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TAUTOMERISM OF AZOPIGMENTS DERIVED FROM BILIRUBIN

F. COMPERNOLLE and S. TOPPET
Laboratory of Macromolecular and Organic Chemistry, University of Leuven, B-3030 Heverlee, Belgium

and

D. W. HUTCHINSON*
Laboratory of Hepatology, Department of Medical Research, University of Leuven, B-3000 Leuven, Belgium

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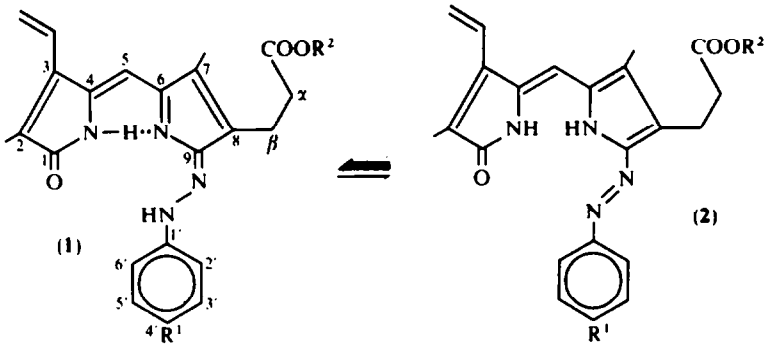
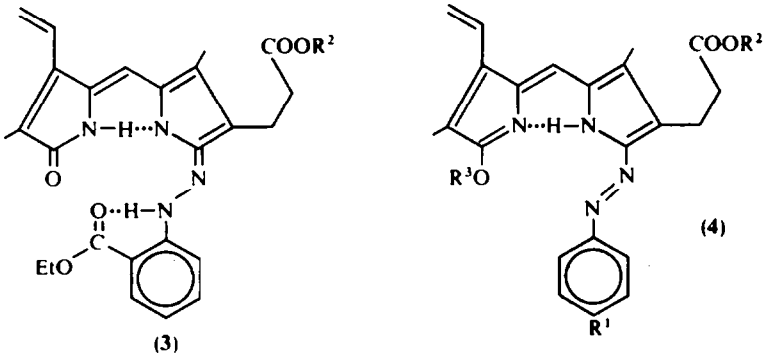
Abstract—^{1}H and ^{13}C n.m.r. data indicate that bilirubin azopigments exist predominantly as phenylhydrazone rather than azobenzene tautomers. Whereas *O*-lactim ethers derived from the azopigments exist predominantly as the azobenzene tautomers.

INTRODUCTION

The reaction of aryl diazonium salts with bilirubin to give dipyrrolic azopigments is the basis of the most commonly used method for the assay of bilirubin and its conjugates in serum or bile.[1] The gross structures of the azopigments have been established by chemical,[2] spectroscopic[2,3] and mass spectrometric means.[2,4,5] We now present evidence based on ^{1}H and ^{13}C n.m.r. data which shows that the commonly assumed azobenzene structure **2** represents only a minor fraction of a tautomeric mixture. The phenylhydrazone form (**1**) is the predominant tautomer in solution at room temperature.





Only *endo*-vinyl isomers shown.

* To whom correspondence should be addressed.
†*Present address:* Department of Chemistry and Molecular Sciences, University of Warwick, Coventry CV47AL, U.K.

EXPERIMENTAL

Azopigments. These were prepared by previously published methods.[5,6] The mixed *endo-* and *exo*-vinyl isomers were isolated by chromatography on silica (elution with chloroform/methanol, 99/1 v/v) and were converted into their methyl esters by brief treatment with ethereal diazomethane.[4] The *endo-* and *exo*-vinyl isomers[7] of the methyl esters were separated by chromatography on silica (elution with toluene/ethyl acetate, 95/5 v/v). *O*-Ethyl lactim ethers were prepared by treating the methyl esters with an excess of triethyloxonium tetrafluoroborate.[8] For example, the methyl ester of the azopigment (**1**, $R^1 = R^2 = H$) (20 mg) in dry dichloromethane (10 ml) was stirred with an excess (50 mg) of triethyloxonium tetrafluoroborate for 3 hrs at room temperature. Examination of the blue solution by t.l.c. showed that a single new compound had been formed in virtually quantitative yield. After destruction of excess triethyloxonium tetrafluoroborate with cold sodium bicarbonate solution, the dichloromethane layer was separated, evaporated to dryness and the residue purified by preparative t.l.c. on silica (developed with toluene/ethyl acetate, 95/5 v/v). The *O*-ethyl ethers were characterised by mass spectrometry (e.g. for (**1**, $R^1 = H$, $R^2 = Me$) m/e 432.2155: $C_{25}H_{28}N_4O_3$ requires m/e 432.2161) and by ^1H and ^{13}C n.m.r. spectroscopy (OCH_2CH_3 at δ 4.62 p.p.m. and δ 65.2 p.p.m.) (Tables 1 and 2). The azopigment (**3**) reacted slowly with triethyloxonium tetrafluoroborate and after 5 hrs less than 50% conversion to the *O*-ethyl ether had occurred. Silylation of the azopigments was carried out as previously described,[4] the mixed *endo-* and *exo*-vinyl isomers of the azopigments (**1**, $R^1 = R^2 = H$) and (**1**, $R^1 = COOEt$, $R^2 = H$) gave *bis*(trimethylsilyl) derivatives as identified by mass spectra at m/e 534 and m/e 606 respectively. The mixed isomers of the azopigment (**3**, $R^2 = H$) gave only a mono(trimethylsilyl) derivative m/e 534 under the same conditions.

N.m.r. Conditions. The ^1H n.m.r. spectra were recorded in deuterochloroform on a Varian XL100-15 spectrometer. ^{13}C n.m.r. spectra were recorded on a Bruker WP80 spectrometer operating at 20 MHz in the FT mode. Initially the ^{13}C n.m.r. spectra were obtained with complete proton noise decoupling. The assignment of the resonance signals due to primary, secondary and tertiary carbon was made by single frequency

Table 1. ^1H n.m.r. chemical shifts at 40°C for azopigments (**1**), (**3**) and (**4**)

Protons	(1), R^1=H R^2=Me endo[a]	(1), R^1=H R^2=Me exo[a]	(1), R^1=COOEt R^2=H endo+exo[a,c]	(1), R^1=COOEt R^2=Me endo[a]	(1), R^1=COOEt R^2=Me exo[a]	(3), R^2=Me endo[a]	(3), R^2=Me exo[a]	(4), R^1=H R^2=Me, R^3=Et endo[a]	(4), R^1=COOEt R^2=R^3=TMS endo+exo[a]
NH-CO	b	10.75[b]	10.60/10.75	11.00[b]	10.80[b]	10.55	10.30	-	-
NH	b	11.60[b]	11,13(+COOH)	11.75[b]	11.60[b]	13.35	13.30	11.90	11.60
H-2',6'	7.20 to	7.20 to	7.54/7.52	7.38	7.30	7.50	7.48	7.70	7.72
H-3',5'			7.95	7.96	7.96	7.97/7.75	7.95/7.74	7.40	8.07
H-4'	7.50	7.50	-	-	-	6.90	6.90	7.40	-
H-5	6.00	5.92	6.12/6.10	5.98	5.84	5.92	5.84	6.52	6.5/6.42
$CH_{2\beta}$	2.95	2.94	2.83	2.94	2.94	2.92	2.88	3.17	3.13
$CH_{2\alpha}$	2.70	2.70	2.52	2.70	2.70	2.70	2.66	2.72	2.70

All spectra recorded in $CDCl_3$ at room temperature. All chemical shifts (in p.p.m.) are relative to tetramethylsilane as standard. For a full analysis of ^1H n.m.r. of bilirubin azopigments see ref. (2).

[a]Endo- or exo-vinyl isomers of azopigments

[b]Not detectable at room temperature, values found at -40°C

[c]Spectrum recorded in [^2H]^6-DMSO

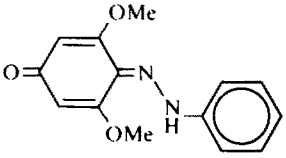
Table 2. ^{13}C n.m.r. chemical shifts for azopigments (**1**), (**3**) and (**4**)

Carbon atoms	(1), R^1=H R^2=Me endo[a]	(1), R^1=H R^2=Me exo[a]	(1), R^1=COOEt R^2=Me endo[a]	(1), R^1=COOEt R^2=Me exo[a]	(3), R^2=Me endo[a]	(3), R^2=Me exo[a]	(4), R^1=H R^2=Me, R^3=Et endo[a]	(5) Ref.(10)	(6) Ref.(10)
C-5	98.5	98.0	98.2	97.3	96.7	96.6	110.4		
CH=	126.5	126.0	126.3	126.0	126.5	126.1	127.9		
CH_2=	123.2	122.0	123.5	122.5	123.1	122.3	119.0		
C-1'	143.8	143.9	146.9	146.7	145.2	145.2	154.0	142.6	152.7
C-2'	116.1	116.2	114.6	114.7	113.4	113.5	122.5	115.3	122.6
C-6'	116.1	116.2	114.6	114.7	114.2	114.3	122.5		
C-3'	129.6	129.6	131.5	131.5	131.6	131.4	129.3	129.5	129.1
C-5'	129.6	129.6	131.5	131.5	134.7	134.7	129.3		
C-4'	124.1	124.2	124.8	124.9	120.9	121.0	129.3	124.3	130.5
C-2	130.9	128.8	131.5	129.5	(131.4)	(129.4)	132.1		
C-8	134.1	134.0	135.9	135.9	136.3	136.3	125.7		
C-3	140.2	140.0	140.6	140.4	140.2	140.3	142.0		
C-7	140.6	140.5	141.3	141.3	140.7	140.7	126.0		
C-4	143.8	144.8	145.5	146.7	146.6	147.6	145.5		
C-6	153.5	153.4	155.0	155.3	157.1	157.3	129.3		
C-9	162.9	162.3	167.3	167.1	168.2	168.2	148.0		
C-1	173.0	171.6	173.2	171.6	172.0	170.9	174.9		

All shifts (in p.p.m.) are relative to tetramethylsilane

[a]Endo- or exo-vinyl isomers of pigments

off-resonance decoupling or by selective decoupling of the corresponding protons. An unambiguous assignment could be made for the quaternary carbon C-1′ of the phenyl ring in the undecoupled spectrum (the signal due to C-1′ is a triplet due to two meta C-H couplings) also by selective decouplings of the meta protons and by comparison with the spectra of azobenzene[9] and 2,6-(**5**) or 3,5-dimethoxy-4-hydroxyazobenzene (**6**).[10] The assignment of the signals due to C-1, C-2, C-3, and C-4 in the lactam ring was made tentatively by a comparison with literature values[3] and by comparing the chemical shifts in the *endo*- and *exo*-vinyl isomers. The assignments for the "pyrrole" ring mainly C-6 and C-9 were made from the variation in chemical shifts of the carbon atoms with substituents on the phenylhydrazo-ring. Carbon C-9 in (**4**) was assigned by selective decoupling of the CH_2 β protons at δ 3.17 p.p.m.



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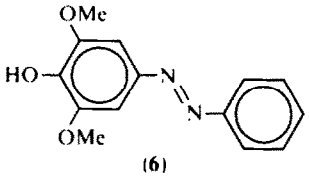

(6)

DISCUSSION

The ^1H and ^{13}C n.m.r. spectra of the *endo*- and *exo*-vinyl isomers of the azopigments are very similar and the isomerisation of the vinyl groups does not appear to affect the tautomerism of the azopigment. There are major differences between the ^1H n.m.r. spectra of (**1**) and (**3**) compared with the spectra of (**4**) (Table 1). This is particularly noticeable for the chemical shifts of the protons attached to the methine bridge (H-5), the protons of the phenyl ring which are ortho to the nitrogen atom (H-2′ and H-6′) and the protons of the methylene groups in the side chain β- to the carbomethoxy group. From the chemical shift differences at three sites, it appears that *O*-ethylation of the lactams induces important structural modifications not only in the lactam rings themselves but also in the two other aromatic rings. Furthermore, the two N–H protons in (**1**) are not observed at room temperature but can be observed at −40°C. On the other hand, the spectra of (**3**) and (**4**) display relatively sharp N–H signals at room temperature suggesting that these protons do not exchange easily presumably because they are intramolecularly hydrogen bonded. When the ^1H n.m.r. spectra of (**1**) are recorded at −40°C the signals of the various protons are markedly broadened whereas the spectra of (**3**) and (**4**) are still well resolved at this temperature. We suggest that compounds (**3**) and (**4**) are present as one tautomer at both room temperature and −40°C while for the compounds (**1**) the freezing of an equilibrium between the two tautomeric forms is excluded from ^{13}C n.m.r. data. Restricted rotation about the (C-5)–(C-6) bond in form (**1**) is a possible explanation for the broadening of the signals in the ^1H and ^{13}C spectra observed at −40°C.

In the ^{13}C n.m.r. spectra of the azopigments (Table 2) there is a marked difference in the chemical shift between the signal due to C-9 in (**1**) or (**3**) (162–168 p.p.m.) and (**4**) (148 p.p.m.). The chemical shifts of the carbon atoms of the aryl ring in the spectra of (**1**) and (**3**) resemble those of a phenylhydrazone[10] while the chemical shifts of the aryl carbon atoms in (**4**) are comparable to those of an azobenzene. Furthermore, the carbon atoms C-6, C-7, C-8, and C-9 in the pyrrole rings of (**1**) and (**3**) resonate at δ values between 134 and 168 p.p.m. Such shift values are unlike those of tetrasubstituted pyrroles[11] which always display in their spectra at least two upfield absorptions (between 115 and 130 p.p.m.) for carbon atoms β to the N atom. The ^{13}C δ values assigned to C-6, C-7, C-8 and C-9 in (**4**) (δ 125.7, 126, 129.3 and 148 p.p.m.) are closer to the values expected for these pyrroles. Finally, there is a substantial difference between the chemical shifts of C-5 in (**1**) or (**3**) (97, 98 p.p.m.) and (**4**) (110 p.p.m.). This downfield shift parallels that observed for H-5 in the ^1H n.m.r. spectrum and reflects the lower electron density at C-5 in the lactim form. This downfield shift is not directly related to the azo-hydrazo tautomerism.

To rationalise the n.m.r. data, we suggest that (**3**) exists entirely in the phenylhydrazo- form stabilised as shown by H-bonding between the NH and carbethoxy groups and that this form is the major tautomer of both the *endo*- and *exo*-vinyl isomers of (**1**) in $CDCl_3$ at room temperatures. On the other hand, compounds (**4**) appear to exist entirely as the azobenzene tautomers. Assuming that the chemical shifts of C-9 in the phenylhydrazo- and azobenzene tautomers are 168 and 148 p.p.m. respectively, it can be estimated that the phenylhydrazo- tautomer represents 75% of (**1**, R^1 = H, R^2 = Me) and 95% of (**1**, R^1 = COOEt, R^2 = Me). Additional evidence for the different tautomeric composition of these two compounds comes from a comparison of the δ values of C-2′ and C-6′ of the aryl rings. As the effect of a COOEt substituent in an aryl ring on the meta carbons is usually negligible[12] the difference (δ 116.1 vs δ 114.6 p.p.m.) may be ascribed to the higher phenylhydrazo-content of (**1**, R^1 = COOEt, R^2 = Me). The ^{13}C n.m.r. spectrum of (**1**, R^1 = H, R^2 = Me, exo-vinyl) in $CDCl_3$ at −35°C shows not only a marked broadening of almost all carbon signals even those having identical chemical shifts in the phenylazo- and phenylhydrazo-tautomers but also a variation in chemical shift for C-9 (+2 ppm), C-1′ (−0.9 ppm) and C-2′, C-6′ (−1 ppm) consistent with a displacement of the fast tautomeric equilibrium towards more phenylhydrazo-form. Recently the tautomerism of 2,6-(**5**) and 3,5-dimethoxy-4-hydroxyazobenzene (**6**) has been studied by ^1H and ^{13}C n.m.r.[10] The ^{13}C chemical shifts of the carbon atoms in benzene rings in (**5**) and (**6**) closely resemble those in (**1**) or (**3**) and (**4**) respectively (Table 2) providing further confirmation for the tautomeric forms of these azopigments. Moreover, the stabilisation by electron withdrawing substituents on the phenyl ring of the hydrazo-form of phenylazo dyes is well known.[13]

In the bistrimethylsilyk derivative (**4**, $R^1 = COOEt$, $R^2 = R^3 = TMS$) carbon atoms C-2′ and C-6′ resonate at the same δ value (122 p.p.m.) as in (**4**, $R^1 = H$, $R^2 = Me$, $R^3 = Et$) and we suggest that these compounds are probably entirely in the phenylazo-form.

Our ^{13}C n.m.r. data are in general agreement with those given by Salmon *et al.*[3] for azopigments of bilirubin and mesobilirubin derived from diazotised aniline, although our assignments for C-6 and C-1′ are different. However, these authors did not consider the possibility of different tautomeric forms even though their ^{13}C n.m.r. data are not consistent with the presence of tetrasubstituted pyrrole. Jansen and Stoll[2] comment on the presence of broad unexpectedly low field signals in the ^1H n.m.r. spectra of (**3**) at δ 13.3 p.p.m. We suggest that these signals are due to intramolecularly hydrogen bonded hydrazo N–H atoms as shown in our structure.

In the IR spectra of the azopigments and their derivatives, the N–H region is broad and the peaks are ill defined. However, the carbonyl region of the spectrum of (**1**, $R^1 = COOEt$, $R^2 = H$) differs considerably from the corresponding region in the spectrum of (**3**). In the IR spectrum (KBr disc) of the former, the carbonyl absorption of the carbethoxy group appears at 1700 cm^{-1} while in the latter the absorption is at 1685 cm^{-1}. When the IR spectra (KBr discs) of the methyl esters of the azopigments are compared, the absorption of the carbethoxy group in (**1**) is at 1715 cm^{-1} and in (**3**) is at 1705 cm^{-1}. These values indicate that the carbethoxy group in (**3**) is involved in hydrogen bonding which would be consistent with formula (**3**).

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