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Synthesis and Characterization of Luciferin Derivatives for Use in Bioluminescence Enhanced Enzyme Immunoassays

New Ultrasensitive Detection Systems for Enzyme Immunoassays, I.¹⁾

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Summary: Derivatives of luciferin, *D*-luciferin methyl ester, *D*-luciferyl-*L*-phenylalanine, *D*-luciferyl-*L*-N^α-arginine, *D*-luciferin-*O*-sulphate and *D*-luciferin-*O*-phosphate, were synthesized for use as highly sensitive substrates for enzyme assays. The luciferin derivatives were characterized by ultraviolet and fluorescence spectrophotometry, by amino acid analysis and by fast atom bombardement mass spectrometry. Enzymatic cleavage of the compounds by enzymes leading to the release of *D*-luciferin was demonstrated. Kinetic constants were determined for the following enzyme/substrate pairs: *D*-luciferin methyl ester/carboxylic esterase, *D*-luciferyl-*L*-phenylalanine/carboxypeptidase A, *D*-luciferyl-*L*-N^α-arginine/carboxypeptidase B, *D*-luciferin-*O*-sulphate/arylsulphatase, *D*-luciferin-*O*-phosphate/alkaline phosphatase. All compounds proved to be acceptable substrates for the respective enzymes, *D*-luciferin-*O*-phosphate being accompanied by an especially high turnover number ($k_{\text{cat}} = 1010 \text{ s}^{-1}$) with alkaline phosphatase.

Introduction

Since the introduction of the radioimmunoassay by *Berson & Yalow* nearly three decades ago (1, 2), this technique has become a widely used tool in medical research and routine diagnosis. It is used for determining and quantifying antigens such as peptide and non-peptide hormones, drugs, enzymes, viruses, bacterial and tumour constituents, and other organic substances of biological and environmental interest (2–6). Radioimmunoassays are relatively simple to handle, and have a remarkably high specificity and sensitivity (4, 5). Disadvantages are the contact with radioactive material, the often short shelf-life of the tracers, and the instability (radiolysis) of labelled antigens (7, 8).

For these reasons immunoassays with non-isotopic labels (e.g. fluorescence quenchers, chemiluminescence precursors) have been investigated and devel-

oped in recent years (9, 10). In particular, enzyme immunoassays (11–16) have proved to be suitable alternatives to radioimmunoassays. The various ways in which enzymes have been employed, either directly or indirectly as immunoassays labels, have been discussed in detail elsewhere (4, 17). Enzyme labels most commonly used in heterogenous enzyme immunoassays are peroxidases,²⁾ alkaline phosphatase and β -*D*-galactosidase. For homogeneous enzyme immunoassays, lysozyme, glucose-6-phosphate dehydrogenase and malate dehydrogenase are most frequently used as enzyme labels (17). These enzymes are very effective catalysts, and their substrates are suitable for spectrophotometric determination (17). A single molecule of enzyme typically converts 10^3 – 10^4 mole-

²⁾ Alkaline phosphatase (EC 3.1.3.1), arylsulphatase (EC 3.1.6.1), carboxylic esterase (EC 3.1.1.1), carboxypeptidase A (EC 3.4.17.1) and carboxypeptidase B (EC 3.4.17.2).

¹⁾ II.: this j. 25, 31–38 (1987).

cules of substrate into product per minute, but for some enzymes this figure can be as high as 10^6 – 10^7 (17). Hence analytes can be detected in enzyme immunoassays down to femtomol levels.

Our aim was the development of a new type of enzyme substrate based on luciferin derivatives, which are highly sensitive in the test systems. These new substrates can be used for unmodified enzymes and for enzyme conjugates, and applied in enzymatic test systems and in enzyme immunoassays, respectively. Briefly, the release of *D*-luciferin (*Photinus pyralis*) from *D*-luciferin conjugates is quantified by a luminometric detection system (25), allowing a determination of antigen concentrations down to 5 fg/l.

Materials and Methods

Synthetic *D*-luciferin (*Photinus pyralis*), *p*-nitrophenyl acetate, *p*-nitrophenyl phosphate, *p*-nitrophenyl sulphate, hippuryl-*L*-phenylalanine, hippuryl-*L*-arginine, *N,N'*-dicyclohexylcarbodiimide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, carboxylic esterase (porcine liver; 100 U/mg), carboxypeptidases A (bovine pancreas; 50 U/mg) and B (bovine pancreas; 175 U/mg), arylsulphatase (*Helix pomatia*; 400–600 U/mg) and alkaline phosphatase (bovine kidney; 200–400 U/mg; 25 °C) were purchased from Sigma, München, FRG.

Luciferase (*Photinus pyralis*; spec. act. 8 mU/mg) was a product of Boehringer, Mannheim, FRG.

L-phenylalanine, *L*-arginine, pyridine/ SO_3 -complex, POCl_3 , silica gel 60 (40–63 μm), ATP, NAD^+ and all solvents were products from Merck, Darmstadt, FRG.

Synthesis and characterization of luciferin derivatives

D-luciferin methyl ester (M_r : 294.35)

D-luciferin methyl ester was synthesized as described by White et al. (18) and further purified using a silica gel 60 column (10 $\times$ 2 cm) equilibrated with benzene/ethyl acetate (1 + 1 by vol.).

Yield: 69% of theory.

Element analysis for $\text{C}_{12}\text{H}_{10}\text{N}_2\text{O}_3\text{S}_2$:
calculated: C 48.97%, H 3.42%, N 9.52%
found: C 49.59%, H 3.77%, N 9.41%.

D-luciferin-*N*-hydroxysuccinimide ester (M_r : 377.39)

30 mg (0.11 mmol) *D*-luciferin and 12 mg (0.11 mmol) *N*-hydroxysuccinimide were dissolved in 1.8 ml tetrahydrofuran and 25 mg (0.12 mmol) *N,N'*-dicyclohexylcarbodiimide, dissolved in 0.1 ml tetrahydrofuran, were added. The reaction mixture was incubated in the dark at room temperature for 3 h. Precipitated urea was removed by filtration, and 21 ml *n*-hexane were added to the filtrate. The newly formed precipitate was collected by filtration and dried in vacuo over potassium hydroxide.

Yield: 33 mg (80% of theory).

Element analysis for $\text{C}_{15}\text{H}_{11}\text{N}_3\text{O}_5\text{S}_2$:
calculated: C 49.68%, H 3.66%, N 10.53%
found: C 49.39%, H 3.77%, N 10.56%.

D-luciferyl-*L*-phenylalanine (M_r : 402.49)

10 mg (25 μmol) *D*-luciferin-*N*-hydroxysuccinimide ester were added to a solution of 4.5 mg (27 μmol) *L*-phenylalanine and 6 μl triethylamine in 0.3 ml dioxane and 0.2 ml water. The reaction mixture was incubated in the dark at 20 °C for 2 h. Thereafter, 1 ml 1 mol/l acetic acid was added and the solution stored overnight at 0 °C. The precipitate formed was collected by centrifugation and dissolved in a small volume of 0.5 mol/l ammonia. *D*-luciferyl-*L*-phenylalanine was further purified by HPLC system I.

Yield: 6.52 mg (63% of theory).

D-luciferyl-*N*^ω-arginine (M_r : 436.52)

12 mg (30 μmol) of *D*-luciferin-*N*-hydroxysuccinimide ester were dissolved in 0.3 ml dioxane and added to 0.2 ml *L*-arginine solution (0.6 mol/l, pH 7.5). The pH was adjusted to pH 6 by adding 2 mol/l NaOH, and the mixture was incubated in the dark at 20 °C for 2 h. The reaction mixture was dried in a rotary evaporator. The dry residue was dissolved in 0.5 mol/l ammonia and further purified by HPLC system II.

Yield: 4.81 mg (37% of theory).

D-luciferin-*O*-sulphate (M_r : 360.39)

20 mg (71 μmol) *D*-luciferin was dissolved in 0.6 ml dry pyridine and added to 16 mg (100 μmol) of SO_3 /pyridine-complex. The reaction mixture was incubated at 20 °C in the dark for 2 h, dried in a rotary evaporator, dissolved in a small volume of 0.5 mol/l ammonia, and further purified by HPLC system III.

Yield: 10.2 mg (36% of theory).

D-luciferin-*O*-phosphate (M_r : 359.39)

50 mg (0.18 mmol) *D*-luciferin and 350 mg magnesium oxide were suspended in 3 ml water and cooled to 0 °C. Thereafter, 92 mg (0.6 mmol) of phosphorus oxytrichloride in 1 ml carbon tetrachloride were added dropwise to this suspension. The reaction mixture was kept at 20 °C for 30 min, filtered and neutralized with acetic acid. After evaporation in vacuo, *D*-luciferin-*O*-phosphate was purified by HPLC system III.

Yield: 21.5 mg (29% of theory).

Spectrometry

Ultraviolet spectra were measured at room temperature using a Zeiss PMQ 3 spectrophotometer.

Fluorescence spectra were taken with a Hitachi fluorimeter 650-40 at room temperature.

Chromatography

Amino acid analyses were performed with a Biotronic amino acid analyser after hydrolysis of samples in 6 mol/l HCl for 24 h at 110 °C.

Thin layer chromatography was performed using the following separation systems:

system I: ethyl acetate: benzene = 1 + 1 by vol. (silica gel 60 plates; Merck, Darmstadt).

system II: ethanol: 1 mol/l ammonium acetate, pH 7.5 = 3 + 1 by vol. (cellulose plates; Merck, Darmstadt, FRG).

Isocratic HPLC systems (column: Hibar EC RP-8, 250 $\times$ 4 mm, Merck, Darmstadt, FRG; flow: 1 ml/min; wavelength: 350 nm):

system I: 42% 0.05 mol/l ammonium acetate (pH 8.0) and 58% methanol.

system II: 50% 0.05 mol/l ammonium acetate (pH 8.0) and 50% methanol.

system III: 65% 0.05 mol/l ammonium acetate (pH 6.5) and 35% methanol.

Determination of enzyme activity

Luciferin was measured luminometrically in a bioluminometer according to the following procedure (Biolumat, Fa. Berthold Typ 9500 T, Wildbad, FRG; 25 °C, volume: 0.5 ml, (24)): 0.4 ml test buffer (30 mmol/l HEPES, 6.6 mmol/l MgCl₂, 0.66 mmol/l EDTA, 0.1 mmol/l dithiothreitol, 5 mmol/l ATP, 1 µg luciferase, pH 7.75) was preincubated at 25 °C for 5 min in a bioluminometer. Thereafter 0.1 ml luciferin solution (standard or incubation mixtures of the immunoassays (25)) were added and light impulses were measured for 10 s.

Luciferase activity was measured using the same, slightly modified procedure: 0.4 ml test buffer (30 mmol/l HEPES, 6.6 mmol/l MgCl₂, 0.66 mmol/l EDTA, 0.1 mmol/l dithiothreitol, 5 mmol/l ATP, pH 7.75, containing no luciferase) and 0.05 ml luciferin solution (0.2 mmol/l) were incubated at 25 °C for 5 min in the bioluminometer. Thereafter 0.05 ml luciferase solution were added and the light impulses integrated over 10 s.

Inhibition of luciferase activity by luciferin derivatives

This was measured by the following procedure: 0.4 ml test buffer (30 mmol/l HEPES, 6.6 mmol/l MgCl₂, 0.66 mmol/l EDTA, 0.1 mmol/l dithiothreitol, 5 mmol/l ATP, 1 µg luciferase (8 mU/mg), pH 7.75) was preincubated at 25 °C for 5 min. Then 0.05 ml of *D*-luciferin and 0.05 ml of *D*-luciferin derivative solution were added and the light signal integrated over 10 s.

Determination of kinetic constants

Michaelis-Menten constant (K_m) and V values were calculated according to *Wilkinson* (26) after graphical inspection of the data in *Lineweaver-Burk* diagrams. The assays were performed fluorimetrically as follows (test volume: 0.5 ml; 37 °C; substrate concentrations: 25, 50, 100, 250, 500, 750, 1000 µmol/l):

Carboxylic esterase (substrate: *D*-luciferin methyl ester; excitation: 330 nm; emission: 541 nm): 0.4 ml 0.05 mol/l Tris/HCl buffer, pH 7.5 and 0.05 ml enzyme solution (0.08 U) were incubated for 5 min. Then 0.05 ml substrate solution was added, and the decrease of relative fluorescence units per minute was measured.

Carboxypeptidase A (substrate: *D*-luciferyl-*L*-phenylalanine; excitation: 332 nm; emission: 543 nm): 0.40 ml 0.05 mol/l Tris/HCl buffer containing 3 g/l LiCl, pH 7.5 and 0.05 ml enzyme solution (2.25 U) were incubated for 5 min. Then 0.05 ml substrate solution was added, and the decrease of relative fluorescence units per minute was measured.

Carboxypeptidase B (substrate: *D*-luciferyl-*L*-N^α-arginine; excitation: 333 nm; emission: 544 nm): 0.40 ml 0.05 mol/l Tris/HCl buffer containing 0.2 mol/l NaCl, pH 7.8 and 0.05 ml enzyme solution (1 U) were incubated for 5 min. Then 0.05 ml substrate solution was added, and the decrease of relative fluorescence units per minute was measured.

Arylsulphatase (substrate: *D*-luciferin-O-sulphate; excitation: 338 nm; emission: 412 nm): 0.40 ml 0.01 mol/l sodium acetate buffer pH 5.0 and 0.05 ml enzyme solution (0.2 U) were incubated for 5 min. Then 0.05 ml substrate solution was added, and the decrease in relative fluorescence units measured.

Alkaline phosphatase (substrate: *D*-luciferin-O-phosphate; excitation: 345 nm; emission: 442 nm): 0.40 ml 0.01 mol/l diethanol-

amine buffer containing 0.5 mol/l MgCl₂, pH 9.8 and 0.05 ml enzyme solution (0.01 U) were incubated for 5 min. Then 0.05 ml substrate solution was added, and the decrease in relative fluorescence units measured.

Precisely quantified luciferin derivatives and luciferin solutions were used as calibration standards for the fluorescence measurements.

k_{cat} values were calculated as described in l.c. (27).

Results and Discussion

The structure of luciferin, a complex heterocyclic chemical compound was elucidated in 1963 by *White* et al. (30). Luciferin is very stable in solution at neutral pH, whereas in acid solution (pH below 2) a rapid degradation to *D*-cysteine and a benzthiazole derivative occurs. In alkaline solution luciferin is oxidized to dehydroluciferin which is a potent inhibitor of firefly luciferase (31). For these reasons synthesis strategies had to be developed which lead to the expected luciferin derivatives, but do not destroy luciferin during synthesis. Luciferin derivatives (fig. 1) were synthesized as described in Materials and Methods.

Luciferin methyl ester was synthesized as described by *White* et al. (18) using diazomethane. The chemical reaction proceeded problem-free, and the byproducts formed (nitrogen and methanol) could be easily removed. Remaining traces of unreacted luciferin were removed by silica gel chromatography. Thus a luciferin methyl ester preparation free of luciferin was obtained in 69% yield.

Luciferyl-*L*-phenylalanine and luciferyl-*L*-N^α-arginine were synthesized via luciferin-N-hydroxysuccinimide. Luciferin-N-hydroxysuccinimide was prepared by reacting luciferin with N-hydroxysuccinimide in the presence of N,N'-dicyclohexylcarbodiimide. Synthesis of luciferyl-*L*-phenylalanine proceeded without problems in relative high yield (63%). Synthesis of luciferyl-*L*-N^α-arginine is more difficult to perform because of the presence of the reactive guanido group. Using slightly acid conditions (pH 6; (31)), luciferyl-*L*-N^α-arginine could be synthesized in acceptable yield (37%).

Luciferin-O-sulphate was prepared by reacting luciferin with SO₃/pyridine complex (32) in the dark. The relatively high selectivity of the reagent (only phenolic OH-groups are attacked; (32)) lead to the expected product in 36% yield.

In the preparation of luciferin-O-phosphate by phosphorus oxytrichloride in the dark and 0 °C, only a relatively low yield (29%) of luciferin-O-phosphate was obtained, because of the relative aggressive reaction conditions.

Fig. 1. HPLC chromatograms of *D*-luciferin, and synthetic *D*-luciferin derivatives. (left: chromatograms of reaction mixture; right: chromatograms of purified *D*-luciferin derivatives; for details see Method) and fluorescence spectra (left: excitation; right: emission).

All synthesized luciferin derivatives were purified by high performance liquid chromatography (see fig. 1). Because of the high sensitivity of the luminometric assay (24) in which traces of luciferin would interfere, all preparations of luciferin derivatives had to be free of luciferin. This was verified by thin layer chromatography (two systems), by several HPLC systems, and by the luminometric assay itself.

The identity of all luciferin derivatives was assessed by ultraviolet (tab. 1) and fluorescence spectroscopy (tab. 2 and fig. 1), and by mass spectrometry (tab. 3). For luciferin methyl ester and luciferin-N-hydroxysuccinimide, elementary analysis were also performed. Amino acid analysis was used to determine the content of phenylalanine and arginine in luciferyl-*L*-phenylalanine and luciferyl-*L*-N^α-arginine. *D*-luci-

Tab. 1. Ultra violet maxima of *D*-luciferin derivatives.

<i>D</i> -luciferin derivative	Solvent	Maxima (nm)
<i>D</i> -luciferin	methanol	269, 332*)
<i>D</i> -luciferin methyl ester	methanol	269, 328
<i>D</i> -luciferyl- <i>L</i> -phenylalanine	0.05 mol/l Tris/HCl, pH 7.5	270, 335
<i>D</i> -luciferyl- <i>L</i> -N ^α -arginine	0.05 mol/l Tris/HCl, pH 7.5	265, 335
<i>D</i> -luciferin-O-sulphate	0.1 mol/l Na acetate, pH 5.0	250, 290
<i>D</i> -luciferin-O-phosphate	0.05 mol/l diethanolamine, pH 9.8	260, 315

*) according to White et al. (18).

Fig. 1. HPLC chromatograms of *D*-luciferin, and synthetic *D*-luciferin derivatives. (left: chromatograms of reaction mixture; right: chromatograms of purified *D*-luciferin derivatives; for details see Method) and fluorescence spectra (left: excitation; right: emission).

Tab. 2. Fluorescence properties of *D*-luciferin derivatives; excitation and emission maxima.

<i>D</i> -luciferin derivatives	Solvent	Excitation (nm)	Emission (nm)
<i>D</i> -luciferin	0.05 mol/l Tris/HCl, pH 7.5	330	531
<i>D</i> -luciferin methyl ester	0.05 mol/l Tris/HCl, pH 7.5	330	541
<i>D</i> -luciferyl- <i>L</i> -phenylalanine	0.05 mol/l Tris/HCl, 1 mol/l NaCl, pH 7.5	332	543
<i>D</i> -luciferyl- <i>L</i> - N^{ϵ} -arginine	0.05 mol/l Tris/HCl, 0.1 mol/l NaCl, pH 7.8	333	544
<i>D</i> -luciferin- <i>O</i> -sulphate	0.1 mol/l Na acetate, 0.25 mol/l NaCl, pH 5.0	338	412
<i>D</i> -luciferin- <i>O</i> -phosphate	0.05 mol/l diethanolamine, 1 mmol/l $MgCl_2$, pH 9.8	345	442

feryl-*D*-phenylalanine had a phenylalanine content of 37.9% (calculated: 38.4%). For *D*-luciferyl-*L*- N^{ϵ} -arginine, an arginine content of 38.7% (calculated: 39.7%) was determined.

Furthermore, quantitative luciferin determinations were performed by the luminometric assay (tab. 4)

and by fluorescence measurements. For this purpose, derivatives were incubated with the corresponding enzyme for a sufficiently long period (approx. 2 h) to release all *D*-luciferin under optimal conditions. The amount of luciferin found by both methods was close to the calculated values. In all these tests satisfactory results were obtained.

Tab. 3. m/z values of luciferin derivatives obtained by fast atom bombardement mass spectroscopy.

Luciferin derivative	M_r	MH^+ (m/z)
<i>D</i> -luciferin methyl ester	294.4	295
<i>D</i> -luciferyl- <i>L</i> -phenylalanine	427.6	428
<i>D</i> -luciferyl- <i>L</i> - N^{α} -arginine	436.5	437
<i>D</i> -luciferin-O-sulphate	360.4	361
<i>D</i> -luciferin-O-phosphate	359.4	360

Tab.4. Enzymatically determined content of *D*-luciferin in *D*-luciferin derivatives.

<i>D</i> -luciferin-derivatives Enzyme*)		<i>D</i> -luciferin content	
		calculated (%)	found (%)
<i>D</i> -luciferin methyl ester	carboxylic esterase	89.5	89.4
<i>D</i> -luciferyl- <i>L</i> -phenylalanine	carboxypeptidase A	61.6	61.3
<i>D</i> -luciferyl- <i>L</i> - N^{α} -arginine	carboxypeptidase B	60.3	60.5
<i>D</i> -luciferin-O-sulphate	arylsulphatase	73.1	72.4
<i>D</i> -luciferin-O-phosphate	alkaline phosphatase	73.3	73.0

*) enzymatic cleavage was performed as described in Materials and Methods.

Tab. 5. Percentage of light impulses measured in the luminometric assay (*Photinus pyralis*) in the presence of *D*-luciferin derivatives.

<i>D</i> -luciferin derivative	c (mol/l)	<i>D</i> -luciferin concentration		
		10^{-9} mol/l	10^{-8} mol/l	10^{-7} mol/l
Control	0	100	100	100
<i>D</i> -luciferin methyl ester	10^{-7}	—	80	96
	10^{-6}	—	34	40
	10^{-5}	—	24	2
<i>D</i> -luciferyl- <i>L</i> -phenylalanine	10^{-6}	100	96	71
	10^{-5}	80	78	65
	10^{-4}	40	40	31
<i>D</i> -luciferyl- <i>L</i> - N^{α} -arginine	10^{-6}	—	80	73
	10^{-5}	—	92	71
	10^{-4}	—	61	58
<i>D</i> -luciferin-O-sulphate	10^{-6}	100	96	73
	10^{-5}	100	89	55
	10^{-4}	100	100	52
<i>D</i> -luciferin-O-phosphate	10^{-6}	100	100	80
	10^{-5}	100	100	69
	10^{-4}	5	46	59

As can be seen in table 3, m/z values found by fast atom bombardement mass spectrometry for luciferin derivatives indicated the expected luciferin compounds.

Before use of the synthesized luciferin substrates in immunoassay detection systems the following questions had to be answered:

- 1) can different amounts of luciferin be quantified in the luminometric assay?
- 2) are the derivatives themselves substrates for luciferase?; is a light emitting reaction induced?
- 3) are the derivatives inhibitors of luciferase?; are the luminometric assays disturbed by these compounds?
- 4) can the derivatives be cleaved by enzymes?, and
- 5) is *D*-luciferin released?

As to the first question luminometric assays (*Photinus pyralis*) are widely used at present for the quantification of ATP. Luciferin and luciferase are constants in these assays, and ATP is the variable measured. In the present detection system, the variables have been reversed (luciferase and ATP are constants, luciferin is the variable). Because of the mechanism of the luciferase reaction of *Photinus pyralis* (no Michaelis-Menten kinetics, but a second order reaction (33)) it should also be possible to measure variable luciferin concentrations by this method. Indeed, this was achieved, as demonstrated in figure 2.

Fig. 2. Determination of *D*-luciferin in the luminometric assay (concentrations in test: 5 mmol/l ATP, 1 μ g luciferase). For details see Materials and Methods.

Enzymatic studies clearly demonstrate that luciferin derivatives applied in the luminometric assay up to 1 mmol/l cause no light emission. In other words, the luciferin derivatives are not substrates for firefly luciferase.

Depending on the concentrations of luciferin derivatives added to the luminometric assay (see tab. 5), luciferin derivatives can inhibit the light emitting reaction. The absence of this interference is essential

Tab. 6. Kinetic data for enzymes and *D*-luciferin substrates.

Enzyme***)	<i>D</i> -luciferin derivative	K_m (mol/l)	V (mol · min ⁻¹ · mg ⁻¹)	k_{cat} (s ⁻¹)	k_{cat}/K_m (l · mmol ⁻¹ · s ⁻¹)
Carboxylic esterase	<i>D</i> -luciferin methyl ester	$1.6 \cdot 10^{-5}$	10.6	11*)	662
Carboxypeptidase A	<i>D</i> -luciferyl- <i>L</i> -phenylalanine	$3.5 \cdot 10^{-3}$	13.6	7.7*	2.2
Carboxypeptidase B	<i>D</i> -luciferyl- <i>L</i> -N ^ω -arginine	$2.0 \cdot 10^{-4}$	12.0	7.0*)	35
Arylsulphatase	<i>D</i> -luciferin-O-sulphate	$7.1 \cdot 10^{-5}$	6.4	24**)	338
Alkaline phosphatase	<i>D</i> -luciferin-O-phosphate	$4.3 \cdot 10^{-5}$	610	1010*)	23500

*) protein concentration was given by manufacturer.

**) protein concentration was determined according to *Bradford* (28). Bovine serum albumin was used as standard.

***) enzyme activity was proven as described in l. c. (19–23).

for the sensitivity of the detection systems of the immunoassays. Therefore, concentrations of luciferin derivatives were later used in the detection systems which did not interfere in the luminometric assay (25).

The most important point was whether the luciferin derivatives can be cleaved by enzymes and whether intact luciferin is indeed released by these reactions. As can be seen from the data in table 6, which have been obtained by following the hydrolytic reactions by the luminometric assay, luciferin derivatives can be cleaved by appropriated enzymes. Substitution of the hydroxyl group of the carboxyl group of luciferin by a methoxy group led to a compound which could be cleaved by carboxylic esterase. Substitution by a phenylalanine residue resulted in a substrate for carboxypeptidase A. Substitution by an arginine residue in N(α) position gave a substrate for carboxypeptidase B. Formation of a sulphate with the hydroxyl group of luciferin led to a substrate which was cleaved by arylsulphatase. Luciferin-O-phosphate was a substrate for alkaline phosphatase. The *Michaelis-Menten* constants and V values determined for the luciferin

substrates and the respective enzymes were in a range typical for other substrates of these enzymes. Especially alkaline phosphatase showed a very high catalytic rate with the substrate luciferin-O-phosphate. The sensitivity of the assay is about sixty-fold higher than with the usual substrate *p*-nitrophenyl phosphate.

Conclusion

In summary, the present luciferin derivatives fulfil all prerequisite conditions for their use in enzyme immunoassays with a bioluminescent detection system as outlined in figure 3. Such assays are described in detail in the accompanying paper (25).

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Fig. 3. Scheme of the bioluminometric detection system for enzyme-labelled antibodies.
a) liberation of luciferin from luciferin derivatives substituted at the carboxyl group;
b) release of luciferin from luciferin derivatives substituted at the hydroxyl group.

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