Preparation of a chemiluminescent imidoester for the non-radioactive labelling of proteins

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Abstract

A chemiluminescent aryl acridinium ester was synthesized which possesses an imidate ester group capable of reacting with proteins under mild conditions. The compound can be detected at levels as low as 5.2×10^{-19} mol using commercially available luminometers and can therefore be used to produce high specific activity labelled antibodies for use in immunochemiluminometric assays. The imidate ester compares favourably with a previously reported *N*-succinimidyl ester in terms of its labelling properties but is easier to synthesize, requiring one less step. The compound was used to label affinity purified sheep antibodies to human parathyroid hormone to demonstrate its utility in a two-site immunochemiluminometric assay for the measurement of intact parathyroid hormone.

Keywords: acridinium ester, chemiluminescence, imidoester, immunoassay, labelled antibodies, luminometry.

1. Introduction

Immunoassay techniques based upon the use of radiolabelled antigens or antibodies are well established though for many reasons attempts have been made to develop immunoassay systems based on non-radioactive labels. The use of chemiluminescent molecules as immunoassay probes has been reported for many years though it is only recently that chemiluminescent immunoassays have been developed which exhibit superior performance to their radioactive counterparts [1].

Chemiluminescent acridinium esters have been used as a basis for the development of high-performance immunochemiluminometric assays using labelled antibodies and to a lesser extent for the development of labelled antigen immunoassays. The preparation of labelled protein tracers has involved

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the use of 4-(2-succinimidyloxycarbonylethyl)phenyl 10-methylacridinium 9-carboxylate fluorosulphonate [2]. This compound possesses an *N*-hydroxy-succinimide ester which permits the rapid labelling of lysine residues, under mild conditions, by forming an amide bond.

Imidoesters have been widely used as a means of labelling or cross-linking proteins and it has been suggested that the subsequent formation of a chargeable amidine bond is less deleterious than the formation of a non-chargeable linkage [3, 4].

Here we describe the synthesis of an imidoester derivative of a chemiluminescent acridinium ester and preliminary studies involving its coupling to antibodies. The imidoester derivatives have amine specificity ($-NH_2$ groups on *N*-terminii or lysine residues) of proteins. It therefore has the potential advantage of yielding a chargeable linking group which will improve the solubility of the label in aqueous environments. These labelled antibodies have then been used as reagents in a two-site immunochemiluminometric assay for intact human parathyroid hormone (hPTH), the performance of which has been compared with an established procedure [5].

2. Experimental details

2.1. Materials

Acridine 9-carboxylic acid and thionyl chloride were obtained from Aldrich Chemical Co. Ltd., Gillingham, Dorset. 3-(4-hydroxyphenyl)propionitrile was obtained from Lancaster Synthesis Ltd., Morecambe, Lancashire. Mouse, rabbit and bovine immunoglobulins were obtained from Sigma Chemical Co Ltd., Poole, Dorset. Bovine serum albumin was from BCL, Lewes, Sussex. All other chemicals were from BDH Ltd., Poole, Dorset.

Immunoaffinity purified sheep antibodies to hPTH(1-34) and monoclonal mouse antibodies to hPTH(44-68) were prepared as described previously [5]. Antibody and protein magnetizable and diazocellulose solid-phases were prepared according to published methods [6, 7].

2.2. Buffers

The following buffers were used in the study. The phosphate labelling buffer consisted of a mixture of 0.1 M solutions of sodium dihydrogen orthophosphate and disodium hydrogen orthophosphate of various proportions to provide a pH value of 8.0. The borate labelling buffer consisted of a 0.1 M solution of disodium tetraborate buffered to pH values of 8.5, 9.0, 9.5 and 10.0 with 2 M sodium hydroxide solution. The assay buffer was a solution of a mixture of sodium dihydrogen orthophosphate and disodium hydrogen orthophosphate (0.1 M, pH 6.3) with sodium chloride (0.15 M), containing sodium azide (0.5 g l^{-1}), and bovine serum albumin (0.1%). The wash buffer consisted of an aqueous solution of sodium dihydrogen orthophosphate (1.8 g l^{-1}) containing sodium chloride (0.15 M), sodium azide (0.5 g l^{-1}), Triton X-100 (1.0%) and bovine serum albumin (0.05%).

2.3. Synthesis of 4-(2-methoxycarbonimidoylethyl)phenyl 10-methylacridinium 9-carboxylate chloride hydrochloride (V)

The label (**V**) was synthesized in the following stages (Fig. 1). Acridine 9-carbonyl chloride (**II**) was synthesized from compound **I** by published procedures [8]. Then to a solution of this acid chloride (**II**) (2.12 g, 8.8 mmol) in anhydrous pyridine (25 ml) was added 3-(4-hydroxyphenyl)propionitrile (1.32 g, 9.0 mmol). The mixture was stirred for 18 h at room temperature. The reaction mixture was poured into iced water (200 ml) and the resulting precipitate was filtered and dried *in vacuo* to give buff coloured crystals of 4-(2-cyanoethyl)phenylacridine 9-carboxylate (**III**) (1.92 g, 62%), melting point 165–166 °C (from ethyl acetate. ν max (KBr disc) 2240 (C≡N) and 1740 cm^{-1} (C=O); δ ($CDCl_3$) 8.46–7.58 (12H, aromatic), 3.06 (2H, t, CH_2) and 2.70 (2H, t, CH_2); *m/e* gave the expected fragmentation pattern and (M^+) was measured as 352.1213 ($C_{23}H_{16}N_2O_2$ is 352.1210).

The ester (**III**) (0.28 g, 0.80 mmol) was suspended in methyl iodide (8 ml) in a thick walled glass tube, cooled in liquid nitrogen and sealed *in vacuo*. The tube was then heated in an oil bath at 100 °C for 24 h. The tube was cooled, seal broken and methyl iodide was removed by evaporation to give brown crystals of 4-(2-cyanoethyl)phenyl 10-methylacridinium 9-carboxylate iodide (**IV**) (0.12 g, 61%), melting point 200 °C (decomp., from ethyl acetate–cyclohexane. ν max (KBr disc) 2260 (C≡N) and 1750 cm^{-1} (C=O); δ (d6-DMSO) 8.36–6.72 (12H, aromatic), 3.52 (3H, s, CH_3), 3.0 (2H, t, CH_2) and 2.82 (2H, t, CH_2); *m/e* (FAB from thioglycerol) gave the

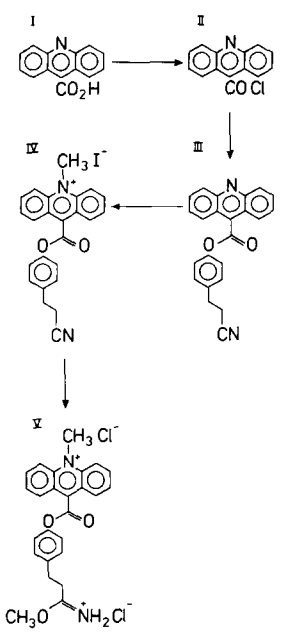


Fig. 1. Synthesis of the chemiluminescent imidoester.

expected fragmentation pattern and (M^+) was measured as 367.1451 ($C_{24}H_{19}N_2O_2$, cation is 367.1447). Compound **IV** (0.069 g, 0.14 mmol) was suspended in anhydrous methanol (10 ml) at 0 °C under nitrogen. A stream of dry HCl gas was bubbled through the reaction mixture for 1 h and then the mixture was left to stand at 0 °C for 2 h. Dry diethyl ether (150 ml) was added to give yellow crystals of 4-(2-methoxycarbonimidoylethyl)phenyl 10-methylacridinium 9-carboxylate chloride hydrochloride (**V**) (0.055 g, 83%), melting point 232 °C (decomp.). ν max (KBr disc) 3500 (C=NH) and 1752 cm^{-1} (C=O); δ (d6-DMSO) 9.14–7.40 (m, aromatic), 4.16 (3H, s, CH_3), 3.5 (3H, s, CH_3) and 3.0–2.8 (4H, m, CH_2); *m/e* (FAB from thioglycerol) gave the expected fragmentation pattern and (M^+) was measured as 400.1769 ($C_{25}H_{24}N_2O_3$, dication is 400.1780).

2.4. Measurement of chemiluminescence

Luminometry was performed using a Magic Lite Analyser (Ciba Corning Diagnostics, Walpole, MA 02032). The emission intensity of compound **V** was determined by initially dissolving **V** in acetonitrile (0.5 mg ml^{-1} solution) and then making further dilutions in assay buffer to give a final dilution of (500 pg ml^{-1}). 10 μl of this solution was measured in the luminometer. Replicate measurements routinely exhibit precision of less than 5%.

2.5. Optimization of antibody labelling

The general procedure for labelling antibody involved the preparation of stock solutions of compound **V** in acetonitrile and the addition of antibody in the appropriate labelling buffer to an aliquot of the stock solution in a glass vial. After the appropriate reaction time at room temperature in the dark, 100 μl of a solution of lysine monohydrochloride (10 mg ml^{-1}) in the same labelling buffer was added, followed by a further incubation period of 5 min. The reaction mixture was purified using a 80×6 mm column of medium-grade Sephadex G50 equilibrated and eluted with assay buffer, 1 ml fractions were collected. A 10 μl aliquot of each fraction was diluted 100-fold in assay buffer and 10 μl of this dilution was used for luminometry. Void volume fractions exhibiting chemiluminescence were pooled. The effects of changing the initial label-to-protein ratio, pH and reaction time were studied as described below.

2.6. Calculation of incorporation ratio

Some initial experiments were performed in which the molar incorporation ratio of acridinium to rabbit gammaglobulin (RIgG) was determined by both chemiluminescence and fluorescence measurements. The latter measurements were made on *N*-methylacridone (NMA), the product of the chemiluminescent reaction which is dissociated from the rest of the original molecule and thus not subject to protein microenvironmental effects such as quenching. 1 ml of phosphate labelling buffer, pH 8.0 containing 250 μg of RIgG was added to 50 μg of 0.5, 1.0, 2.0 and 4.0 mg ml^{-1} stock solution of compound **V** respectively and the reaction and purification performed as described above

except that protein-free assay buffer was used. The molar concentration of acridinium in the pooled fractions was determined by first calibrating the luminometer with known amounts of compound **V** and then interpolating the light yield obtained from a 10 μl aliquot of the pool. Protein determinations were performed by measuring the optical density of the pool at 280 nm and calibrating the spectrophotometer with known concentrations of RIgG.

The pools of labelled antibody were ultimately oxidized by adding 1 ml of an aqueous solution of 1 M sodium hydroxide containing 1% hydrogen peroxide (100 volume) such that the final concentration of these reagents was 0.2 M and 0.2% respectively. The fluorescence emission intensity of the NMA produced by the oxidation was measured using a spectrophotofluorimeter which had previously been calibrated using known amounts of NMA ($\lambda_{ex} = 395$ nm, $\lambda_{em} = 430$ nm).

2.7. Effect of pH on the labelling reaction

The general labelling protocol described above was performed using 10 μl of a 0.5 mg ml^{-1} stock solution of compound **V** and 50 μg of RIgG in borate labelling buffer (200 μl at pH 8.5, 9.0, 9.5 and 10.0 respectively). Following each reaction and purification the total void volume chemiluminescence was calculated. In a further experiment, incorporation ratios were calculated for reactions at pH 8.0 and 9.5 using 0.5, 1.0, 2.0 and 4.0 mg ml^{-1} stock solutions of compound **V**.

2.8. Time course of labelling reactions

Labelling reactions were carried out at pH 9.5 using 10 μl of the 0.5 mg ml^{-1} stock solution of compound **V** and 50 μg of RIgG in 200 μl of buffer with incubation times of 0, 15, 30, 60, 120 and 240 min. Following each reaction and purification the total void volume chemiluminescence was calculated.

Ultimately, 50 μg of antibody in 200 μl of borate labelling buffer pH 9.5, was reacted with 10 μl of a solution of compound **V** (0.5 mg ml^{-1}) in acetonitrile. The reaction was allowed to proceed in the dark for 1 h at room temperature prior to quenching and purification.

2.9. Immunochemiluminometric assay (ICMA) for intact (1-84) human parathyroid hormone (hPTH)

Affinity purified antibodies to hPTH(1-34) were labelled with compound **V** as described above. The pooled fractions from the purification were absorbed with diazocellulose-linked non-immune mouse IgG for 5 min to remove any non-specific binding components from the preparation and stored in aliquots at -20 °C. Tracer quality was initially assessed by its binding to diazocellulose-linked bovine serum albumin and diazocellulose-linked bovine PTH, in either the presence or absence of 10^{-10} M hPTH(1-34), for 1 h at room temperature.

Immunoassays were carried out in a similar fashion to those described previously [5]. Briefly, PTH standard in horse serum (100 μl) was dispensed into 12×75 mm polystyrene tubes. 100 μl of labelled antibody in assay

buffer (30 ng ml^{-1}) were then added and the tubes, vortex-mixed and incubated overnight at room temperature. Magnetizable particle-linked anti-hPTH (44-68) (50 μl, 15 μg of antibody) was then added and the tubes vortexed then incubated for a further 1 h at room temperature. Separation was achieved using a magnetic separating rack (Ciba-Corning Diagnostics, E. Walpole, MA 02032) as follows. 1 ml of wash buffer was added to the tubes and the rack placed on the separator for 3 min. The supernatants were decanted to waste and the rack removed from the separator. After a further wash and separation the tubes were placed in turn into the luminometer in order to quantify the chemiluminescence emission from the solid-phase immune complexes. Photon counts were integrated over 2 s following initiation of the chemiluminescent reaction. The assays were performed in duplicate using standards consisting of known concentrations of hPTH in horse serum. The dose–response relationship of the assay was expressed as the specific chemiluminescence emission intensity plotted with respect to the hPTH concentration.

3. Results and discussion

The emission intensity of compound **V** was calculated as 2.1×10^5 relative light units (RLU) per picogram which gives a minimum detection limit of 5.2×10^{-19} mol at two standard deviations above the reagent background.

The molar incorporation ratio at pH 8.0 as determined by both acridinium chemiluminescence (CL) and NMA fluorescence (F) measurements, predictably increased as the initial ratio of acridinium ester to protein increased (see Table 1).

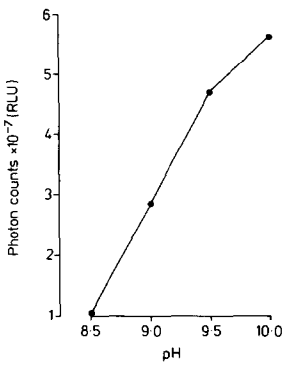
The uptake of label by IgG increased with increasing pH linearly up to pH 9.5 (Fig. 2). At this pH there was no overall loss of chemiluminescence efficiency over the time period of the labelling reaction (data not shown). The uptake of label was substantially greater over a range of initial reactant ratios at pH 9.5 than was observed in the presence of high concentrations of the imidoester at pH 8.0. At an initial ratio of 272 mol label per mol

TABLE 1

Incorporation of acridinium ester into immunoglobulin at pH 8.0

Initial mass of acridinium (μg)	Incorporation (mol per mol)	
	CL calculation	F calculation
25	0.24 (3.2)	0.29
50	0.30 (6.0)	0.40
100	0.63 (9.6)	0.74
200	1.20 (18.6)	2.12

The figures in parentheses refer to the incorporation of acridinium ester into immunoglobulin at pH 9.5.



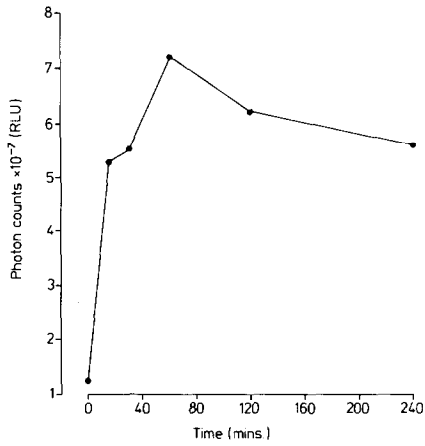


Fig. 2. Effect of pH of the reaction mixture on the uptake of the chemiluminescent imidoester by rabbit IgG. Incorporation is represented by the total chemiluminescence in the exclusion volume fractions of gel columns used to isolate protein bound label from the reaction mixture.

Fig. 3. Effect of incubation time on the uptake of the chemiluminescent imidoester by rabbit IgG. Incorporation is represented by the total chemiluminescence in the exclusion volume fractions of gel columns used to isolate protein bound label from the reaction mixture.

IgG there was 94% loss of protein. This is in contrast to the situation at lower initial ratios where protein recovery was routinely 75%. At pH 9.5, uptake of label was rapid (approximately 30 min) with little increased uptake at reaction times in excess of 1 h (Fig. 3). The label hydrolyses at alkaline pH, hence yielding loss of chemiluminescence activity. Thus long incubation times for the coupling reaction result in an apparent decrease in chemiluminescence.

The quality of the labelled anti-hPTH antibodies produced by this method was comparable with that observed for an acridinium *N*-succinimidyl ester studied previously in terms of non-specific binding, specific binding and displacement from solid-phase antigen by liquid phase antigen. The figures for the imidate derived labelled antibodies were 0.29%, 42.9% and 7.6% (expressed as percentage binding of the total added counts) respectively, compared with 0.24%, 43.2% and 10.5% for the *N*-succinimidyl ester derived labelled antibodies. The dose–response curves obtained for the PTH–ICMAs using both types of labelled antibodies exhibited similar characteristics in terms of sensitivity, slope and dynamic range (Fig. 4) though a rigorous comparison has not been made at this stage. Assays were performed in duplicate and routinely gave coefficients of variation of less than 10%.

It is apparent that the protein loss occurs by precipitation at high label-to-protein ratios at pH 9.5. Under such conditions the label is presumably not as water soluble as might be expected for two possible reasons. Firstly the imidate group (pKa = 6) is not protonated and secondly the label exists as a carbinol base (pseudo-base) at high pH [2]. This is less soluble than the acridinium ester which is the predominant form at low pH. The initial

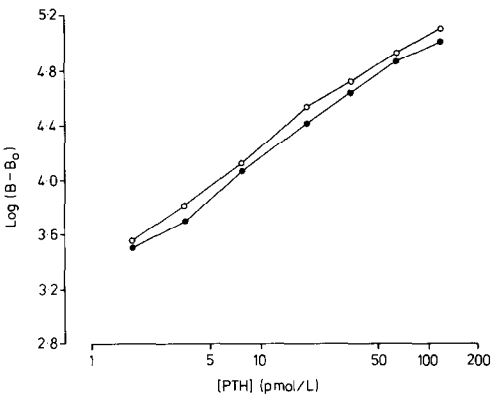


Fig. 4. Dose–response relationships of immunochemiluminometric assays for intact parathyroid hormone. Affinity purified sheep (anti-PTH(1-34)) antibodies were labelled with the chemiluminescent imidoester (●) and a chemiluminescent *N*-succinimidyl ester (○). The solid phase antibody consisted of monoclonal mouse (anti-PTH(44-68)) IgG coupled to paramagnetic particles. The ordinate axis is plotted in terms of the difference between the specifically bound activity, at a given concentration of PTH, and the non-specific binding at zero concentration.

high pH used for labelling is replaced by a low pH environment (pH 6.3) upon purification of the label. Provided that extensive precipitation does not occur, then the immunoreactivity of the antibodies is not affected in this instance by loss of chargeable amine groups. However, there may be occasions where retention of chargeability is an important factor in dictating the immunoreactivity of the labelled reagent such as in the case of small proteins and peptides. In such situations the availability of the chemiluminescent imidoester described here may prove valuable for the production of chemiluminescent tracers.

4. Conclusions

In conclusion, we have synthesized an alternative acridinium ester label based on an imidoester derivative which has the similar amine specificity as its *N*-succinimidyl counterpart. This label was used for the production of labelled antibodies to human PTH. Studies on the immunoreactivity of these reagents and their subsequent use in a two-site immunochemiluminometric assay show that they behave in a similar manner to the *N*-succinimidyl label. The effects of overlabelling and loss of assay performance are also comparable [6]. However the synthetic route to the imidate ester involves less steps, which will save time and labour, and the yields of the products in each step are higher. Imidates are generally highly crystalline compounds and they are more hydrophilic than their *N*-succinimidyl counterparts.

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