Synthesis and interaction with human serum albumin of the first 3,18-disubstituted derivative of bilirubin

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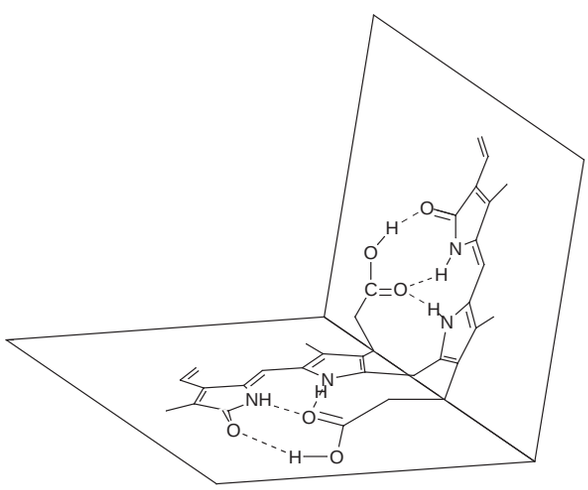
Addition of excess (>500 equiv.) of *p*-fluorothiophenol to bilirubin in the presence of toluene-*p*-sulfonic acid catalyst afforded 3,18-didevinyl-3,18-bis[2-(*p*-fluorothiophenyl)ethyl] bilirubin 2 from anti-Markovnikov addition to both the *exo* and *endo* vinyl groups of bilirubin. Toluene-*p*-sulfonic acid is not essential as *p*-fluorothiophenol acts as the acid and nucleophile in the reaction. In contrast, in the presence of toluene-*p*-sulfonic acid, regioselective Markovnikov addition of thioacetic *S*-acid to the *exo* vinyl group occurs, in agreement with previous studies of the addition of a range of oxygen and sulfur nucleophiles to bilirubin (P. Manitto and D. Monti, *Experientia*, 1973, 29, 137). Binding of 2 to human serum albumin was measured by circular dichroism. The two bulky *p*-fluorothiophenyl groups do not appear to impede interaction with the protein. This result supports a model in which the reactive methylene bridge of bilirubin, that connects rings B and C, points into the binding pocket of human serum albumin.

Introduction

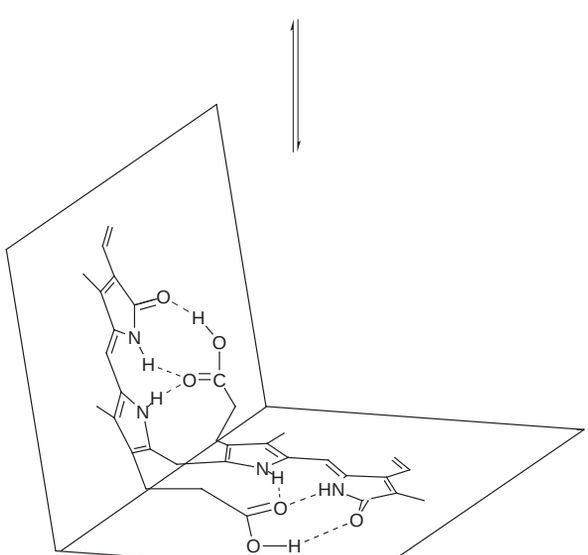
(4*Z*,15*Z*)Bilirubin IXα **1** is the light orange, hydrophobic tetrapyrrole pigment that is produced from heme catabolism.[1,2] Abnormalities in bilirubin formation, transport and excretion are usually manifested in jaundice in neonates. The pigment predominantly exists as the dianion at physiological pH (7.4) while at pH < 7, rotation about the methylene bridge and hydrogen bonding between the polar groups forms the water insoluble diacid 'ridge-tile' structure, which exists as two helical enantiomers, **1**(*M*) and **1**(*P*) in both the solution[3,4] and the solid state.[5,6] In the body, bilirubin is transported in plasma primarily by association with human serum albumin (HSA).[7,8] Once binding reaches saturation, the unbound bilirubin levels increase and bilirubin crosses the blood–brain barrier causing kernicterus in newborn infants.[7,8] While the exact binding site, and the detailed molecular structure of the HSA–bilirubin complex are unknown, it has been suggested that the methylene bridge points into the binding pocket[9] and that hydrogen bonding and salt linkages play an important role in stabilising the right-handed folded conformation in the protein binding site.[10]

We were interested in introducing a fluorine label into bilirubin, as an NMR probe, for studying bilirubin interactions with biomolecules, in particular human serum albumin. To our knowledge there is only one report of a fluorinated derivative of bilirubin, an intermediate in the synthesis of hydroxybilirubin, that was not fully characterised.[11] More recently, the total synthesis of the symmetrical difluorinated bilirubin analogue, α,α′-difluoromesobilirubin XIIIα, has been reported;[12] fluorines were introduced α to the carboxylic acids in an attempt to modify the acidity and hence strength of the hydrogen bonding in the 'ridge-tile' conformation, but poor solubility and unexpected properties were observed for this analogue.[12] We chose to modify the vinyl groups of bilirubin since they are not involved in the 'ridge-tile' hydrogen bonding, and hence introduction of groups at these positions should minimise any changes to the overall shape and conformation of bilirubin, and hence the interaction of the fluorinated bilirubin derivative with HSA. In addition, previous HSA binding studies on 18-substituted bilirubin derivatives[13] suggested that quite large groups can be tolerated at this position on bilirubin without inhibition of HSA binding.

In the course of our fluorination studies we have synthesised



1(*M*)



1(*P*)

and fully characterised the first 3,18-disubstituted bilirubin derivative **2** prepared directly from bilirubin. In contrast to all other derivatives prepared by reaction of bilirubin with nucleophiles in the presence of an acid catalyst,[11,14–16] anti-Markovnikov addition to both vinyl groups was observed with *p*-fluorothiophenol. Binding studies of derivative **2** with HSA have provided new information regarding the nature of the bilirubin–HSA complex and suggest that considerable steric bulk may be incorporated at positions 3 and 18 of bilirubin without preventing interaction with HSA.

Results and discussion

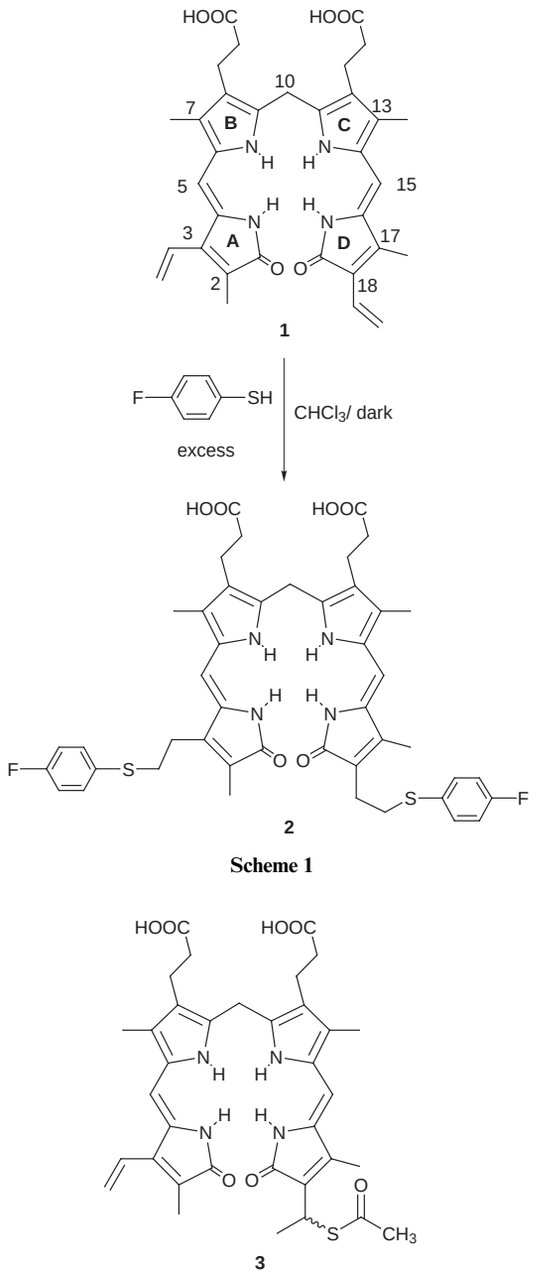
Synthesis

Due to the 'ridge-tile' conformation of bilirubin in chloroform,[4,17] direct modification of the vinyl groups in chloroform and other non-polar solvents, without protection of other functional groups in the molecule, is possible. The *exo* and *endo* vinyl groups of bilirubin have different chemical properties, and under acid catalysis,[11,14–16] or photochemical conditions,[18,19] regioselective Markovnikov addition of alcohols, thiols and thioic *S*-acids to the 18-vinyl group, in moderate to high yield, has been reported. This chemistry has been used to develop methods for selective protection and deprotection of one vinyl group under mild conditions and has been used in methods to prepare bilirubin IIIα from bilirubin IXα **1** in high yield.[20,21] To our knowledge, there are no examples of derivatives from addition reactions to both the 3 and 18 vinyl groups of bilirubin.[22]

The incorporation of fluorine into bilirubin was investigated by addition of fluorinated thiols to bilirubin and followed the general procedure reported for the synthesis of 18-devinyl-18-(1-acetylthio)ethylbilirubin **3** from the addition of thioacetic *S*-acid (CH_3COSH) to bilirubin.[15] Bilirubin is acid and oxygen sensitive due to the central methylene bridge which is prone to electrophilic and oxidative attack.[9,23] It has a tendency to autooxidise and decompose in solution, particularly in the presence of acids, oxygen and light.[24,25] Acid catalysed isomerisation to other bilirubin isomers can occur, as well as degradation to dark green biliverdinoid type products, that may be removed on work-up by extraction into methanol.[24,25] Hence, all experiments were carried out under a nitrogen atmosphere, and a large excess (500–1000 equiv.) of the thiol was used to trap the intermediate carbocation. In agreement with previous studies,[15] despite the use of a large excess of thioacetic *S*-acid (500 equiv.), exclusive regioselective addition to the 18-vinyl group of bilirubin occurred to give racemic **3**. Markovnikov addition is readily apparent from the characteristic downfield quartet in the ^{1}H NMR spectrum of **3**.[3]

In contrast, under similar reaction conditions used for the preparation of **3**, addition of *p*-fluorothiophenol (500–1000 equiv.) to bilirubin **1** in the presence of toluene-*p*-sulfonic acid catalyst afforded exclusively the disubstituted derivative **2** (Scheme 1). This reaction was monitored by ^{1}H NMR analysis of aliquots of the reactions, and within 3 hours, the signals due to both the 3-vinyl and 18-vinyl groups had disappeared. 400 MHz ^{1}H NMR spectra of **2** were recorded in $CDCl_3$, in which the 'ridge-tile' conformations are present, and d_6-DMSO in which the intramolecular hydrogen bonding conformation is destabilised, and hence simplifies the spectrum. While significant spectral overlap in the region 1–3 ppm prevented full peak assignments in $CDCl_3$, all signals were fully resolved in d_6-DMSO, and the two new spin systems for the $–CH_2CH_2S–$ groups at positions 3 and 18 were assigned. A NOESY spectrum (τ_m 400 ms) allowed full assignment of all protons; in particular H5 showed NOEs to the 7-CH_3 and the α-CH_2– on ring A ($–CH_2CH_2S–$), while H15 showed NOEs to both the 13-CH_3 and the 17-CH_3. As expected, two fluorine signals were detected in the proton decoupled ^{19}F NMR spectra recorded in both $CDCl_3$ and DMSO.

Attempts to synthesise the corresponding mono-substituted



Scheme 1

derivative from addition to only the 18-vinyl group, by varying the reaction time and the amount of *p*-fluorothiophenol present (1–6 equiv.), resulted in either no reaction (1 equiv.) or the formation of a mixture of products that were not isolated and characterised, along with the desired monosubstituted product. The reaction was also repeated in the absence of toluene-*p*-sulfonic acid catalyst, as excess *p*-fluorothiophenol may also act as the proton source, and also in the presence of oxygen. Under both sets of reaction conditions, the disubstituted derivative **2** was produced in good yield.

Addition of trifluorothioacetic *S*-acid to bilirubin was also investigated. Trifluorothioacetic *S*-acid has a low boiling point (35.5 °C) and is volatile, highly corrosive and very acidic. When trifluorothioacetic *S*-acid (400–500 equiv.) was added to bilirubin **1** in chloroform for 24 hours, analysis of the ^{1}H NMR spectrum of the crude product and mass spectroscopy were consistent with the formation of the disubstituted product. However, a number of other products were also present, including dark green biliverdinoid type by-products. Reducing the number of equivalents of thiol also resulted in a complex mixture of products which were not isolated and characterised.

Derivative **2** is the first example of a 3,18-disubstituted bilirubin derivative prepared directly from bilirubin in one

step, and to our knowledge the only example of an anti-Markovnikov addition product prepared from bilirubin. The addition of a range of thiol (and oxygen) nucleophiles to bilirubin has been reported, including 2-mercaptoethanol, glutathione, *N*-acetyl-L-cysteine and several thioic *S*-acids.[11,14–16] With all these thiols, regioselective Markovnikov addition to the 18-vinyl group has been reported, *via* an electrophilic addition reaction, with no reaction at the 3-vinyl group. This regiospecificity has been rationalised previously on the basis of the UV spectrum of bilirubin in the presence of toluene-*p*-sulfonic acid, which is consistent with the formation of a partially protonated dipyrromethane type bilirubin cation.[14]

While the 18-vinyl group of bilirubin is the most reactive under electrophilic addition conditions, addition to the 3-vinyl group was observed with *p*-fluorothiophenol. Thiols add to alkenes by electrophilic, nucleophilic or radical mechanisms.[26,27] Anti-Markovnikov addition is generally observed in radical reactions, while electrophilic addition in the presence of a proton catalyst follows Markovnikov's rule, but there are exceptions in each category. We conclude that addition of *p*-fluorothiophenol most probably involves a radical mechanism, while other thiol and oxygen nucleophiles previously studied[11,14–16] add *via* an electrophilic addition reaction mechanism. The different mechanism in the case of *p*-fluorothiophenol is supported by the fact that this reaction occurred under normal oxygenated conditions, and addition to both vinyl groups occurred. However, anti-Markovnikov addition due to steric effects of the bulky nucleophile, *via* an electrophilic addition mechanism cannot be ruled out; slow reaction at the *endo* vinyl group may occur due to the excellent nucleophile, *p*-fluorothiophenol, which is only weakly acidic and hence does not catalyse competitive rearrangement and degradation reactions of bilirubin **1**.

HSA-binding studies

Circular dichroism (CD) has been shown to be a powerful technique to study the interaction of bilirubin with HSA.[10,28,29] The role of HSA in binding and transporting bilirubin is to cause the molecule to unfold, breaking the hydrogen bonds and holding it in a tight, highly twisted orientation in a locus protected from the surrounding medium. There is an instantaneous association of bilirubin with HSA at the primary binding site, and then a series of relaxation processes as the final configuration is attained.[29] It is the rotation of the two dipyrrole halves relative to each other that appears to be occurring during the slow relaxation processes, and it is this rotation that causes the chirality observed by CD.[29]

Fig. 1 shows the molar dichroic absorption spectrum of bilirubin **1** and disubstituted derivative **2** added to solutions of HSA. Due to the limited aqueous solubility of derivative **2**, DMSO was used as a carrier solvent to study the interaction of the derivative **2** with HSA,[10] and for comparison, the spectrum of bilirubin **1** was measured under the same conditions. The presence of DMSO does not alter the maxima or minima values of the binding. In the case of bilirubin **1**, the CD spectrum is characterised by two oppositely signed Cotton effects, at 465 and 410 nm (Fig. 1, dashed line). Derivative **2** showed a similar CD spectrum (Fig. 1, solid line), but with slightly shifted peaks at 445 and 395 nm and reduced signal intensities.

Although it is known that the primary binding site of bilirubin involves residues 198–308, on the second subdomain of HSA, the exact binding site is unknown.[29] Proposals for the exact primary binding site have been suggested, including Lys-240 and Arg-222.[29] A number of monosubstituted derivatives of bilirubin have been shown to interact with HSA.[13] The fact that a CD spectrum was observed with derivative **2**, despite the two rather bulky groups substituted at the 3- and 18-vinyl positions, shows that stabilisation of one enantiomeric form of **2** is occurring on interaction with the protein.

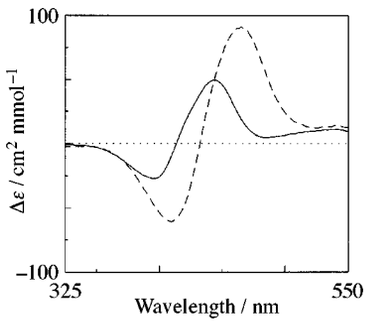


Fig. 1 Circular dichroism spectra obtained on addition of 5% DMSO–10% NaOH (0.02 M) water solutions of bilirubin **1** (1.7 mM) (dashed line) and derivative **2** (2.4 mM) (solid line) to a solution of HSA (0.4 mM) in 50 mM phosphate buffer at pH 7.3

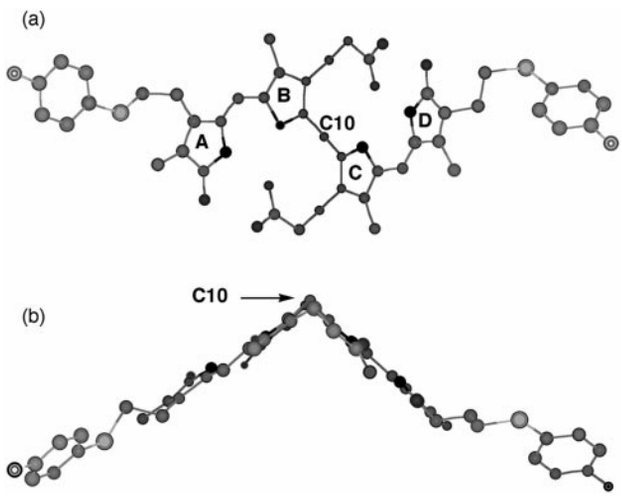


Fig. 2 3D model of the 'ridge-tile' conformation of **2**, generated from X-ray coordinates of **1** showing (a) folded conformation with *p*-fluorothiophenyl substituents attached to rings A and D (b) bent shape of the molecule and methylene bridge at C10; only one enantiomer shown, hydrogen atoms omitted for clarity

Fig. 2 shows a three-dimensional representation of the disubstituted derivative **2** in the 'ridge-tile' conformation generated from the X-ray coordinates of bilirubin.[6] The CD data support a previous hypothesis[9] for the methylene bridge of bilirubin pointing into the binding pocket of HSA as incorporation of the two bulky groups at the 3 and 18 positions of bilirubin does not impede the interaction of the derivative with HSA. These results suggest that considerable bulk may be incorporated at these positions without preventing interaction with HSA, as this still allows the methylene bridge of bilirubin, that connects rings C and D, to insert into the binding pocket of HSA. The crystal structure coordinates of HSA[30] provides the opportunity to model this interaction and provide further insight into the nature of the bilirubin–HSA complex.

Experimental

General

Bilirubin was purchased from ICN Biochemicals and was used without further purification. No other isomers or biliverdin impurities were detected by NMR spectroscopy or thin layer chromatography. Deoxygenation of all solvents and reagents was carried out by saturation with nitrogen by several cycles of vacuum at room temperature followed by nitrogen saturation. Water was purified using a Millipore Alpha-Q filter and purifying system. Chloroform was deacidified immediately prior to use by treatment with potassium carbonate. Ultraviolet (UV) spectra were recorded on an Hitachi 150-20 spectrophotometer using a 1.0 cm cell. Infrared (IR) spectra were recorded on a BIO RAD FTS-7 spectrometer. ^{1}H NMR spectra were recorded

on a Bruker AMX 400 or an AC 200 NMR spectrometer. Spectra were recorded in the solvent indicated, locked on solvent deuterium and referenced to TMS. *J* Values are given in Hz. ^{19}F NMR spectra were recorded on a Bruker AMX 400 spectrometer at 376.54 MHz and were referenced to fluorobenzene. Electron ionisation mass spectra (EI MS) were obtained on an A.E.I. MS-902 spectrometer at 70 eV. High resolution mass spectra were recorded on a LSIMS at The Central Services Laboratory, Tasmania. Thin layer chromatography was carried out using silica gel aluminium sheets $60F_{254}$ (0.25 mm thick) precoated UV sensitive plate. Melting points were determined using a hot plate microscope and are uncorrected.

3,18-Didevinyl-3,18-bis[2-(*p*-fluorothiophenyl)ethyl] bilirubin 2

Three crystals of toluene-*p*-sulfonic acid were added to a stirred solution of bilirubin **1** (50 mg, 85 mmol) and *p*-fluorothiophenol (4.5 cm^3, 42.2 mmol) in chloroform (60 cm^3). The mixture was left under nitrogen at room temperature in the dark for 24 h. A stream of nitrogen was passed over the reaction mixture to remove the solvent and methanol (100 cm^3) was added to the crude dark burgundy coloured oil. The precipitate was filtered and washed with methanol (200 cm^3) and dried under high vacuum for 4 h to give 3,18-didevinyl-3,18-bis[2-(*p*-fluorothiophenyl)ethyl] bilirubin **2** as an orange solid (53 mg, 73%), mp (decomp.) 253 °C; R_f (MeOH–C_6H_6–$CHCl_3$ 1.5 : 100 : 50) 0.2; (MeOH–C_6H_6–$CHCl_3$ 1 : 10 : 5) 0.76; δ_H(400 MHz, d_6-DMSO) 1.84 (s, 2-CH_3), 2.00 (s, 7-CH_3), 2.06 (m, CH_2CH_2COOH), 2.11 (s, 13-CH_3 and 17-CH_3), 2.51 (m, CH_2CH_2COOH), 2.67 (t, *J* 7.2, 17-CH_2CH_2S-), 2.86 (t, *J* 7.2, 3-CH_2CH_2S-), 3.19 (t, *J* 7.2, 3 and 17-CH_2CH_2S-), 4.06 (s, 10-CH_2), 5.89 (s, H5), 6.08 (s, H15), 7.28 (t, *J* 8.8, H *ortho* to S), 7.51–7.58 (m, H *ortho* to F), 9.94 (s, H24), 9.96 (s, H21), 10.46 (br s, H22, 23), 12.02 (br s, 2 × COOH); δ_F(376.54 MHz, $CDCl_3$) 2.3 (s), 3.8 (s); *m*/*z* (CI) 841 (M^+, 100%), 429 (18), 415 (44), 129 (56) (Calc. $C_{45}H_{46}F_2N_4O_6S_2$: 840.2826. Found: 840.2815); ν_{max}(KBr)/cm^{-1} 3415, 3265, 1689, 1641, 1618; λ_{max}($CHCl_3$)/nm 442 (ε/M^{-1} cm^{-1} 65 250).

Circular dichroism

Circular dichroism measurements were recorded on a Jasco J-710 spectropolarimeter. The instrument was calibrated using a standard solution of aqueous ammonium D-camphor-10-sulfonate (0.6 mg cm^{-3}) in a 1 cm cell (CD = 190.4 mdeg at 290.5 nm). Spectra were recorded using a 0.1 cm cell and the following parameters; range 300–600 nm, accumulation 10 scans, temperature 20 °C, step 0.5 nm, speed 50 nm min^{-1}, response 2 s and bandwidth 1.0 nm.

Human serum albumin (HSA), Fraction V was purchased from Sigma and was defatted and dialysed (Selby type 453103 dialysis tubing) according to the literature procedure.[31] Protein concentration was determined by UV spectroscopy (extinction coefficient of 36 500 M^{-1} cm^{-1}). pH Measurements were made using a Beckman F 11 meter and a Mettler NMR tube pH probe.

Bilirubin **1** (1 mg, 1.7 mmol) was dissolved in sodium hydroxide (0.02 M, 0.5 cm^3) and diluted with water (9 cm^3) in the dark at room temperature. This solution (7.5 cm^3) was added to HSA (2 mL, 0.4 mM) and the final pH was adjusted to 7.3. CD measurements were recorded immediately after the addition. For experiments requiring DMSO as a carrier solvent, derivative **2** (1–2 mg) was dissolved in DMSO (0.5 cm^3) and aqueous sodium hydroxide (0.02 M, 0.5 cm^3) was slowly added with stirring. The solution was diluted with water (9 cm^3) in the dark at room temperature. This solution of **2** (7.5 cm^3) was added to HSA (2 cm^3, 0.4 mM) and the final pH was adjusted to 7.3. CD measurements were recorded immediately after the addition. Reference spectra of HSA and bilirubin were recorded and showed no CD signals.

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