On the development of NAD(P)H-sensitive fluorescent probes

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In contrast to current, multi-reagent assay systems, the development of a single reagent that can be used to assay NAD(P)H is described. The reagent eliminates the fluorophore 4-methylumbelliferone from a quinoxalinium adduct upon reduction and the chemistry of this process is described.

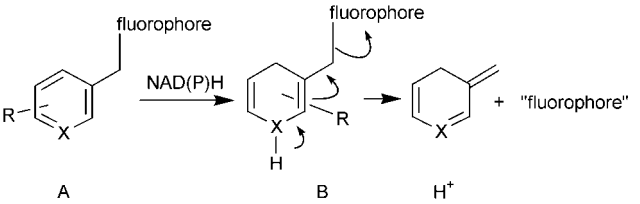
1,4-Dihydronicotinamide adenine dinucleotide (NADH) and its phosphate ester, NADPH, are ubiquitous reducing agents in Nature, and measurement of intracellular turnover of these vectors is often used to indicate cellular viability and activity.[1] In rapidly proliferating cervical cancer cells the turnover of NAD(P)H is greater than in normal cells and thus it can be used as a biological marker for the detection of cancer.[1] One therefore requires a means for the detection and estimation of NAD(P)H levels in cells.[2]

NAD(P)H concentrations can be monitored by direct measurement of their incipient fluorescence but, since it is rapidly turned over [reoxidized to $NAD(P)^+$], absolute levels do not necessarily reflect the level of enzymic and metabolic activity occurring within the cell. Furthermore, interference from background signals from other biochemical entities in cells can also obscure any reliable estimation.

As a consequence of these problems there is considerable interest in using reagents that themselves are selectively reduced by NAD(P)H with the formation of either a coloured or fluorescent signal. Few dye precursors react directly with NAD(P)H and, in order to overcome this problem, a catalyst usually has to be used. The catalyst is either an enzyme, such as diaphorase,[3] or a known electron carrier, such as methylphenazinium methyl sulfate **1**.[4] Thus NADH reduction of the colourless or pale yellow tetrazolium salts, such as iodonitrotetrazolium blue, does not occur by direct interaction but can be catalysed by the enzyme diaphorase to form the intensely coloured formazan dye. The product formazans are generally highly insoluble and non-fluorescent.[5] Use has also been made of the weakly fluorescent dye resazurin **2**, which is reduced by NADH in the presence of either diaphorase[6] or the phenazinium salt **1**[7] to give, in this case, the strongly fluorescent dye resorufin **3**.

The mechanism of the catalysis with the phenazinium salt **1** proceeds with formation of the dihydrophenazine species **4**, which is extremely unstable and rapidly undergoes further redox reactions. This instability is probably because the intermediate dihydrospecies **4** is formally anti-aromatic.

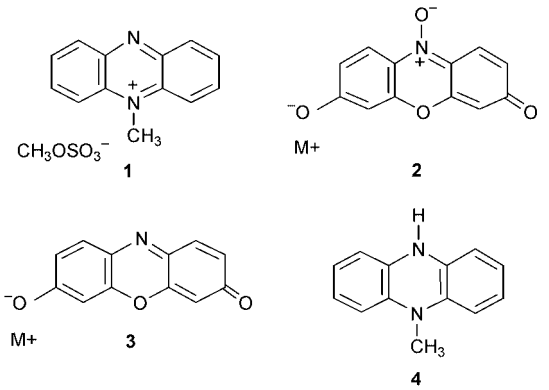
In order to avoid the need for a two-component system, involving a reducible fluorophore and a catalyst, we considered the possibility of using a reducible substrate that could release a masked fluorophore after reduction with NAD(P)H. The general scheme was as depicted in Scheme 1. The first step was



Scheme 1 Reduction of a protected fluorophore **A** should lead to an intermediate dihydro-compound **B** that collapses spontaneously with liberation of the free fluorophore.

to generate masked fluorophore substrates, such as **A**, that would interact with NAD(P)H to produce a reduced species, such as **B**, that would spontaneously collapse with the liberation of the fluorophore.

Apart from work with methylphenazinium methyl sulfate, surprisingly little work has been reported on the use of NADH as a direct reductant of other organic substrates, *i.e. not* involving an enzyme-mediated process. Substrate requirements are for a system that is sufficiently oxidizing to drive the equilibrium with NADH over to the NAD^+ state, preferably without being too strong an oxidant so as not to interfere with other components in any assay. Also, since NADH reductions generally involve a face-to-face interaction with the substrate, thus encouraging a net hydride delivery, planar systems should initially be investigated.



Results and discussion

In this work two systems have been explored, the phenazinium and quinoxalinium systems. Phenazine derivatives of the type **6**, upon N-methylation, should give the phenazinium system, related to **1** and known to be rapidly reduced with NADH.[4] 4-Methylcatechol was oxidized with sodium periodate to produce the *ortho*-quinone **5** which was immediately treated with 1,2-diaminobenzene to produce 2-methylphenazine **6** in good yield (see Scheme 2). The methyl group could be brominated, using *N*-bromosuccinimide (NBS), to give the monobromide **7**, characterized as its phenyl ether **8**. Reaction of the monobromide with 4-methylumbelliferone produced the required

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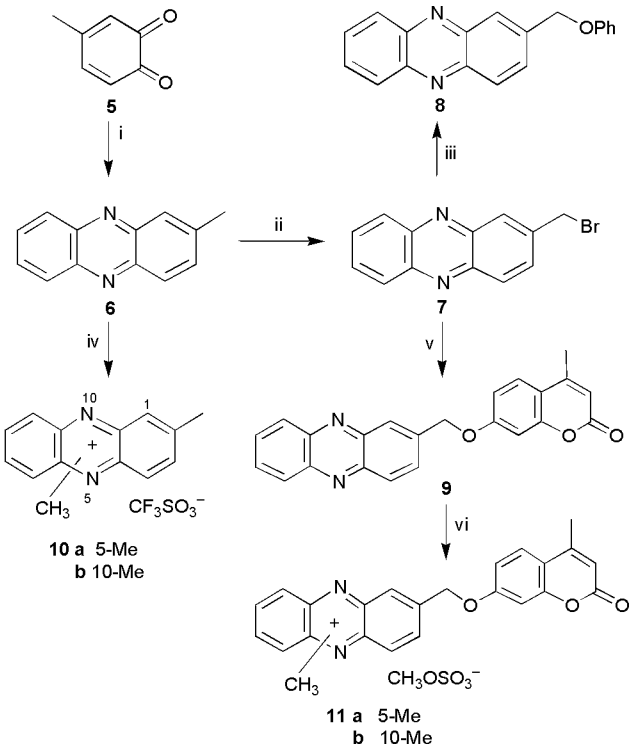
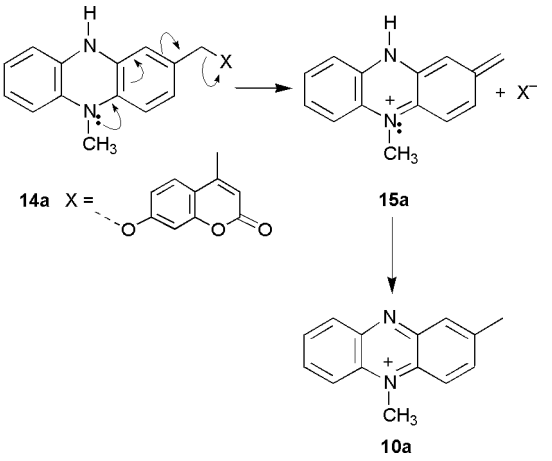


Scheme 2 *Reagents*: i, 1,2-diaminobenzene; ii, NBS; iii, sodium phenoxide; iv, methyl triflate; v, 4-methylumbelliferone, base; vi, dimethyl sulfate.

conjugate **9**. Reaction of the parent phenazine **6** with methyl triflate gave mixtures of the 5- and 10-methyl regioisomers, **10a** and **10b**, not easily separable, and means to overcome this problem could not be found. As a consequence the qualitative behaviour of these mixtures towards NADH was studied.The conjugate **9** was only sparingly soluble in organic solvents and attempts to make its triflate derivative failed. Instead this conjugate was methylated with dimethyl sulfate to produce the regioisomeric mixture **11**.

A solution of the yellow salts **10** in 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES) buffer at pH 7.5 was treated with an excess of a freshly prepared solution of NADH, using salt concentrations in the range 10^{-8} mol dm^{-3} to 10^{-7} mol dm^{-3}. Reaction was monitored by disappearance of the yellow colour (λ_{max} 390 nm), the solutions going colourless within minutes. When only a slight excess of the NADH reductant was used, the initial bleaching of the colour was followed by its reappearance.[7] This observation is in agreement with the observations of Davis and Thornalley,[8] Zaugg,[9] and Chew and Bolton[10] on the parent phenazinium methyl sulfate. They suggested that aerial reoxidation of the reduced intermediate **13** (*cf.* **4**) occurs. Attempts to isolate the dihydro-species **13** (X = H) were unsuccessful, the compound being readily oxidized in air,[11] only small samples of the demethylated phenazine **6** being obtained from the complex reaction mixture. Aerial photo-oxidation of the phenazinium system is well recorded[12] and results in formation of products such as pyocyanin and **12**.

NADH reduction of the yellow-green conjugate **11** also resulted in a rapid decoloration followed by reappearance of the colour. Examination of the products again showed a complex mixture, amongst which were small quantities of the fluorescent 4-methylumbelliferone. A fluorescence study also showed that some of the fluorophore was freed during reduction but the yields obtained were small (<10%). A possible mechanism for this elimination is shown in Scheme 3; the



Scheme 3

reduced intermediate (**14a**, or its regioisomer, **14b**) would be expected to eliminate the fluorophore to produce the intermediate **15** and hence re-form the phenazinium species, *i.e.* **10a**(**b**), before further reduction with NADH.

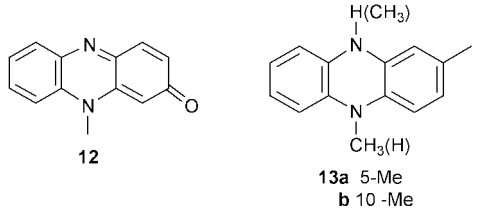
It is clear that, for the phenazinium series, the intermediate dihydro-species, such as **4** and **13**, were too sensitive to competing aerial oxidation and redox reactions[7] to be of value in estimating NADH. Encouraged by the appearance of small quantities of the free fluorophore, our attention was turned to exploring other heterocyclic substrates. Of these, the quinoxalinium series was considered, since, it was argued, the corresponding reduced system should be less susceptible to these oxidative side reactions.

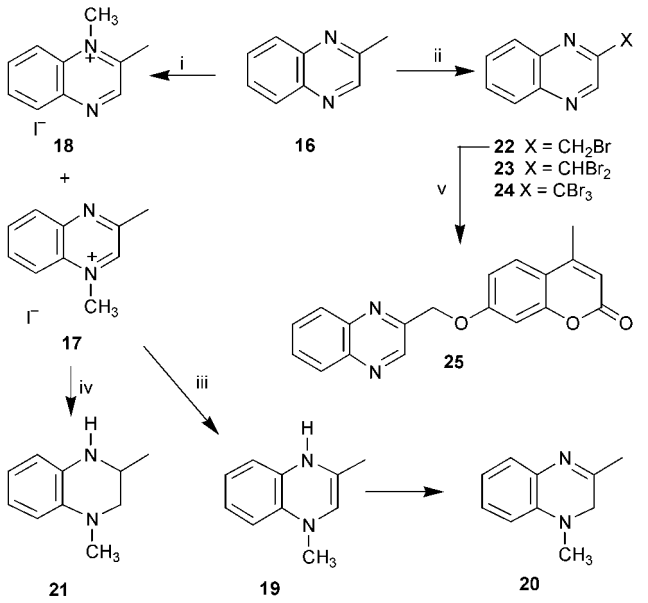
Condensation of 1,2-diaminobenzene with pyruvaldehyde gave 2-methylquinoxaline **16** in good yields.[13]

In contrast to the phenazine system, the quinoxaline was found to react regioselectively with methylating agents, such as methyl iodide or methyl triflate, to afford the corresponding 1,3-dimethylquinoxalinium salt **17**. ^{1}H NMR spectral examination showed that a less than 5% yield of the 1,2-dimethyl regiosomer **18** was formed, and the major isomer was readily purified by recrystallization. Reduction of these salts with NADH at pH 7.5 rapidly produced a dihydro-compound (see Scheme 4). Whilst mechanistic studies with NADH predicted that this would be the 1,4-dihydro-isomer **19**,[14] this tautomer was not isolated since it rapidly rearranges to the 1,2-dihydro-isomer **20**, isolated in high yield (>90%). Again, in contrast to the dihydrophenazine intermediates, the dihydroquinoxaline **20** is relatively stable, although, upon exposure to air it is oxidized to give a variety of highly coloured products. It is to be noted that compound **19**, like **4**, is also formally anti-aromatic and this undoubtedly contributes to its chemical behaviour. Whereas the dihydroquinoxaline **19** has the potential to tautomerize to the more stable isomer **20**, the dihydrophenazine system cannot undergo a similar tautomerism to a more stable form.

Reduction of the salt can also be effected with $NaBH_4$, although with an excess of this reagent further reduction to the tetrahydroquinoxaline system **21** was observed. None of the tetrahydro-product was observed upon the reduction of **17** with NADH.

Because of its bimolecular nature, the rate of reduction of the quinoxalinium salt **17** is concentration dependent. At concentrations in the micromolar range the reduction takes





Scheme 4 *Reagents*: i, CH_3I; ii, NBS; iii, NADH; iv, $NaBH_4$; v, 4-methylumbelliferone, base.

several hours to complete but, at millimolar concentrations, the reaction is complete within seconds.

In order to generate a useful fluorescent probe, 2-methylquinoxaline **16** was brominated at the methyl group using NBS. Selective monobromination proved difficult to control and samples of the dibromo- and tribromo-compounds **23** and **24** always accompanied the required monobromide **22**. The monobromide was unstable to heat, general decomposition occurring to form a range of dark green/blue polar materials. The monobromide was conjugated with 4-methylumbelliferone to form the derivative **25**, which was a stable compound. Methylation of this conjugate with methyl triflate afforded the triflate salt **26**. Neither compound **25** nor **26** showed any significant fluorescence. An aqueous solution of the probe, maintained at pH 7.6 and kept in the dark at room temperature, was stable over several days, no free methylumbelliferone being released.

Reduction of the salt **26** with an excess of NADH was studied under a range of conditions. In a quantitative experiment at pH 7.5, reduction to the (unstable) intermediate **27** occurred and an emission spectrum characteristic of the free umbelliferone species **29** immediately started to form. After reduction was complete, the free umbelliferone could be isolated and a recovery of >85% was obtained. In the absence of NADH no liberation of 4-methylumbelliferone was observed, confirming that the liberation was a direct result of reduction by NADH. The rate of the reaction is concentration dependent. At concentrations of both probe and reductant in the micromolar range, reaction was extremely slow. However, in the lower millimolar concentration range an acceptable rate of reduction was observed. Fig. 1 shows the emission results of a typical reduction. The increase in 4-methylumbelliferone **29** fluorescence is obtained by mixing a solution of the salt **26** at 1×10^{-4} mol dm^{-3} with NADH at 1×10^{-3} mol dm^{-3} in tris(hydroxymethyl)methylamine (TRIS) buffer at pH 7.5, taking aliquots at 2 min intervals and diluting 100-fold in buffer (to give a probe concentration of 1×10^{-6} mol dm^{-3}) before measuring the fluorescence emission spectrum (λ_{ex} 350 nm), monitoring the methylumbelliferone signal at λ_{em} 450 nm. Reaction takes less than 24 min under these conditions and, whilst not fast, is adequate for assaying NADH concentrations. Using an excess of the quinoxalinium salt, a linear relationship between emission intensities and NADH concentrations could be obtained in the range 5×10^{-7} to 10^{-4} mol dm^{-3} range.

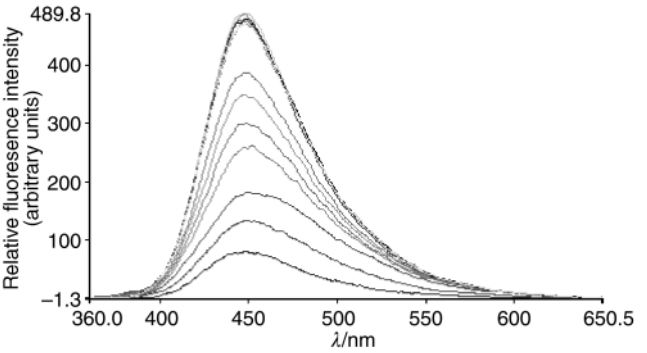
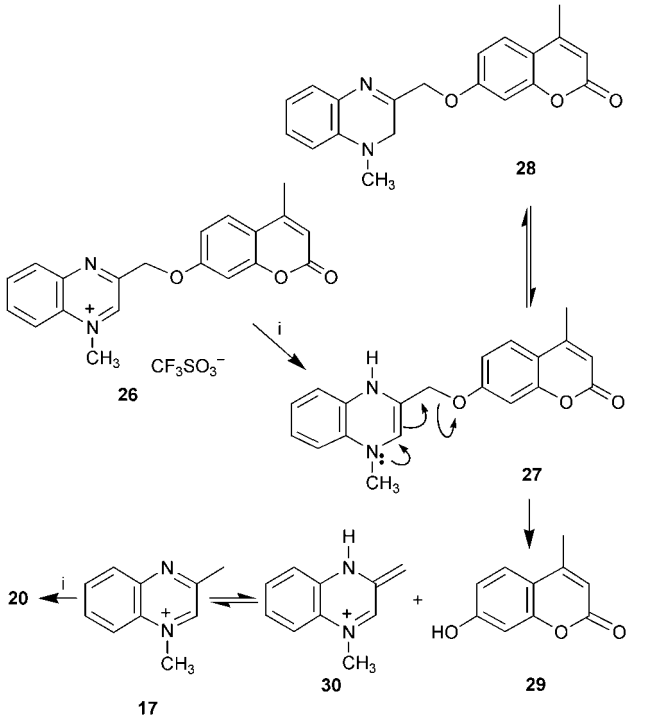


Fig. 1 Increase in umbelliferone fluorescence by addition of NADH to the probe **26**. NADH, 1×10^{-3} mol dm^{-3} mixed with probe, 1×10^{-4} mol dm^{-3}, in TRIS buffer at pH 7.6 sampled at 2 min intervals, aliquots diluted 100-fold before measurement; λ_{ex} 360 nm; λ_{max} reached within 24 min.

The fate of the quinoxalinium moiety has also been explored and the dihydro-species **20** was also isolated from the NADH reduction mixture. Formation of the latter is explained as in Scheme 5. Initial reduction by NADH is assumed to give the



Scheme 5 *Reagent*: i, NADH.

1,4-dihydro-intermediate **27**. We speculate that rather than tautomerizing to the isomer **28**, the intermediate **27** ejects 4-methylumbelliferone forming the species **30**, which can tautomerize back to the parent quinoxalinium species **17**, itself a substrate for further reduction with NADH to give the dihydro-species **20**. In principle, the initial reduction product **27** could be in an equilibrium with its tautomer **28**, the reaction ultimately leading to the same products. However, by monitoring reactions in D_2O, no evidence for the formation of substantial amounts of the tautomer **28** could be obtained.

From the reduction process it was noted that the strength of the fluorescence emission signal at 450 nm was not as high as that obtained for a control sample of 4-methylumbelliferone **29** at the same concentration, indicating some quenching process. This was assumed to be by the co-product dihydroquinoxaline **20**.

A Stern–Volmer quenching experiment confirmed this assumption (Fig. 2). At the concentrations used in fluorescence

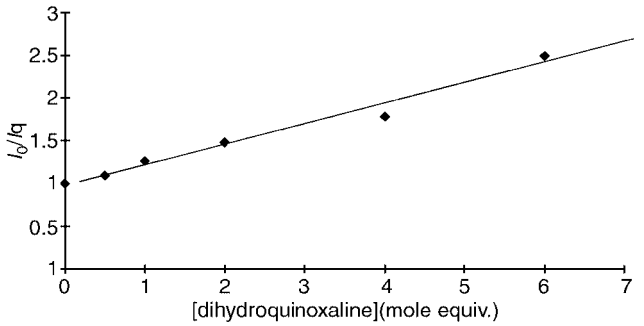


Fig. 2 Stern–Volmer quenching of 4-methylumbelliferone **29** fluorescence by dihydroquinoxaline **20** in TRIS buffer at pH 7.6. [**29**] = 1×10^{-6} mol dm^{-3}; λ_{ex} 360 nm; λ_{em} 450 nm.

measurements for the NADH-reduction experiment, this quenching accounts for almost all of the observed reduction in fluorophore signal intensity.

Little is known about the factors responsible for increasing the kinetic rate of direct reduction of heterocyclic substrates with NAD(P)H. For example, it was noted that the observed rate of reduction of the quinoxalinium derivative **17** was much slower compared with that for methylphenazinium methyl sulfate **1**. Presumably the increased aromatic footprint of the latter enhances the initial interactions between the two species. Studies on such factors, and also to define the scope of such reductions, are in progress.

Experimental

Mps were determined on a Kofler hot-stage apparatus and are uncorrected. IR specta were recorded on a Perkin-Elmer System 2000 FTIR spectrometer, for samples either as films, Nujol mulls or solutions in chloroform. UV spectra were determined on a Perkin-Elmer Lambda 9 spectrophotometer for sample solutions in 10 mm cuvettes. ^{1}H NMR spectra were recorded on either a Bruker 300 MHz spectrometer or a JEOL FX270 MHz instrument, for samples as solutions in deuteriochloroform, unless otherwise stated, using tetramethylsilane as internal reference. Mass spectra were obtained from the EPSRC National Mass Spectrometry Service Centre, Swansea, and microanalytical determinations were by MEDAC Ltd, Englefield Green, Surrey. All fluorescence spectra were obtained on a Perkin-Elmer LS50B spectrofluorimeter loaded with FLwinlab software.

TLC was carried out on glass plates precoated with 0.25 mm Kieselgel 60 GF_{254} and column chromatography was through Merck Kieselgel 60G. Solvents were distilled before use and solvent ratios are in volumes before mixing. Light petroleum refers to the fraction of boiling range 60–80 °C and ether refers to diethyl ether. Brine is a saturated aqueous solution of sodium chloride. Reagents and chemicals obtained from external sources were checked by TLC and NMR prior to use. Solvents were purified by the methods described in Perrin.[15] Solutions were dried over anhydrous sodium sulfate unless otherwise specified.

Methylphenazinium methyl sulfate 1

This was prepared according to the literature method,[16] mp 158–160 °C (decomp.).

2-Methylphenazine[17] 6

2-Methylcatechol (3.72 g, 30 mmol) was dissolved in water (300 cm^3) at room temperature and sodium periodate (6.73 g, 33 mmol) added. The mixture was stirred vigorously for 1 min before extraction with methylene dichloride (2 × 70 cm^3). The organic extract was immediately added to a solution of 1,2-diaminobenzene (3.24 g, 30 mmol) in methylene dichloride (40 cm^3) with stirring. To the solution was then added, dropwise over a period of 10 min, glacial acetic acid (20 cm^3) and the mixture was stirred for a further 30 min at room temperature before being heated to reflux for 4 h. The solution was cooled, and washed successively with water (100 cm^3), aq. sodium hydrogen carbonate (2 × 100 cm^3) and finally brine (100 cm^3). The organic layer was dried, filtered, and evaporated to dryness to afford an orange solid. Filtration through a plug of alumina, using methylene dichloride as solvent, afforded the product as a yellow solid (3.08 g, 60%), mp 117 °C (lit.,[17] 117 °C); δ_H 2.66 (3 H, s), 7.64 (1 H, dd, *J* 7, 2 Hz), 7.83 (2 H, m), 7.99 (1 H, s), 8.13 (1 H, d, *J* 7 Hz), 8.22 (2 H, m).

2-(Bromomethyl)phenazine 7

2-Methylphenazine (1.63 g, 8.3 mmol) was dissolved in CCl_4 (70 cm^3) and NBS (1.48 g, 8.3 mmol, freshly recrystallized from water) and benzoyl peroxide (50 mg) were added. The mixture was heated to a gentle reflux for 2 days, with addition of two further portions of benzoyl peroxide (50 mg) after 20 and 30 h. The mixture was cooled to room temperature, filtered, and the filtrate washed successively with water (50 cm^3) and brine (50 cm^3), before drying and removal of solvent under reduced pressure. The brown solid was purified by chromatography through silica gel (1:5 ethyl acetate–light petroleum) to afford the *title compound* as a yellow solid (0.43 g, 80%), mp 156–162 °C; δ_H 4.74 (2 H, s, CH_2Br), 7.80–7.88 and 8.21–8.28 (7 H, m, ArH); δ_C 143.8, 143.7, 143.05, 140.0, 131.1, 130.7, 130.5, 129.65, 129.6, 129.5, 128.85, 32.75 (2 overlapping signals); *m*/*z* (EI) 274 ($M^+ - Br$), 193.

2-(Phenoxymethyl)phenazine 8

To a solution of phenol (0.846 g, 9.1 mmol) in 50:1 acetonitrile–water (51 cm^3) was added potassium hydroxide (0.512 g, 9.1 mmol) and the solution was heated to reflux for 30 min and then cooled slightly before addition of (bromomethyl)-phenazine **7** (0.5 g, 1.82 mmol) and heating of the solution for a further 4 h. The reaction mixture was diluted with ethyl acetate (100 cm^3) and washed successively with water (2 × 70 cm^3), 5% w/v aq. sodium hydroxide (70 cm^3) and brine (50 cm^3) before separation of the organic layer, drying, filtering and removal of solvent to give the product as a brown solid. The material was purified by column chromatography (5:1 light petroleum–ethyl acetate) to afford the *title compound* (0.43 g, 80%), mp 118–119 °C; δ_H 5.37 (2 H, s, CH_2O), 7.00 (1 H, t, *J* 7 Hz, ArH), 7.06 (2 H, d, *J* 8.4 Hz, ArH), 7.32 (2 H, dd, *J* 7, 8.4 Hz, ArH), 7.87–7.84 (2 H, m, ArH), 7.91 (1 H, dd, *J* 1.8, 8.9 Hz, ArH), 8.24–8.29 (3 H, m, ArH), 8.32 (1 H, s, ArH); *m*/*z* (EI) 286 ($M^+ - Ph$), 210 ($M^+ - OPh$) (Found: C, 79.6; H, 4.9; N, 9.7. $C_{19}H_{14}N_2O$ requires C, 79.7; H, 4.9; N, 9.8%).

2-(4-Methylcoumarin-7-yloxymethyl)phenazine 9

Freshly recrystallized 4-methylumbelliferone **29** (0.58 g, 3.3 mmol) and sodium hydroxide (0.132 g, 3.3 mmol) were dissolved in 50:1 acetonitrile–water (51 cm^3) and the solution was heated to reflux for 10 min before cooling, and addition of (bromomethyl)phenazine **7** (0.45 g, 1.65 mmol). The mixture was heated to reflux for 4 h before cooling, and refiltering off of the light green solid (0.45 g, 40%). A sample was recrystallized from ethanol to give pale green crystals of the *title compound*, mp 255–257 °C; δ_H 2.39 (3 H, s, CH_3), 5.42 (2 H, s, CH_2O), 6.12 (1 H, s, C*H*CO), 6.95 (1 H, s, ArH), 7.03 (1 H, d, *J* 8.2 Hz, ArH), 7.53 (1 H, d *J* 8.1 Hz, ArH), 7.86–7.88 (3 H, m, ArH), 8.26–8.31 (4 H, m, ArH); *m*/*z* 368 (M^+), 193 (Found: M^+, 368.1161. $C_{23}H_{16}N_2O_3$ requires *M*, 368.1161).

2,5(10)-Dimethylphenazinium trifluoromethanesulfonate 10

2-Methylphenazine **6** (48.5 mg, 0.25 mmol) was dissolved in dry toluene (2 cm^3) under a dry nitrogen atmosphere in a flask

wrapped in aluminium foil. Methyl trifluoromethanesulfonate (41 mg, 0.25 mmol) added dropwise at room temperature and the mixture was stirred overnight. The crystalline precipitate that formed was collected by filtration, washed with ether, and dried in the dark under reduced pressure to give a mixture of the two *isomeric title compounds* as a bright yellow solid (36 mg, 40%), mp 104–106 °C; δ_H 2.92 and 2.79 (3 H, s, CH_3), 5.16 and 5.12 (3 H, s, CH_3), 8.02–8.88 (7 H, m, ArH) (Found: C, 50.1; H, 3.6; N, 7.7. $C_{15}H_{13}F_3N_2O_3S$ requires C, 50.3; H, 3.7; N, 7.8%).

5(10)-Methyl-2-(4-methylcoumarin-7-yloxymethyl)phenazinium methyl sulfate 11

The methylphenazine **6** (48.5 mg, 0.25 cm^3) was added to freshly distilled dimethyl sulfate (2 cm^3) and the mixture was heated to 110 °C for 10 min. The solution that formed was cooled to room temperature and acetonitrile (3 cm^3) was added. To the brown solution was added ether (10 cm^3) to afford a brown solid precipitate, which was isolated by filtration. The material was re-dissolved in acetonitrile and re-precipitated with dry ether to afford the *title salt* (25 mg, 32%), mp 231–232 °C (Found: $M^+ - CH_3O_4S$ 383.1406. $C_{24}H_{19}N_2O_3^+$ requires *m*/*z*, 383.1396). This material was used directly in the reactions with NADH.

2-Methylquinoxaline[13] 16

Sodium metabisulfite (23.2 g, 0.122 mol) was added to a solution of pyruvaldehyde (26 g, 0.36 mol) in water (85 cm^3; 23% w/v) and, after 10 min, the solution was added to a vigorously stirred solution of 1,2-phenylenediamine (10.2 g, 94 mmol) in water (52 cm^3). After 18 h at room temperature the solution was adjusted to pH 10 by the addition of sodium carbonate (20 g), and the mixture was extracted with ether (3 × 30 cm^3). The extract was dried, the solvent removed under reduced pressure, and the residue purified by vacuum distillation to afford the *title quinoxaline* as a colourless oil (12.38 g, 91%), bp 125–126 °C/11 mmHg (lit., 245–247 °C/760 mmHg); δ_H 2.75 (3 H, s, CH_3), 7.65–7.74 (2 H, m, ArH), 7.9–8.07 (2 H, m, ArH), 8.72 (1 H, s, 3-H).

Methylation of 2-methylquinoxaline

2-Methylquinoxaline **16** (1.0 g, 7 mmol) was added to neat, freshly distilled methyl iodide (3 cm^3, 48 mmol) and the solution was stirred in the dark at room temperature under argon. After 18 h a green crystalline precipitate had formed. This solid was collected by filtration, a portion (0.1 g) kept for NMR examination and the rest was dissolved in a minimal amount of dry acetonitrile (≈2 cm^3) and then precipitated by the addition of ether, to afford as a yellow powder, *1,3-dimethylquinoxalinium iodide* **17** (1.52 g, 82%), mp 125–127 °C (decomp.). The purified product showed δ_H 2.92 (3 H, s, ArCH_3), 4.67 (3 H, s, NCH_3), 7.23–7.27 (2 H, m, ArH), 8.41 (1 H, d, *J* 8 Hz, ArH), 8.56 (1 H, d, *J* 8 Hz, ArH), 9.61 (1 H, s, 3-H) (Found: C, 41.9; H, 3.9; N, 9.7. $C_{10}H_{11}IN_2$ requires C, 42.0; H, 3.9; N, 9.8%).

In its ^{1}H NMR spectrum the crude methylation product showed the above signals and small signals (<5%) at δ 3.12 and 4.52 corresponding to the methyl signals for the 1,2-methylated regioisomer **18**; these were virtually absent in the re-precipitated salt.

1,3-Dimethyl-1,2-dihydroquinoxaline 20

A biphasic mixture of a solution of NADH (0.16 g, 0.22 mmol) in water (2 cm^3) and chloroform (6 cm^3) was stirred vigorously in the dark, at room temperature, whilst adding, dropwise, over a period of 5 min, a solution of the salt **17** (60 mg, 0.2 mmol) in water (2 cm^3). The emulsion was stirred for a further 20 min before the phases were separated, collection of the organic solution, drying, filtering and removing the solvent under reduced pressure to afford the *title compound* as a red powder (18 mg, 50%), mp 96–99 °C; δ_H 2.13 (3 H, s, CH_3), 2.76 (3 H, s, NCH_3), 3.73 (2 H, s, NCH_2), 6.61 (1 H, d, *J* 8 Hz, ArH), 6.75 (1 H, t, *J* 8 Hz, ArH), 7.08 (1 H, t, *J* 8 Hz, ArH), 7.21 (1 H, d, *J* 8 Hz, ArH) (Found: C, 74.9; H, 7.3; N, 17.5. $C_{10}H_{12}N_2$ requires C, 75.0; H, 7.55; N, 17.5%).

1,3-Dimethyl-1,2,3,4-tetrahydroquinoxaline 21

The salt **17** (0.286 g, 1 mmol) was dissolved in water (5 cm^3) in the presence of methylene dichloride (5 cm^3) and sodium borohydride (0.076 g, 2 mmol, 8 equiv.) was added. The mixture was stirred for 1 h before addition of more methylene dichloride (5 cm^3), separation of the two layers, washing the organic extract with water (2 × 5 cm^3), drying, filtering and evaporation to dryness to afford a reddish gum (0.117 g, 72%); δ_H 1.17 (3 H, d, *J* 6.3 Hz, CH_3), 1.54 (1 H, br s, exch. D_2O, NH), 2.85 (3 H, s, NCH_3), 2.92 (1 H, dd, *J* 8.3, 10.6 Hz, C*H*HN), 3.15 (1 H, dd, *J* 2.7, 10.6 Hz, CH*H*N), 3.60 (1 H, m, NC*H*CH_3), 6.47 (1 H, m, ArH), 6.56 (2 H, m, ArH), 6.69 (1 H, m, ArH).

This was characterized as its *N-benzoyl derivative*, prepared by reaction of the isolated amine with benzoyl chloride (0.25 cm^3) in pyridine (0.5 cm^3), and work-up in the normal manner. The product was obtained as a viscous gum; δ_H 1.21 (3 H, d, *J* 6.9 Hz, CH_3), 3.0 (1 H, s, NCH_3), 3.08 (1 H, dd, *J* 2, 11.3 Hz, NC*H*H), 3.67 (1 H, dd, *J* 4.6, 11.3 Hz, CH*H*), 5.02 (1 H, br s, NC*H*CH_3), 6.35 (1H, m, ArH), 6.67 (1 H, m, ArH), 6.98 (1 H, m, ArH), 7.2–7.5 (6 H, m, ArH) [Found: M^+ (EI-MS) 266.1417. $C_{17}H_{18}N_2O$ requires *M*, 266.1419].

2-(Bromomethyl)quinoxaline 22

A mixture of the quinoxaline **16** (2.16 g, 15 mmol), NBS (freshly recrystallized from water; 6 g, 33 mmol) and benzoyl peroxide (0.415 g) were added to carbon tetrachloride (75 cm^3) and the mixture was heated to 80 °C with stirring. The reaction was monitored by TLC. After 5 h, when all the starting quinoxaline had reacted, the mixture was cooled to room temperature and filtered. After removal of the solvent under reduced pressure, the residue was immediately chromatographed through silica gel (methylene dichloride as eluant) to yield, initially, the *title compound* (2.10 g, 62%) as a pale blue, crystalline solid, mp 73–74 °C; ν_{max} ($CHCl_3$) 2990, 1559, 1494, 1216, 1201, 982, 757, 667 cm^{-1}; δ_H 4.74 (2 H, s, CH_2), 7.77–7.78 (2 H, m, ArH), 8.05–8.14 (2 H, m, ArH), 9.00 (1 H, s, 3-H) (Found: C, 48.6, 3.4; N, 12.7. $C_9H_7BrN_2$ requires C, 48.5; H, 3.2; N, 12.55%).

Also isolated, in order of elution, were 2-(dibromomethyl)-quinoxaline **23** (0.13 g, 3%), mp 101–103 °C; δ_H 6.77 (1 H, s, $CHBr_2$), 7.85–7.79 (2 H, m, ArH), 8.05–8.17 (2 H, m, ArH), 9.39 (1 H, s, 3-H); *m*/*z* (FAB-MS) 301, 303, 305 (proportions 1 : 2 : 1, MH^+) and 2-(tribromomethyl)quinoxaline **24** (0.11 g, 2%), mp 83–84 °C; δ_H 7.48–7.68 (2 H, m, ArH), 8.09–8.09 (2 H, m, ArH), 9.7 (1 H, s, 3-H); *m*/*z* 379, 381, 383, 385 (proportions 1 : 3 : 3 : 1, MH^+).

2-(4-Methylcoumarin-7-yloxymethyl)quinoxaline 25

To a stirred solution of 7-hydroxy-4-methylcoumarin **29** (0.8 g, 4.5 mmol) and sodium hydroxide (0.18 g, 4.5 mmol), in 1 : 19 water–tetrahydrofuran under an atmosphere of nitrogen, was added 2-(bromomethyl)quinoxaline **22** (1.01 g, 4.5 mmol) and the solution was heated to reflux in the dark for 16 h. The bulk of the solvent was removed under reduced pressure and the residue extracted into methylene dichloride. The organic extract was washed successively with 2 M aq. sodium hydroxide (30 cm^3) and brine (2 × 30 cm^3) before drying, filtering and concentration under reduced pressure to give a dark red solid, which was purified by silica gel chromatography (1 : 49 methanol–methylene dichloride as eluant) and recrystallization from methanol to give the *title compound* as colourless crystals (0.91 g, 64%), mp 211–213 °C; δ_H 2.40 (3 H, s, CH_3), 5.49 (2 H, s, CH_2), 6.16 (1 H, s, ArH), 6.98 (1 H, s, ArH), 7.04 (1 H, d, *J* 9

Hz, ArH), 7.52 (1 H, d, *J* 9 Hz, ArH), 7.78–7.82 (2 H, m, ArH), 8.10–8.16 (2 H, m, ArH), 9.03 (1 H, s, ArH) (Found: C, 71.8; H, 4.45; N, 8.6. $C_{19}H_{14}N_2O_3$ requires C, 71.7; H, 4.4; N, 8.8%).

N-Methyl-2-(4-methylcoumarin-7-yloxymethyl)quinoxalinium trifluoromethanesulfonate 26

To a solution of the quinoxaline **25** (0.25 g, 0.79 mmol) in dry chloroform (5 cm^3) under argon, in the dark, was added methyl trifluoromethanesulfonate (0.76 cm^3, 3.2 mmol, 4 equiv.). The mixture was stirred at room temperature for 24 h, the solvent removed under reduced pressure, and a few drops of acetonitrile added to form a mobile solution before addition of dry ether (20 cm^3) to precipitate the *title compound* as a yellow powder (0.317 g, 83%), mp 195–198 °C; δ_H 2.42 (3 H, s, CH_3), 4.78 (3 H, s, NCH_3), 5.77 (2 H, s, CH_2), 6.27 (1 H, s, ArH), 7.21 (1 H, d, *J* 9 Hz, ArH), 7.25 (1 H, s, ArH), 7.80 (1 H, d, *J* 9 Hz, ArH), 8.27–8.35 (2 H, m, ArH), 8.51 (1 H, d, *J* 8 Hz, ArH), 8.64 (1 H, d, *J* 8 Hz, ArH), 9.85 (1 H, s, ArH) (Found: C, 52.2; H, 3.5; N, 5.6; S, 6.7. $C_{21}H_{17}F_3N_2O_3S$ requires C, 52.6; H, 3.55; N, 5.8; S, 6.65%); *m/z* (FAM-MS) 334 ($M^+ + H^+$).

Preparative-scale reduction of the quinoxalinium conjugate 26

The conjugate salt (100 mg, 0.207 mmol) was dissolved in acetonitrile (2 cm^3) and added to a stirred solution of NADH (1.42 g, 2.07 mmol, 10 equiv.) in TRIS buffer (10^{-2} mol dm^{-3}; pH 7.6; 30 cm^3). Stirring was continued for 2 h, in the dark, before extraction of the reaction mixture with chloroform (2 × 30 cm^3), washing of the organic extracts with water (30 cm^3), drying and evaporation of the solvent under reduced pressure. The residue was chromatographed through silica gel (chloroform, then 1:9 methanol–chloroform as eluant) to afford methylumbelliferone **29** (32.4 mg, 89%) and the dihydroquinoxaline **20** (22 mg, 65%).

Spectroscopic measurements

Freshly prepared stock solutions of reagents, at 1×10^{-2} mol dm^{-3}, were **17** made up daily before use. NADH was made up in distilled water, and the salts **17** and **26**, dihydroquinazoline **20** and 4-methylumbelliferone **29** in acetonitrile. Aliquots were diluted with a solution of TRIS buffer at 1×10^{-2} mol dm^{-3} adjusted to pH 7.6. Excitation was at λ 350 nm and emission measured at λ 450 nm, the emission maximum for methylumbelliferone. A control experiment showed that NADH fluorescence emission under the concentration regimes used is minimal (<10 units at 1×10^{-5} mol dm^{-3}). Similarly the starting salts **17** and **26** showed only weak emission signals.

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