ChemComm

A fluorescent probe for the detection of NAD(P)H

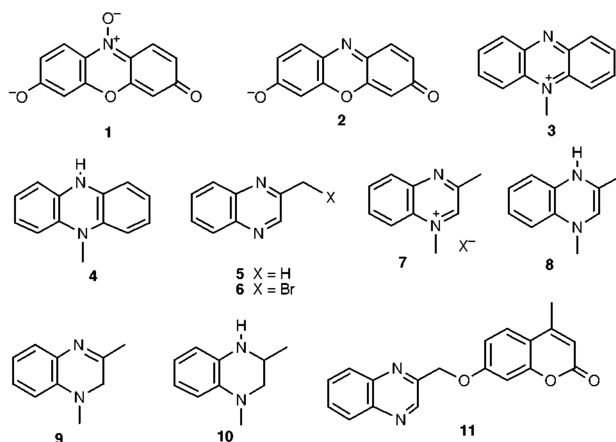
Carl A. Roeschlaub, Nicola L. Maidwell, M. Reza Rezai and Peter G. Sammes*

Molecular Probes Unit, Department of Chemistry, School of Physical Sciences, UNIS, Guildford, Surrey, UK GU2 5XH. E-mail: p.sammes@surrey.ac.uk

Received (in Cambridge, UK) 22nd June 1999, Accepted 16th July 1999

NAD(P)H may be monitored by using reduction to release the fluorophore umbelliferone from a precursor conjugate with a quinoxalinium salt.

NADH and its phosphate ester NADPH are ubiquitous reducing agents in nature. 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 general biological marker for cancer.[1] A potential fluorescent probe for the detection of these aberrant cells has been described[2] that uses the reduction of the weakly-fluorescent dye resazurin **1** to the fluorescent derivative



resorufin **2**. A disadvantage of this system is that direct reduction of resazurin to resorurufin does *not* occur and the reaction has to be 'catalysed' by an electron carrier such as phenazinium methosulfate **3** or an enzyme such as diaphorase.[3] The mechanism of this reduction has been investigated[2] and, for the catalyst phenazinium methosulfate, shown to proceed *via* initial formation of the dihydrophenazine derivative **4**, which is extremely unstable and rapidly undergoes further redox reactions.

NAD(P)H is also co-produced by a number of enzyme-based redox systems, such as glucose-6-phosphate dehydrogenase[4] and is used in a number of enzyme linked immunosorbent assays (ELISA).[5] In these assays a catalyst has also to be used, often another enzyme such as diaphorase, to act as the electron carrier in conjunction with either resazurin, reduced to the fluorescent resorufin,[3] or of a leuco-dye, such as a tetrazolium salt, which is reduced to the corresponding, highly coloured formazan.[6]

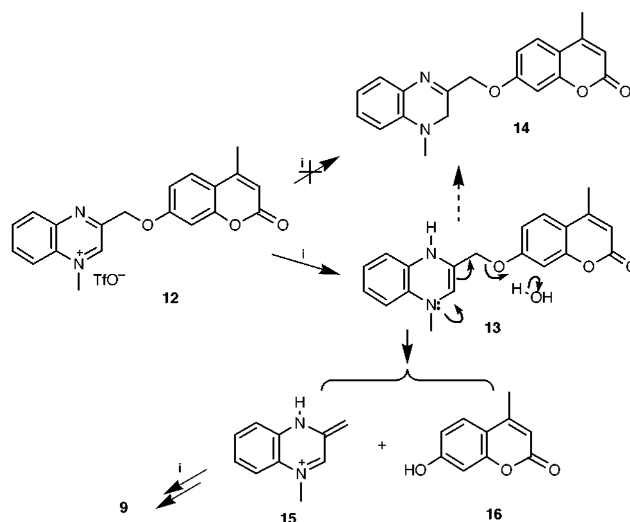
In order to avoid the need for these two-component systems involving an electron carrier, conjugation of a masked fluorophore to a reducible heteroaromatic system was considered, in which the reduction would unmask the fluorophore. After studying several systems we focused on use of derivatives of the quinoxalinium system. 2-Methylquinoxaline **5** was found to react regioselectively with methylating agents, such as MeI and MeOTf, to produce the corresponding 1,3-dimethylquinoxalinium salt **7**.† Reduction of these salts with NADH at pH 7.5 rapidly produces a dihydro compound. Whilst mechanistic studies with NADH predict that this will be the 1,4-dihydro isomer **8**,[7] this was not obtained since it efficiently tautomerises to the 1,2-dihydro isomer **9,** isolated in high yield (>90%). In contrast to the dihydrophenazine **4,** the dihydroquinoxaline **9** is relatively stable, although, upon exposure to air it is oxidised to give a variety of highly coloured products. It is to be noted that both **4** and **8** are both formally anti-aromatic and this undoubtedly contributes to their chemical reactivity. Reduction of the salt **7** can also be effected with $NaBH_4$, although with an excess of this reagent, over-reduction to give the tetrahydroquinoxaline **10** was observed. None of **10** was observed upon reduction of **7** with NADH.

Because of its bimolecular nature, the rate of reduction of the quinoxalinium salt **7** by NADH is concentration dependent. At concentrations in the micromolar range the reduction takes several hours to complete but at millimolar concentrations reaction is complete within seconds.

In order to generate a useful fluorescent probe, 2-methylquinoxaline **5** was brominated at the methyl group, using NBS, and the bromomethyl compound **6** then conjugated with umbelliferone **16** to produce the derivative **11**. Methylation of this base with MeOTf afforded the triflate salt **12**. Neither of the compounds **11** nor **12** showed any significant fluorescence.

Reduction of the salt **12** with NADH was effected by adding a solution of the salt to a stirred solution of NADH at pH 7.5 and immediately re-measuring the emission spectrum. A signal corresponding to that of free umbelliferone **16** formed almost immediately. That this was due to liberated umbelliferone **16** was also confirmed by TLC and direct isolation; in a quantitative experiment >85% **16** could be extracted from the solution. In the absence of NADH no formation of umbelliferone **16** was observed, confirming that the liberation was a direct consequence of reduction by NADH.

The fate of the quinoxalinium moiety has also been explored. Reduction is assumed to give, initially, the 1,4-dihydro intermediate **13**. Rather than tautomerising, in the manner observed for the conversion of the unsubstituted quinoxalinium salt (**7** to **8** to **9**), to give **14**, which has not been observed, elimination of umbelliferone occurs to give the quinoxalinium



Scheme 1 *Reagents and conditions*: i, NADH, pH 7.5.

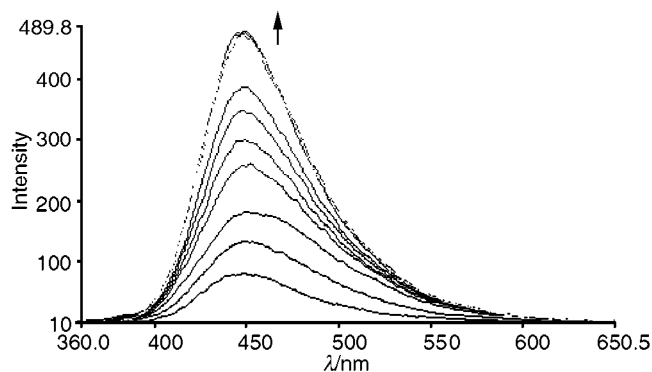


Fig. 1 Increase in emission with time; scans at 2 min. intervals; maximum reached in < 24 min. See text for conditions.

tautomer **15**, which then reforms the quinoxalinium species **7**, followed by a second NADH reduction step to produce the imine **9**, which has also been isolated and characterised (see Scheme 1).

Fig. 1 shows the results of a typical reaction. The increase of umbelliferone **16** fluorescence is obtained by mixing a solution of the salt **12** at 1×10^{-4} mol dm^{-3} with NADH at 1×10^{-3} mol dm^{-3} in TRIS buffer at pH 7.5, taking aliquots at 2 min intervals and diluting 100-fold in buffer (to give a sample at 1×10^{-6} mol dm^{-3}) before measuring the fluorescence spectrum, (λ_{ex} 350 nm), monitoring the umbelliferone emission peak at λ_{em} 450 nm. Under these conditions reduction is observed in under 24 min. Control fluorescence measurements on pure umbelliferone, in the presence of 1 equiv. of the imine **7,** indicate that release of the umbelliferone from the salt **12** is quantitative.

We thank the EPSRC for support of this work and the EPSRC National Mass Spectrometry Service Centre, Swansea, Wales, for accurate mass measurements.

Notes and references

† All new compounds gave satisfactory microanalyses and/or accurate mass measurements.

1 S. K. Jonas, C. Benedetto, A. Flatman, L. Micheletti, C. Riley, P. A. Riley, D. Spargo, M. Zonca and T. F. Slater, *Br. J. Cancer*, 1992, **66**, 185.

2 L. P. Candeis, D. P. S. MacFarlane, S. L. W. McWhinnie, N. L. Maidwell, C. A. Roeschlaub, P. G. Sammes and R. Whittlesey, *J. Chem. Soc., Perkin Trans. 2*, 1998, 2333.

3 D. B. Cook and C. H. Self, *Clin. Chem.*, 1993, **39**, 965.

4 H. Shah, A. M. Saranko, M. Harkonen and H. Adlercreutz, *Clin. Chem.*, 1984, **30**, 185.

5 For a review see A. Voller and D. E. Bidwell, *Enzyme Immunoassays*, in *Alternative Immunoassays*, ed. W. P. Collins, Wiley, Chichester, 1985, pp. 77–86.

6 E. Seidler, *The Tetrazolium-Formazan System: Design and Histochemistry,* in *Progress in Histochemistry and Cytochemistry,* Gustav Fischer Verlag, Stuttgart, 1991, vol. 24, pp. 1–86.

7 G. Blankenhorn, in *Pyridine Nucleotide Dependent Dehydrogenases*, ed. H. Sund, W. de Gruyter & Co., Berlin, 1997, pp. 185–205.

Communication 9/04971A