrate, pH 5.0 (Wood & DeLuca, 1987), or phosphate-buffered saline (PBS), pH 7.3. The A_{600} of each bacterial suspension was standardized to a value of 2.0 (about 1.6×10^9 colony-forming units·ml^{-1}). Cell suspension (150 μl) was added to the wells of a black polystyrene microtitre plate (DynaTech Labs Ltd., Billingshurst, Sussex, U.K.) and 100 μl of the appropriate ester from a 1 mM stock solution was then added, in either citrate solution or PBS, keeping the DMSO concentration to 1% (v/v). The luminescent signal was imaged by the CCD camera for 10 s every min for 10 min and the images were then analysed by

counting the mean light output from a 23 × 23 pixel box positioned over each well.

RESULTS

Five esters of luciferin were synthesized which varied in their hydrophobicity and possibly their ability to act as hydrolytic enzyme substrates. These esters were the luciferin 2-hydroxyethyl ester, 3-hydroxypropyl ester, ethoxyvinyl ester, ethyl ester and decyl ester (Fig. 1.).

Analysis of the luciferin esters with purified luciferase

The esters were assessed in a luminometer for their ability to act as substrates for purified luciferase. The esters did not appear to be efficient substrates for the enzyme and the means (±S.E.M.) from triplicate observations showed that the light output from the luciferin ethyl, 3-hydroxypropyl and decyl esters relative to that from a similar concentration of luciferin (= 100%) was < 0.02%, the minimum level detectable. The 2-hydroxyethyl ester and the ethoxyvinyl ester gave low levels of luminescence, 0.2±0.1% and 6.6±1.7% respectively. Analysis by t.l.c. suggested that contaminating luciferin was probably the cause of the luminescence in these two ester preparations (results not shown).

An important property of the esters was their ability to undergo enzymic hydrolysis. Four of the esters were cleaved by a purified esterase (from rabbit liver), resulting in high luminescence in the presence of luciferase in luminometer assays (Table 1). Only the ethoxyvinyl ester did not appear to be cleaved by the esterase. The esters were also analysed in cell-free assays for their ability to inhibit a control luciferin and luciferase reaction, and inhibition was observed with all esters, except the decyl ester (Table 2), whose properties were probably affected by its poor solubility. Similar studies using a purified luciferase have previously been described with a methyl ester of luciferin (Miska & Geiger, 1987).

Ester studies in live, intact mammalian cells

The intensity of the luminescent image from single cells expressing luciferase was previously found to be related both to the levels of luciferase expression and to the extracellular luciferin concentration added to the cells (White *et al.*, 1990). In the present study, a CCD camera was used to visualize and quantify the luminescent signal from luciferase-expressing *cos* cells grown on Petri dishes.

The *cos* cells, transiently expressing luciferase, showed a similar punctuate pattern of luminescence with both the luciferin esters and luciferin (Fig. 2). The analysed data were quantified by measuring the average luminescent signal from the central area of each plate in a 65 × 65 pixel box (about 45% of the area of each plate). The luminescent signal obtained with a representative ester, the 2-hydroxy ethyl ester, and a similar level of luciferin (0.01 mM), clearly showed that this ester gave a higher luminescent intensity than luciferin (Fig. 2). All of the esters tested, excluding the luciferin decyl ester, supported significant luminescence from the transfected cells. Within the period tested, the ethoxyvinyl ester showed a rapid decrease in light after the first 5 min integration period; however, the luminescence from other esters appeared to build up more slowly than that from either the ethoxyvinyl ester or luciferin (Fig. 3).

Control experiments, in which the luciferin esters were incubated in assay buffer and then assayed with luciferase, showed that the degree of uncatalysed hydrolysis of the esters was insignificant under the assay conditions. This suggested that the luminescence obtained when the esters were added to the cells

Table 1. Effect of purified esterase on light output from luciferin esters with purified firefly luciferase

Each ester was pre-incubated at 37 °C with esterase for 15 min and then assayed, with ATP and luciferase, for luminescence. Results shown are means±S.E.M. of triplicate observations and were corrected for background luminescence (in the absence of esterase), then expressed as a percentage of the luminescence obtained with luciferin (= 100%).

Luciferin ester	Luminescence (% of luciferin control)
2-Hydroxyethyl	30.6±1.5
3-Hydroxypropyl	15.0±0.3
Ethoxyvinyl	−0.6±0.5
Ethyl	35.0±0.8
Decyl	8.2±0.5

Table 2. Effect of luciferin esters on light output from the reaction of luciferin with purified firefly luciferase

Each ester was pre-incubated with ATP and luciferase before injection of luciferin to 29 μM. The luminescent values shown are means±S.E.M. of duplicate observations from two separate experiments and are expressed as a percentage of the control (standard reaction mix) value of 100%. N.D., not determined.

Luciferin ester	Luciferin/ester ratio...	Luminescence (% of luciferin control) 1	2	10
2-Hydroxyethyl		47±2	59±1	86±1
3-Hydroxypropyl		29±2	44±2	80±5
Ethoxyvinyl		64±1	76±4	105±2
Ethyl		40±3	54±1	84±2
Decyl		102±1	N.D.	N.D.

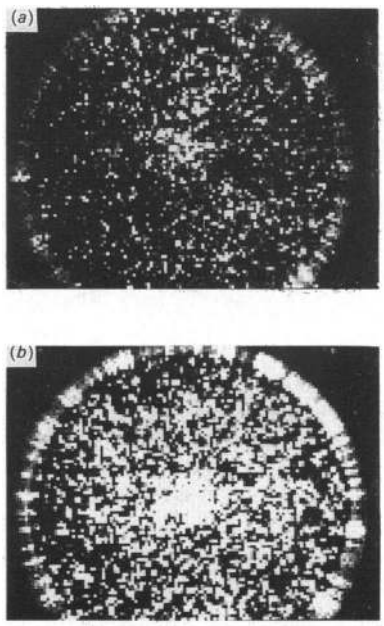

Fig. 2. Luminescence from intact *cos* cells on a Petri dish after addition of 0.01 mM- (*a*) luciferin or (*b*) the 2-hydroxyethyl ester

The Figures shown are 5 min exposures taken on the CCD camera immediately after addition of the appropriate substrate.

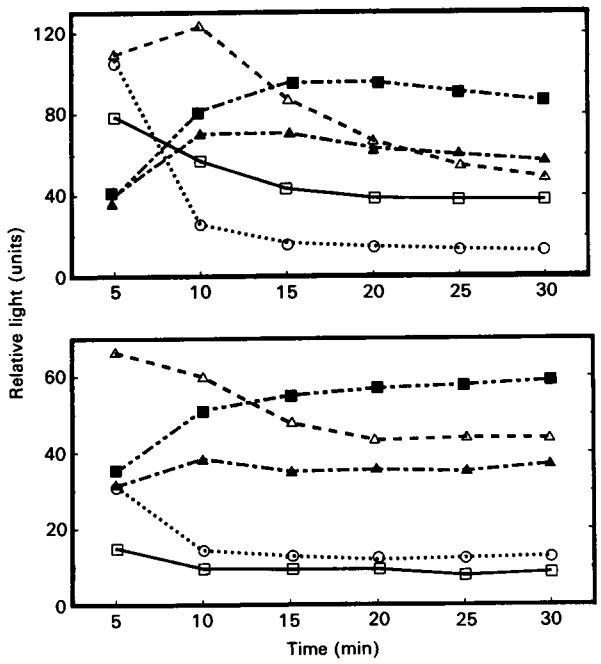

Fig. 3. Kinetics of light output from intact *cos* cells with luciferin and the luciferin esters at a concentration of (*a*) 0.1 mM or (*b*) 0.01 mM

The Figure shown is representative of four separate experiments. □, Luciferin; △, 2-hydroxyethyl ester; ○, ethoxyvinyl ester; ■, ethyl ester; ▲, 3-hydroxypropyl ester.

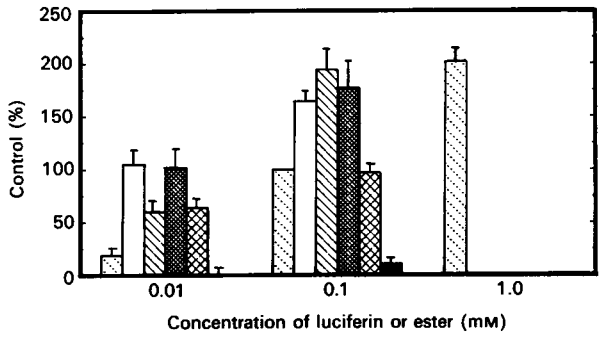

Fig. 4. Peak luminescent value obtained with intact *cos* cells after addition of luciferin or the luciferin esters

The data are means of four separate observations and are expressed relative to the luminescent peak obtained with the 0.1 mM concentration of luciferin (= 100%). Bars represent the S.E.M. of the sample. ▨, Luciferin; □, 2-hydroxyethyl ester; ▨, ethoxyvinyl ester; ▩, ethyl ester; ▨, 3-hydroxypropyl ester; ■, decyl ester.

was caused by the activity of cellular esterases and luciferase. Taking peak light levels, the esters gave up to six times more light than luciferin at concentrations of 0.01 mM (Fig. 4). However, at the 0.1 mM concentration, the difference between the esters and luciferin was less marked. Insolubility precluded the use of most esters at 1 mM, although the 2-hydroxyethyl ester was soluble at this concentration and gave about 3-fold less light than the level observed at 0.1 mM.

Ester studies in live, intact bacterial cells

Previous studies showed that lowered pH (pH 5.0) facilitated luciferin entry into bacterial cells (Wood & DeLuca, 1987). We assayed luciferase-expressing *E. coli* cells with the esters at pH 5.0 in 0.1 mM-sodium citrate and, alternatively, in an iso-osmotic buffer, (PBS, pH 7.3). None of the esters gave significant luminescence at either pH over that expected from free luciferin contamination in two of the ester preparations (see above). Luciferin gave more luminescence at the lower pH (approx. 50-fold), in agreement with the observations of Wood & DeLuca (1987).

DISCUSSION

Various reports have proposed that luciferin entry into both eukaryotic (DeWet *et al.*, 1987) and prokaryotic (Wood & DeLuca, 1987) cells is limiting. Supporting this hypothesis, the peak luminescence obtained from luciferase in mammalian cell lysates was shown to be greater than that from intact cells (DeWet *et al.*, 1987; White *et al.*, 1990). Previously it has been suggested that non-ionic luciferin derivatives may be taken up into intact cells more easily (Gould & Subramani, 1988).

In the present study, we have demonstrated that luciferin esters can be used for analysis of luciferase expression in intact mammalian cells. The luciferin esters were more effective than luciferin at low concentrations, when the substrate was probably limiting (White *et al.*, 1990). This suggested enhanced uptake of the luciferin esters compared with luciferin at low concentrations and subsequent release of free luciferin inside the cells due to cellular esterase activity. At higher concentrations the difference in the light output was less; this may have been due to the inhibitory effect of the esters on luciferase or to the fact that at high concentrations the level of unmodified luciferin entering the cells was no longer limiting.

The assay for luciferase expression in intact cells utilized intracellular oxygen and ATP, but required the addition of extracellular luciferin. The aerobic conditions were previously found to be an additional parameter which influenced the luminescence from *E. coli* cells expressing luciferase (Wood & DeLuca, 1987). This may also be an important factor for optimal luminescence in intact mammalian cells. Other factors which may limit the luminescence from intact cells include the intracellular ATP concentration, compartmentalization of luciferase to the peroxisomes (Keller *et al.*, 1987) and attenuation of the luminescent signal by the intact cell organelles.

A previous study suggested that luciferin uptake into *E. coli* cells was improved using a buffer at low pH (Wood & DeLuca, 1987). This agrees with our present study, where we compared the use of sodium citrate (pH 5.0) with a more physiological buffer (PBS, pH 7.3). No significant light output was noted from *E. coli* with the luciferin esters. The reason for this was probably a lack of appropriate esterase activity within the cells. Differences in esterase specificity have been observed in bacteria (Goullet & Picard, 1990), and it is possible that these luciferin esters may be hydrolysed by other bacterial species.

Preliminary results indicated that the luciferin esters also give significant luminescence from HeLa cells expressing luciferase (results not shown). Lymphocytes and human erythrocytes cannot cleave the ethyl ester of calcium chelators such as quin-2 (Tsien, 1981; Tsien *et al.*, 1982), although in the present study the ethyl ester of luciferin was cleaved by both *cos* and HeLa cells. In addition, an apparent difference in esterase specificity was observed, with the purified esterase being able to cleave all esters except the ethoxyvinyl ester. Thus the ability to cleave esters may reflect differences in esterases in the cell type used and in the structure of the esterified substrate.

The luciferin esters used in the present study could be useful for analysis of luciferase expression in intact cells when luciferin access is limited, for example in tissues (DiLella *et al.*, 1988) or

whole plants (Ow *et al.*, 1986). These luciferin esters could also be useful in other applications such as in dual-enzyme immunoassays which use esterase cleavage as the first enzymic step (Miska & Geiger, 1987). Alternative luciferin derivatives may have improved properties, allowing more sensitive detection of luciferase expression in various cell types. The approach of using esters of charged enzyme substrates to increase their uptake appeared to be successful and could be applied to analysis of the activity of other reporter enzymes in intact cells.

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