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Structural characterisation of Nitrazine Yellow by NMR spectroscopy

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Abstract

Nitrazine Yellow is an indicator used in microscopy with variations of absorption visible spectrum in pH range 6.0–7.0. The stated structure, deriving from the attack of 2′,4′-dinitrobenzenediazonium salt to ortho hydroxy position of 1-naphthol-3,6-disulphonic acid, appears incoherent with the alochromic behaviour. Nmr analysis and, in particular, HMQC and HMBC two-dimensional techniques showed that the real structure comes from an attack of the diazonium salt to para hydroxy position.

Keywords: Nitrazine Yellow; Azodyes; Alochromism; Diazo coupling; NMR spectroscopy; 2D-COSY; ^{1}H–^{1}H NOESY; ^{1}H–^{13}C-HMQC; ^{1}H–^{13}C-HMBC

1. Introduction

In recent decades, organic colour chemistry is undergoing very exciting development as a result of the opportunities presented by dye applications in high technology fields: electronic devices, linear and non linear optics, reprography, sensors, biomedical uses [1]. The importance of functions performed by dyes, beyond the simple provision of colour, coined for them the term "functional", so they are in general referred to as "functional dyes" [2].

Recently, we have been interested in developing a sensor, useful for on-line blood gas measurement systems. This kind of analysis is often required in modern diagnosis and during the treatment of critically ill patients and has come to stand for the measurement of pCO_2, pO_2 and pH of blood. By optical fibres, a film is lighted and the relative optical answer read. If the halochromic film is in direct contact with the blood, its variation in optical properties gives information about the pH of the physiological liquid. Alternatively, a membrane, permeable to blood gases (CO_2 or O_2), is placed over a chamber to isolate it from the blood. The gas permeates the membrane and reacts with a liquid or liquid-containing media in the chamber to shift the pH, that is monitored again by a

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halochromic film in contact only with the liquid placed in the chamber.

It is evident that the choice of the indicator structure is fundamental because it has to allow: i) the variation of optical properties over the physiological pH range (6.8–7.8), ii) absorption of red LED light to guarantee the economy of device, iii) the linking formation to useful membrane, iv) photostability, v) stability during the required gamma or ethylene oxide sterilisation process. Studies on useful indicators sensible to pH variation, dependent on luminescence [3,4] or absorptive [5–7] properties, in physiological fields have been made by several investigators.

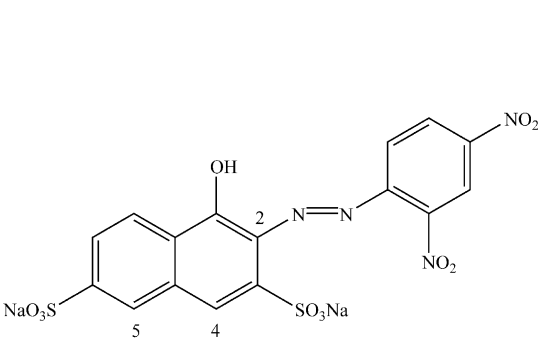
In the course of our work, azo dyes appeared a particularly promising class of indicators for meeting the stated requirements [8]. The pioneering work of Wenker [9] on indicator properties of azodyes proved very informative. In particular, Wenker observed that changes in the absorption/transmission occurred near the physiological pH range (6.8–7.8) if the diazonium salt of 2,4-dinitroaniline or 2,6-dinitroaniline, and relative sulfonic acids, reacts in position 4 of 1-naphthol, and relative 6-, 7-, 8-sulfonic and 3,6-disulfonic acids. On the contrary, if the coupling occurs in position 2, e.g. with the 1-naphtol-4-sulfonic acid, alochromism is observed in more basic conditions. The Wenker conclusion is in agreement with the general consideration that unwelcome halochromism in textile fabrics is shifted to the highest pH values if ortho coupling on naphthol systems occurs. The formation of a strong hydrogen bond, involving azo/hydrazone equilibrium, in *o*-hydroxyazo compounds causes them to be much weaker acids than their *p*-isomers [10].

Keeping Wenker's conclusions in mind, our attention was preliminary focused on commercially available dyes to evaluate in host–guest systems the magnitude of interactions of dyes with cellophane, chosen as polymeric support for the sensor. In fact, possible interactions of dye with the polymer can influence the alochromic behaviour of dye in a manner only experimentally determinable. Several structures are available commercially; in particular, Nitrazine Yellow appeared interesting. It is a useful indicator for microscopy also reported in Colour Index (C.I. 5423-07-4) and in Beilstein [11]. Surprisingly, structure **1**, instead of **2**, is in general assigned to Nitrazine Yellow, corresponding to attack of 2′,4′-dinitrobenzenediazonium salt in position 2 of 1-naphthol-3,6-disulphonic acid, incoherently with its changes in the visible spectroscopic properties that occur in the pH range 6.0–7.0.

The biological interest of this dye and its use as reference for our work on sensors suggested elucidating the relative structure.

2. Results and discussion

In Fig. 1 the high frequency region of the ^{1}H nmr spectrum of commercial Nitrazine Yellow in



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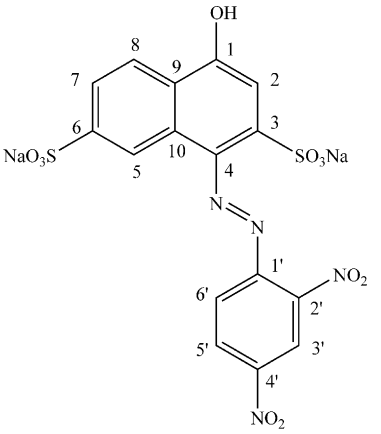

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DMSO-d_6 is reported, while ^{1}H and ^{13}C chemical shifts are given in Table 1. A singlet whose intensity corresponds to one proton is detectable at 14.12 ppm, attributable to the OH group. The chemical shift value of this signal already appears incoherent with structure **1**, for which an azo-hydrazone equilibrium shifted to naphthoquinone form can be invoked reasonably (Scheme 1). Both intramolecular hydrogen bond formation and the combination of an electronic donor (NH) and two

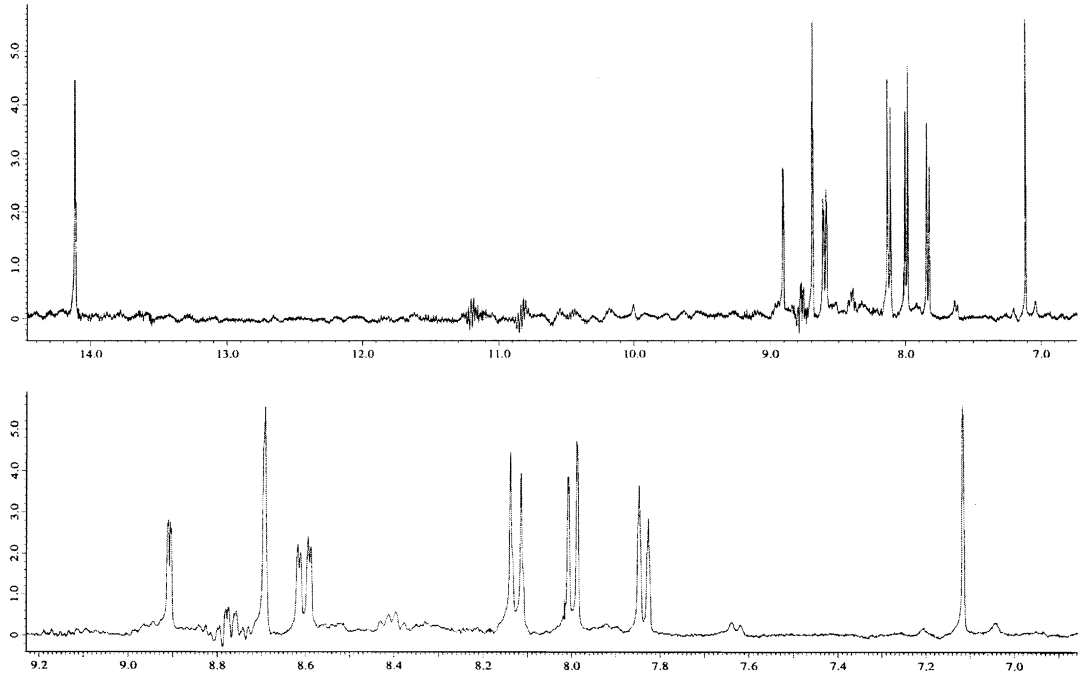
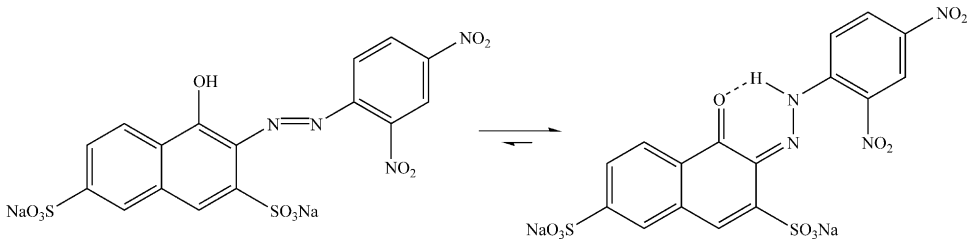


Fig. 1. ^{1}H NMR spectrum of Nitrazine Yellow.

Table 1
^{1}H and ^{13}C chemical shifts of Nitrazine Yellow in DMSO-d_6

H/C number	δ (^{1}H)	Multiplicity	J (Hz)	δ (^{1}C)
OH	14.12	s	–	–
1	–	–	–	138.08
2	7.12	s		129.35
3	–	–	–	147.59
4	–	–	–	133.41
5	8.69	s	–	123.27
6	–	–	–	152.76 or 185.05
7	7.84	d	8.1	127.35
8	8.01	d	8.1	126.26
9	–	–	–	130.79
10	–	–	–	152.76 or 185.05
1′	–	–	–	144.37
2′	–	–	–	134.55
3′	8.91	d	2.0	123.53
4′	–	–	–	140.97
5′	8.60	dd	2.0, 9.6	130.69
6′	8.13	d	9.7	119.34



Scheme 1.

acceptor (NO_2) groups act as stabilising the hydrazone form [10].

Lyčka et al. reported chemical shifts values in the range 15.5–16.2 ppm for the OH group of dyes obtained by coupling of benzene diazonium salt with a series of aminohydroxynaphthalenesulfonic acids (J-, H- and γ acids) [12], involved in the azo-hydrazone equilibrium with strongly prevailing naphthoquinone form.

The assignment of ^1H resonances of Nitrazine Yellow is easily accomplished by means of 2D-COSY and by analysis of chemical shifts and signal multiplicity. The signals of benzene protons can be first identified by comparison with the spectra of other dyes containing the same benzene moiety [13]. The H3′ signal appears as a doublet with J_m of 2.0 Hz at 8.91 ppm, while H5′ appears as a doublet of doublets with J_0 of 9.6 Hz and J_m

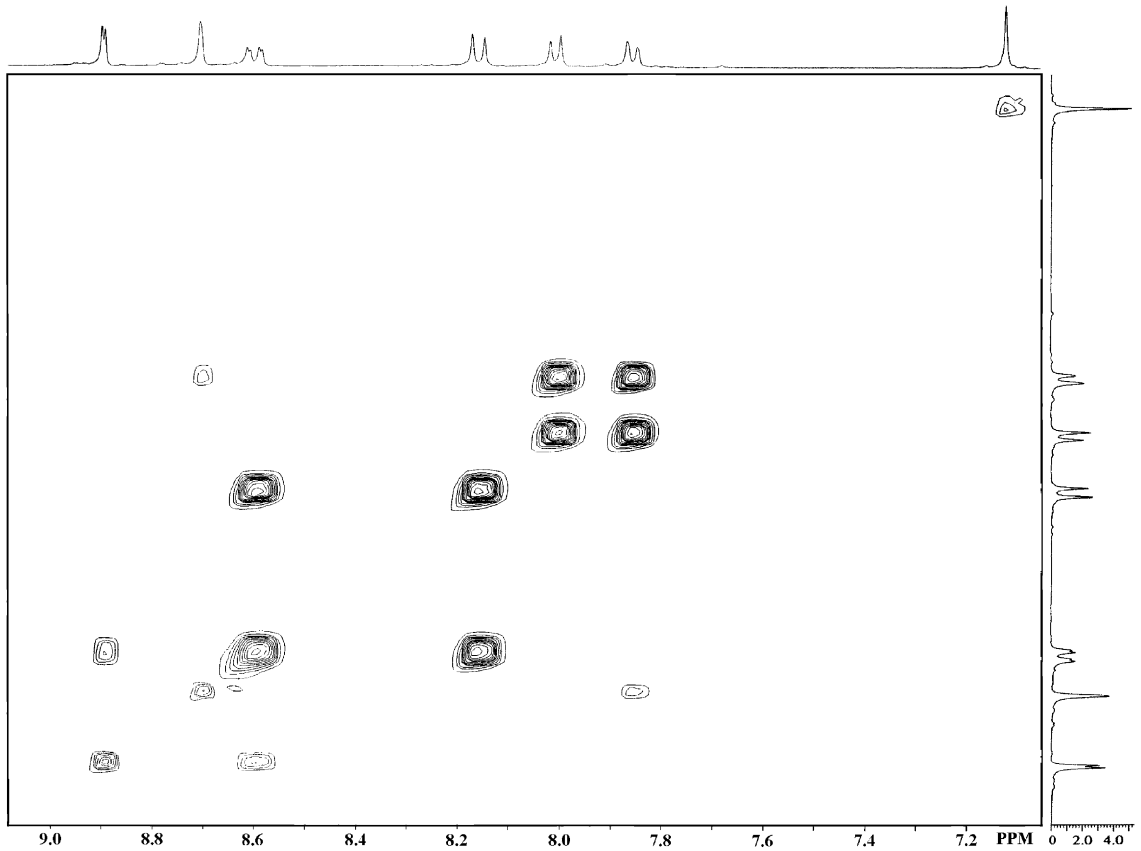


Fig. 2. 2D-COSY spectrum of Nitrazine Yellow.

of 2.0 Hz at 8.60 ppm. The doublet (J_0: 9.6 Hz) at 8.13 ppm is assigned to the remaining benzene H6′ as it gives a strong correlation with the signal of H5′ at 8.60 ppm in the 2D-COSY spectrum (Fig. 2) [14a]. The ^{1}H NMR signals of the naphthalene moiety can be assigned as follows. The 2D-COSY spectrum (Fig. 2) shows two cross peaks involving this group of signals: the first one is between the doublets at 8.01 ppm (J_0: 8.1 Hz) and 7.84 ppm (J_0: 8.1 Hz) and the second one is between the doublet at 7.84 ppm and the singlet at 8.69 ppm. This allows to assign unequivocally the signals at 8.01, 7.84 and 8.69 ppm to H8, H7 and H5 protons respectively. The singlet at 7.12 ppm did not show any through-bond correlation and can be assigned either to H2 in structure **2** or to H4 in structure **1**, without giving particular information about the attack of diazonium salt.

The 2D-NOESY spectrum (Fig. 3) is much more informative. This two-dimensional NMR technique provide a straightforward means to assess the spatial closeness between pairs of spins through the detection of Nuclear Overhauser Effect (NOE) cross peaks [14b]. Lyčka et al. observed a cross peak between the signals of H4 and H5 protons of azodyes obtained from J-, H- and γ acids suggesting that benzenediazonium ion attacked position *ortho* to hydroxy group [12]. On the contrary, no NOE cross peak involving the singlet at 7.12 ppm is found, suggesting that no proton is present in position 4 of naphthalene and that the 2′,4′-dinitrobenzeneazo moiety rather

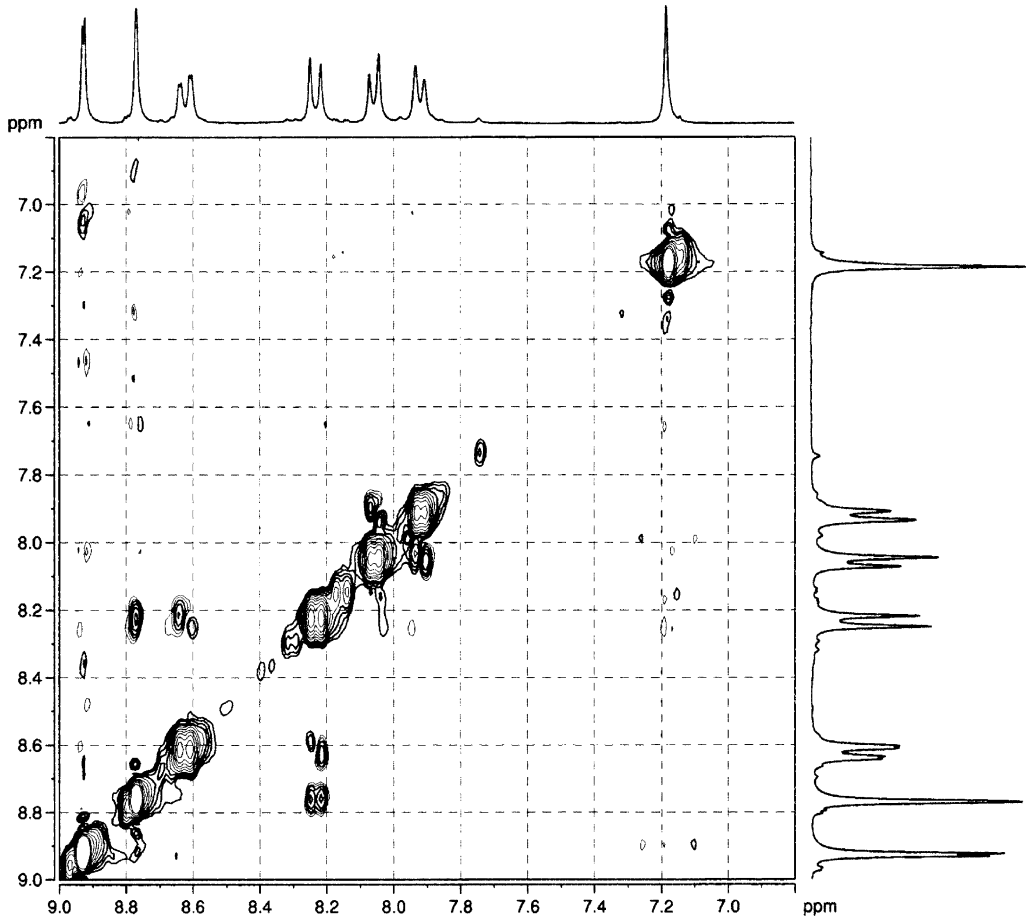


Fig. 3. ^{1}H–^{1}H NOESY spectrum of Nitrazine Yellow.

takes this position. Moreover, the 2D-NOESY spectrum shows a strong cross peak between naphthalene H5 and benzene H6′ protons, that strongly supports the insertion of 2′,4′-dinitrobenzeneazo moiety in position 4 of naphthalene.

The ^{13}C NMR spectrum alone was not so useful to solve the structural problem. In fact, chemical shift in the range 176.2–181.5 ppm for C1 carbon has been adopted as reference for naphthoquinone azodyes [12,15]. The presence of a signal at 185.05 ppm, seems to suggest a hydrazone form, more compatible with structure **1**. A more realistic hypothesis is that the signal so downfield shifted is not due to the carbon atom linked to oxygen. A complete assignment of ^{13}C NMR resonances was therefore performed by the combined analysis of Heteronuclear Multiple-Quantum Coherence (HMQC) and Heteronuclear Multiple-Bond Correlation (HMBC) spectra. The HMQC techniques, providing direct (one-bond) heteronuclear shift correlations, allows the assignment of non-quaternary carbon atoms for which ^{1}H assignment is available [14b]; such a spectrum, shown in Fig. 4, allows the unambiguous assignment of C5, C7, C8, C3′, C5′, and C6′. On the other hand, the HMBC technique allows the identification of proton–carbon connectivities through multiple bond couplings (typically over two to four bonds, $^{n}J_{HC}$, $n = 2$–4) thus providing a route for the assignment of quaternary carbon atoms [14c]. The HMBC spectrum of Nitrazine Yellow is reported in Fig. 5. To confirm that the structure of Nitrazine Yellow

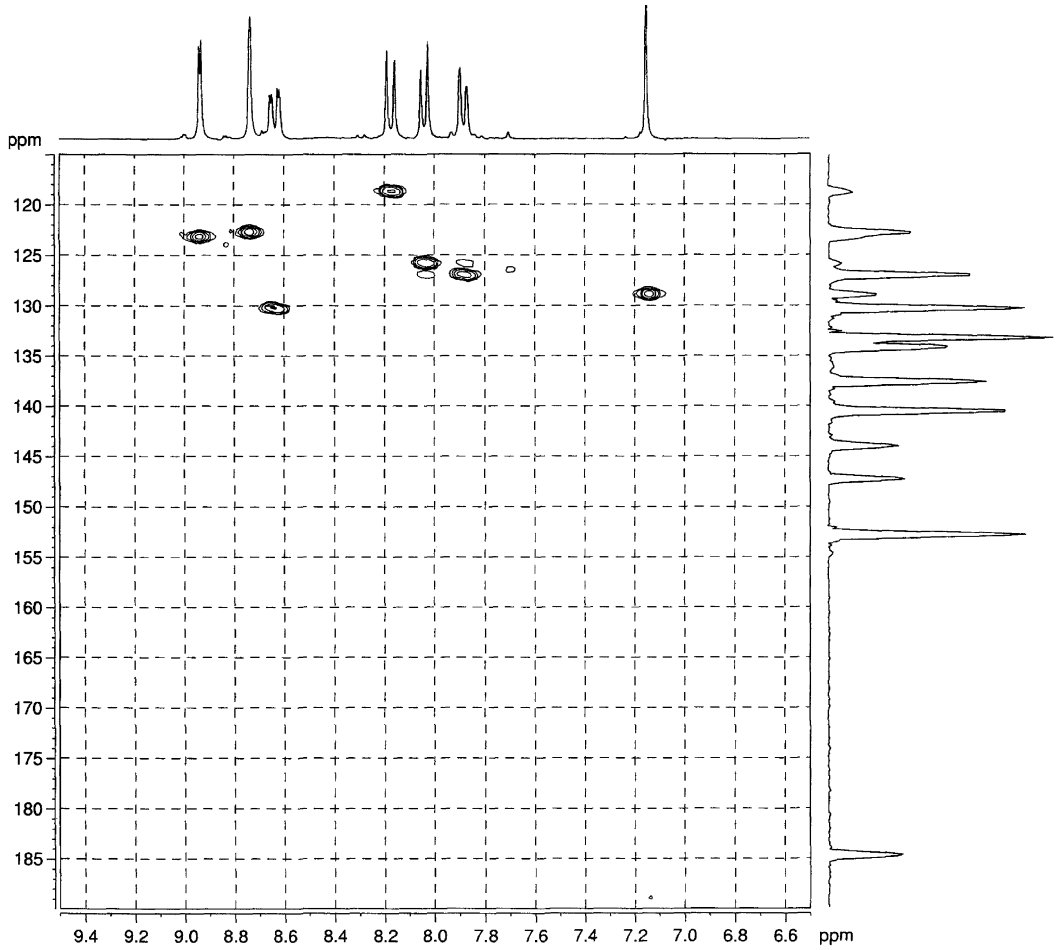


Fig. 4. ^{1}H–^{13}C-HMQC spectrum of Nitrazine Yellow. The ^{13}C-NMR projection is taken from the HMBC spectrum (see also Fig. 5).

is **2** rather **1**, C2 and C4 carbon atoms must be assigned and it must be demonstrated that the former is directly attached to a hydrogen whereas the latter is quaternary. In the hypothesis that structure **2** is correct, correlations over three-bonds are then expected between H2 and C4/C9. C9 can be assigned to the signal at 130.79 ppm on the basis of the cross peaks detected between such carbon atom and H5/H7 (^3J correlations). Moreover the H5 atom shows cross peaks with C7, C9 (already assigned) and with a carbon atom whose signal falls at 133.41 ppm. This signal is unambiguously assigned to the C4. It is worth noting the presence of a W-type correlation between H8 and C4 ($^4J_{H8–C4}$). Usually, $^4J_{1H–13C}$ are small and not detectable but connectivities through four bonds in a planar zig-zag arrangement (W coupling) increase the coupling constant [14b,16]. The observation of the H8/C4 long range correlation through the $^4J_{H8–C4}$ supports unequivocally the assignment of C4. The fact that C4 is quaternary (it does not correlate with a directly bound hydrogen atom) allows to conclude that the 2′,4′-dinitroazobenzene moiety is linked in para position to the naphthalene hydroxy group, confirming the assigned structure **2** and that the signal at 7.12 ppm is due to the H2. Consistently, the expected H2/C4 and H2/C9 correlations are detected.

Heterocorrelated experiments also allowed to assign the signal at 138.08 ppm to the C1 on the basis of the detection of cross peaks between H8/C1, H5/C1, H7/C1 and H2/C1 (the latter three ones are J_w type correlations). The chemical shift

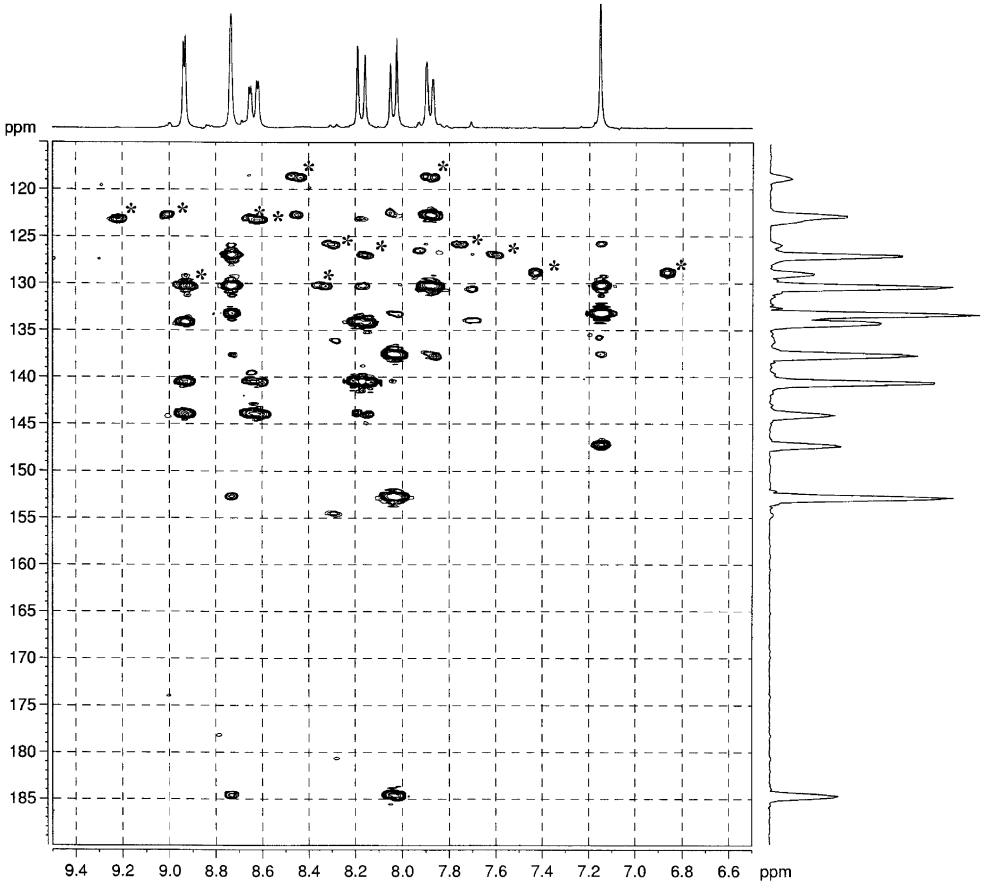


Fig. 5. ^{1}H–^{13}C-HMBC spectrum of Nitrazine Yellow. The peaks marked with asterisks are due to direct correlations which were incompletely suppressed by the low-pass filter.

value of C1 suggests the prevailing in DMSO-d_6 solution of azo form [15]. The assignment of H2 allows to assign the signal at 129.35 ppm and 147.59 ppm to the C2 (HMQC spectrum, Fig. 4) and C3 (HMBC spectrum, Fig. 5) respectively. The signals falling in the downfield region (152.76 ppm and 185.05 ppm) can be assigned to the C6 and C10 carbon atoms, but it is not possible to identify which is which.

The signals of benzene carbons were assigned as it follows. C3′, C5′ and C6′ carbon atoms were directly assigned on the basis of the HMQC spectrum; C2′ was assigned to the signal at 134.55 ppm by considering that it gives HMBC cross peaks with H3′ and H6′ (couplings over two- and three-bonds respectively) but not with H5′, as this proton is too far in the covalent structure. Finally, C1′ and C4′ were assigned to the resonances at 144.37 ppm and 140.97 ppm respectively by considering that ^3J are larger than ^2J and by comparing the intensities of HMBC cross peaks arising from H6′ and H5′.

3. Experimental

The ^{1}H NMR (400 MHz) spectra were recorded in DMSO-d_6 solution (99.8%) using DMSO as reference ($\delta_H = 2.52$ ppm). ^{1}H and ^{13}C spectra were recorded on a Jeol EX400 NMR spectrometer operating at 9.4T (corresponding to ^{1}H and ^{13}C Larmor frequencies of 400 and 100 MHz) and the two-dimensional homonuclear proton NOESY and COSY experiments performed with a spectral width of 4800 Hz over 1024 data points. The mixing time of 2D-NOESY was set at 50 ms. The spectrum (16 scans) was obtained after multiplying the data with a sine square bell function in both dimensions. The heterocorrelated HMQC and HMBC experiments were carried out on a Bruker Avance300 spectrometer operating at 7 T (corresponding to ^{1}H and ^{13}C Larmor frequencies of 300 and 75 MHz respectively) equipped with a *Z*-axis-PFG probe optimised for gradient-enhanced inverse spectroscopy. ^{13}C chemical shifts have been calibrated using the resonance of DMSO-d_6 at 40.45 ppm as reference. ^{1}H–^{13}C HMQC and ^{1}H–^{13}C HMBC spectra were obtained with the standard gradient-enhanced pulse sequences in the phase-insensitive mode. Typical instrumental settings included: spectral width ^{1}H 10 ppm, spectral width ^{13}C 100 ppm, recycle delay 2.5 s, 1024 and 256 complex data points for the t_2 and t_1 dimensions respectively, 8–16 scans for t_1 increment, delay for evolution of long-range couplings 70 ms. The data matrix was zero-filled in the indirect direction to 512 data points and apodized with a sine bell function in both dimensions prior to FT and baseline correction.

4. Conclusions

Visible spectroscopic properties of commercially available Nitrazine Yellow are incoherent with the stated structure, deriving from attack of 2′,4′-dinitrobenzenediazonium salt to *ortho* hydroxy position of 1-naphthol-3,6-disulphonic acid. NMR analysis and in particular HMQC and HMBC experiments showed that the real structure comes from an attack of diazonium salt to para hydroxy position. Henry Wenker originally described indicator properties of Nitrazine Yellow in 1935 [17]. In this paper he described the dye as the result of an ortho hydroxy attack of diazonium salt to coupling agent. As such the indicator was regularly reviewed from Beilstein. One year after, in another paper on similar azodyes Wenker, commenting the visible spectroscopic properties of Nitrazine Yellow and of a red dye isolated from its synthesis suggested an attack in position para and ortho to hydroxy group for Nitrazine Yellow and the red dye respectively: "Probably this red dye, which also turns blue in alkaline solution (pH range 9.6 to > 10), is the ortho azo dye, while Nitrazine Yellow is the para isomer" [9]. The Wenker correction was newer reported on Beilstein.

Acknowledgements

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