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THE SYNTHESIS OF [10-^{13}C]BILIRUBIN IXα

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SUMMARY

The total synthesis of [10-^{13}C]bilirubin IXα , the principal waste product of haem degradation, is described. Site specific labelling was accomplished by the Vilsmeier formylation of one of the dipyrrolic fragments using [1-^{13}C]dimethylformamide. The penultimate dehydrohalogenation reaction was complicated by a competing elimination reaction which yielded a bridged biliverdin derivative. [10-^{13}C]bilirubin IXα was prepared in an overall yield of 6 % (from the step where ^{13}C was introduced) with quantitative isotopic incorporation.

Key words: [10-^{13}C]bilirubin IXα; [10-^{13}C]biliverdin IXα; ^{13}C labelling; labelled bile pigment.

INTRODUCTION

Bilirubin IXα, the major product of haem degradation is poorly excreted in the newborn and accumulation may lead to damage of the central nervous system. In an effort to elucidate the pathophysiological mechanisms involved in various disorders of bilirubin metabolism, numerous studies have been carried out involving the labelling of bilirubin and its use in tracer experiments.[1,2,3] The labelling procedures which have been used include the partial[4] and total synthesis[5] of the tetrapyrrole as well as the biosynthetic approaches,[6,7] using labelled haem precursors. To date kinetic studies have used radiolabelled bilirubin and for ethical reasons these have not involved neonates. A stable non-radioactive isotope was required for this purpose. We have thus synthesised[10-^{13}C]bilirubin using a convergent route adapted from previously published work.[8]

DISCUSSION

Although the synthesis of bilirubin has been reported by various groups, only Wray *et al.*[9] have prepared a ^{13}C-enriched bilirubin. In their work, [^{13}C]mesobilirubin and its derivatives were used for the unequivocal assignment of the quaternary pyrrole carbons of their ^{13}C NMR spectra. For the preparation of unsymmetrically substituted biliverdins and bilirubins the approach *via* di-*t*-butyl *b*-bilene-1,19-dicarboxylates is efficient and can readily be adapted for the insertion of an isotopic label.

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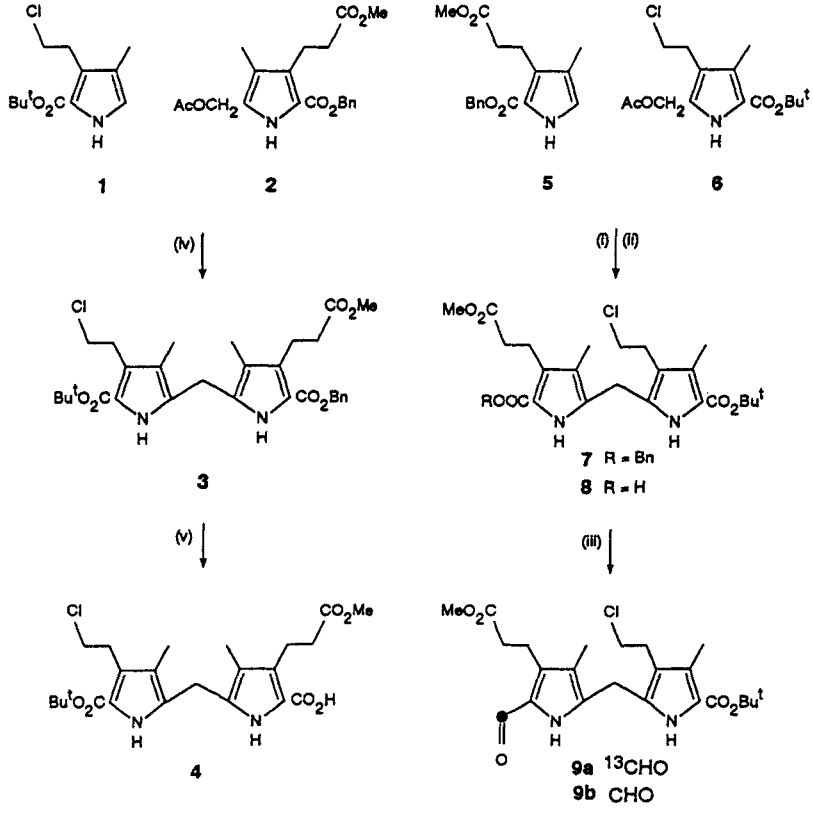
The pyrroles (1,2,5 and 6) required for the syntheses of the respective dipyrromethane intermediates (3, 7) were prepared using reported synthetic routes.[8] In each case synthesis of the dipyrromethanes by the condensation of a 5-acetoxymethyl pyrrole and a 5-unsubstituted pyrrole gave the desired product (3,7). This condensation reaction had previously been effected using acetic acid containing a catalytic quantity of *p*-toluenesulphonic acid.[8] More recent work has exploited the advantages of the aluminosilicate, montmorillonite.[10,11] We used a nickel substituted synthetic-mica montmorillonite (SMM). The incorporation of nickel results in a significant increase in stable surface area of the catalyst. This is in contrast to the loss of surface area when SMM is impregnated with metal salts.[12] We found the NiSMM to be more efficient than the commercially available Montmorillonite K10. Mild calcination of the NiSMM results in deamination and converts the material to the Bronsted acid form. However, calcination at higher temperatures converted most of the Bronsted acid sites in the octahedral layer to Lewis acid sites and in this state the catalyst was not as effective.



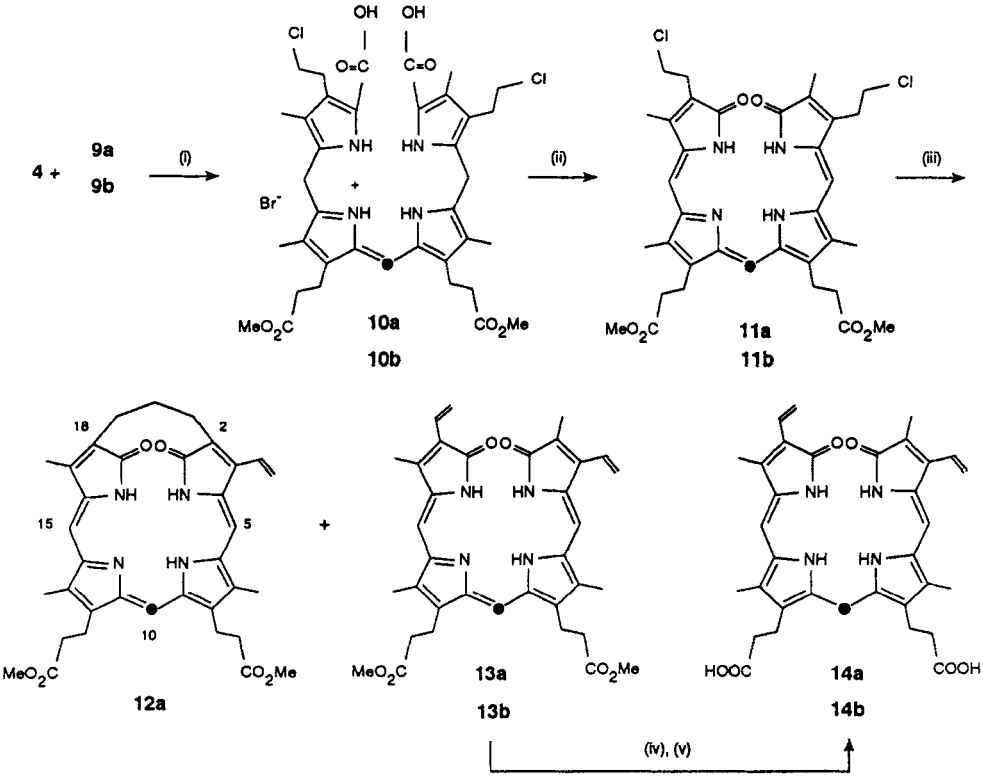
Scheme 1 (i) & (iv) NiSMM, CH_2Cl_2 (ii) & (v) Pd-C, Et_3N, THF (iii) CH_2Cl_2, $POCl_3$, [1-^{13}C]DMF.

The acetoxymethyl pyrrole (2) was added in small portions (to prevent any self condensation) to the unsubstituted pyrrole (1) and two mass equivalents of NiSMM in dichloromethane (scheme 1). After 1 h at 20°C the aluminosilicate was filtered off to give

the dipyrromethane (**3**) in nearly quantitative yield. The molecular ion of M^+ 566 together with the NMR data was in accordance with that previously reported. The dipyrromethane (**3**) was catalytically debenzylated to give (**4**). The reaction was monitored using TLC and the deprotected product (**4**) was used immediately in the synthesis of the *b*-bilene (**10**), owing to its lability. In an analogous procedure coupling of pyrroles (**5**) and (**6**) gave the dipyrromethane (**7**) in 83% yield. Hydrogenolysis of the dipyrromethane (**7**) afforded the dipyrromethane carboxylic acid (**8**).

The key step for our purposes was the subsequent decarboxylation–formylation of the dipyrromethane (**8**) with 1-^{13}C-dimethylformamide in order to incorporate a ^{13}C-label. This reaction afforded the 5-formyldipyrromethane (**9a**) in 62% yield. The NMR spectrum of ^{13}C-formyl dipyrromethane (**9a**) showed a doublet for the formyl proton with a chemical shift which was consistent with that of the unlabelled equivalent. The large coupling constant of 170.2 Hz is in accordance with the theoretical value predicted for ^{1}H-^{13}C coupling.[13]

Condensation of the dipyrromethanes (**4** and **9**) (scheme 2) in the presence of *p*-toluenesulphonic acid gave the *b*-bilene hydrobromide (**10**) after counterion exchange using hydrogen bromide gas. The spectral data of (**10b**) were identical to that reported;[8] however,



Scheme 2 Series (a) refers to labelled (10-^{13}C) and series (b) to unlabelled compounds.
(i) TsOH, CH_3OH, CH_2Cl_2 (ii) TFA, Br_2, N_2 (iii) C_5H_6N, NaOH (iv) CH_3OH, 1M NaOH, EDTA (v) $NaBH_3CN$, C_2H_5OH

the ^{13}C enriched compound (**10a**) gave a doublet at δ 7.6 (*J* 160.4 Hz) for the C-10 proton. Treatment of the hydrobromide (**10a**) with a slight excess of bromine in trifluoroacetic acid, afforded the [10-^{13}C]bis-(2-chloroethyl)-biliverdin (**11a**). Once again the NMR spectrum of (**11a**) only differed from that of the unlabelled material in so far as the C-10 methine proton showed a doublet at δ 6.75 (*J* 155.5 Hz). The field desorption mass spectrum (FDMS) showed a molecular ion of 683 and that the ^{13}C enrichment was in excess of 99.5%. It is essential to exclude oxygen from the reaction mixture to obtain the quoted yields of the biliverdin (**11**). In the presence of oxygen the major product was purple in colour. Smith and Kishore[14] established that this was a bilipurin compound. This type of reaction would undoubtedly be due to formation of the biliverdin cation-radical (with bromine as oxidant) and further reaction and fragmentation with oxygen.[15,16]

The penultimate step in the synthesis entailed formation of biliverdin IXα dimethyl ester (**13**). Initial attempts to carry out the dehydrochlorination of (**11**) using pyridine and aqueous potassium hydroxide at 115°C gave poor yields and the desired product was isolated using multiple development preparative layer chromatography (PLC). Most of the starting material was converted to an intractable highly polar by-product. Lower temperatures and shorter reaction times resulted in incomplete elimination of the chloroethyl substituents. The presence of products, impurity and starting material was an additional complication with respect to chromatography and characterisation. A further disadvantage of the use of aqueous pyridine is the problem of monitoring the reaction due to the simultaneous hydrolysis of the methyl esters

In the light of the foregoing problem the use of non-nucleophilic bases such as 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and 1,5-diazabicyclo[4,3.0]non-5-ene (DBN) was considered. Treatment of the β and δ isomers of the bischloroethyl biliverdin (**11**) with DBU gave the extended biliverdins of the neopterobilin type (by nucleophilic attack of the pyrrole nitrogen) in good yield.[17] The position of the α-isomer's C(4)-C(5) and C(15)-C(16) double bonds as well as the the *Z-syn* conformation at C-5 and C-15 precludes any intramolecular alkylation and we thought this would be a propitious approach for the preparation of biliverdin IXα. The bischloroethyl biliverdin (**11**) was treated with DBU at room temperature and the reaction monitored using normal phase HPLC. Reaction products from incomplete dehydrohalogenation were not sufficiently resolved for the application of a semi-preparative HPLC procedure. Similar results were obtained when DBN was used and again the reaction products were not isolated.

Owing to the difficult purification of the reaction products from the DBU or DBN dehydrohalogenation, we reverted to the original method described in the literature.[8] When the reaction was scaled up using aqueous sodium hydroxide in pyridine, the 400 MHz NMR spectrum of the product, eluting as a single band on PLC, revealed the presence of a second reaction product. The two compounds were resolved by means of HPLC.

The less polar fraction was easily identified as the desired biliverdin IXα dimethyl ester (**13a**) by its retention time as well as its spectroscopic data. A doublet at δ 6.78 was observed for the C-10 proton with a coupling of 156.87 Hz. The FDMS showed a molecular ion of 611 consistent with the molecular formula $^{13}C_1C_{34}H_{38}N_4O_6$.

The more polar compound proved to be more complicated. The proton NMR spectrum showed the disappearance of the high field C-2 methyl singlet at δ 1.86. Only one vinyl group was observed implicating the other chloroethyl in some unexpected side reaction. Three high field signals which together integrated for six protons suggested the presence of three methylene groups. The ^{13}C spectrum was very similar to that of biliverdin IXa dimethyl ester (**13a**) but lacking one methyl carbon and only one set of vinylic carbons was present. Three signals at 23.08, 23.46 and 30.15 ppm provided confirmatory evidence that the unassigned signals in the proton NMR were in fact six methylene protons. The high resolution mass spectrum showed a molecular ion of 611. On the basis of the above evidence a 2,18-bridged biliverdin derivative (**12a**) (scheme 2) was proposed. The COSY as well as the HETCOR spectrum showed connectivities which confirmed the presence of three methylene groups of the propano bridge. However, no long range coupling between the bridge protons and the substituents of rings A and D was apparent from the spectra.

The NOE data, X-ray crystallographic structure and proposed mechanism for the formation of the bridged biliverdin derivative (**12a**) are described in a recent communication.[18]

The final step in the total synthesis necessitated the saponification and reduction of biliverdin dimethyl ester (**13**) to give bilirubin IXα (**14**). Earlier methods[19,20] involved sodium borohydride reduction to the intermediate bilirubin ester followed by the hydrolysis of the ester to give the free acid. More recently sodium cyanoborohydride has been used for the reduction of bilirubin IXα and its isomers.[21] The steric and electronic effects of the cyano substituent greatly influence the reactivity of the borohydride ion. Thus sodium cyanoborohydride with its strongly electron-withdrawing group is a milder and more selective reducing agent than sodium borohydride. Also, the stability of sodium cyanoborohydride in protic solvents at low pH permits reductions to be carried out under conditions that would rapidly hydrolyse sodium borohydride. When the saponification of the biliverdin esters was carried out prior to the reduction overall yields of 90-92% were obtained on a 500-800 nmol scale. This procedure obviates the isolation and storage of the photolabile bilirubin ester which may undergo disproportionation[22] and provides a more favourable route to bilirubin isomers in the acid-free form. Thus the biliverdin dimethyl ester (**13**) was treated with 1M NaOH containing 1mM EDTA at 37°C for 30 min. Sodium cyanoborohydride was used for the virtually quantitative reduction of the free acid to bilirubin IXα (**14**). Extreme care was taken to exclude oxygen from the reaction mixtures by purging with argon. The 400 MHz proton NMR of [10-^{13}C]bilirubin (**14a**) showed a doublet with a coupling constant of 151.1 Hz for the 10-CH_2. This was consistent with the signals observed for the ^{13}C-verdin intermediates. The other spectral assignments were in accordance with previously published data.[23] A weak molecular ion at 585 was observed in the electron impact mass spectrum. The spectrum showed an intense pair of dipyrrolic fragment ions at m/e 286 and 300 arising from cleavage at the central methylene bridge.

The total synthesis of [10-^{13}C]bilirubin was carried out and the label was introduced prior to the coupling of the dipyrromethanes using [1-^{13}C]dimethylformamide. The poor yield of 6 % (with respect to the step at which the ^{13}C was introduced) can be accounted for by the unexpected and unprecedented competing elimination reaction which was

encountered in the penultimate step. For these reasons this route would not not seem to be the most propitious approach and the use of phenylselenylethyl groups as protected vinyl substituents[24,25] may well be more appropriate. The novel bridged biliverdin derivative which was isolated and characterised is the first naturally occurring IXα isomer in which the 2- and 18- positions are bridged with a propano-tether. Recently there has been a lot of interest in bridged biliverdins as model compounds for studies of the relationship between basicity and conformation in biliverdins.[26,27]

ACKNOWLEDGEMENTS

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EXPERIMENTAL

All reagents, including the [1-^{13}C]DMF, were obtained from Aldrich Chemical Company (except where noted). Melting points were determined on a Reichert-Jung hot-stage apparatus and are quoted uncorrected. NMR spectra were recorded on a Varian VXR-200 (200 MHz spectrometer), or a Varian Unity (400 MHz spectrometer), for solutions in deuteriochloroform, unless otherwise specified. Tetramethylsilane (TMS) was used as internal standard. The chemical shifts (δ) are given in ppm relative to TMS (δ 0.00). Mass spectra were recorded on a VG Micromass 16 F mass spectrometer (operating at 70 eV with an accelerating voltage of 4 kV). UV spectra were obtained on a Hitachi U-3200 spectrophotometer. All reactions were monitored by thin layer chromatography (TLC) using Merck F^{254} precoated silica gel plates. Column chromatography was carried out on slurry-packed columns using silica gel (Kieselgel 60, Merck).

Benzyl 9-*t*-butoxycarbonyl-8-(2-chloroethyl)-2-(2-methoxycarbonylethyl)-3,7-dimethyldipyrromethane-1-carboxylate (3) Benzyl 5-acetoxymethyl-3-(2-methoxycarbonylethyl)-4-methylpyrrole-2-carboxylate (**2**)[8] (300 mg, 1.23 mmol) was added, in small portions, to a well stirred solution of *t*-butyl 3-(2-chloroethyl)-4-methylpyrrole-2-carboxylate (**1**)[28] (420 mg, 1.13 mmol) in dichloromethane (30 ml) containing Ni-SMM (1.8 g). The Ni SMM was calcined at 300°C for 16 h prior to use The reaction was monitored by TLC and after 1 h the Ni-SMM was filtered off and washed with dichloromethane. The combined filtrates were evaporated to dryness and the residue was purified on a silica gel column [elution with ethyl acetate-hexane (1:4)]. The dipyrromethane (**3**) was obtained after recrystallisation from dichloromethane-hexane (0.52 g, 83%), m.p. 109°C (lit.,[8] m.p. 110°C); δ 1.52 (9H, s, *t*-Bu), 1.95 and 1.97 (each 3H, s, 3- and 7-Me), 2.46 and 3.00 (each 2H, t, *J* 7.6 Hz, 2^1- and 2^2-CH), 3.10 and 3.54 (each 2H, t, *J* 7.8 Hz, 8^1- and 8^2-CH), 3.60 (3H, s, OMe), 3.81 (2H, s, 5-CH_2), 5.24 (2H, s, $CH_2C_6H_5$), 7.31 (5H, m, C_6H_5), 9.20 and 9.48 (each 1H, br s, NH); (Found: M^+, 556. $C_{30}H_{37}ClN_2O_6$ requires *M* 556).

9-*t*-Butoxycarbonyl-8-(2-chloroethyl)-2-(2-methoxycarbonylethyl)-3,7-dimethyldipyrromethane-1-carboxylic acid (4) The foregoing dipyrromethane (**3**) (100 mg, 0,179 mmol) in tetrahydrofuran (6.3 ml) and triethylamine (7 ml) was hydrogenated

over 10% palladium on carbon (11 mg) at room temperature until uptake of hydrogen ceased. The catalyst was removed by filtration through Celite. The filtrate was evaporated and the residue was purified by flash chromatography [elution with ethyl acetate-hexane (2:3)] to yield the dipyrromethane carboxylic acid (4). The dipyrromethane (4) could not be crystallised and was precipitated out as an amorphous powder (79 mg, 95 %) which was used immediately in the *b*-bilene synthesis due to its lability.

Benzyl 9-*t*-butoxycarbonyl-7-(2-chloroethyl)-2-(2-methoxycarbonylethyl)-3,8-dimethyldipyrromethane-1-carboxylate (7) *t*-Butyl 5-acetoxymethyl-4-(2-chloroethyl)-3-methylpyrrole-2-carboxylate (6)[8] (500 mg, 1.58 mmol) was added to a mixture of benzyl 3-(2-methoxycarbonylethyl)-4-methylpyrrole-2-carboxylate(5)[8] (514 mg, 1.71 mmol) and Ni-SMM (1.9 g) in dichloromethane (30 ml), as described for the synhtesis of dipyrromethane (3). Chromatography on a silica gel column [elution with ethyl acetate-hexane (1:4)] and evaporation of the eluate gave an oil (831 mg, 94%) which could not be induced to crystallise. δ 1.5 (9H, s, *t*-Bu), 1.98 and 2.23 (each 3H, s, 3- and 8-Me), 2.46 and 3.01 (each 2H, t, *J* 7.6 Hz, 2^1- and 2^2-CH_2), 2.82 and 3.40 (each 2H, t, *J* 7.4 Hz, 7^1- and 7^2-CH_2), 3.86 (2H, s, 5-CH_2), 5.24 (2H, s, $CH_2C_6H_5$), 7.30 (5H, m, C_6H_5), and 9.20 and 9.48 (each 1H, br s, NH); (Found: M^+, 556. $C_{30}H_{37}ClN_2O_6$ requires *M* 556).

9-*t*-Butoxycarbonyl-7-(2-chloroethyl)-2-(2-methoxycarbonylethyl)-3,8-dimethylpyrromethane-1-carboxylic acid (8) The dipyrromethane (7) (100 mg, 0.214 mmol) in dry tetrahydrofuran (3.3 ml) containing triethylamine (7 ml) and 10% palladised charcoal (1.0 mg) was hydrogenated at room temperature as described for the preparation of (4). The filtrate was evaporated and the residue was purified by flash chromatography [elution with ethyl acetate-hexane (2:3)] to yield the dipyrromethane carboxylic acid (7) (66.6 mg, 67%) which was used directly in the next reaction.

***t*-Butyl 3-(2-chloroethyl)-9-formyl-8-(2-methoxycarbonylethyl)-2,7-dimethyldipyrromethane-1-carboxylate (9)** (a) The dipyrromethane carboxylic acid (8) (653 mg, 1.40 mmol) in dry dichloromethane (27 ml) was stirred with *p*-toluenesulphonic acid monohydrate (653 mg, 3.48 mmol) in dry methanol (13 ml) for 40 min at 22°C. The mixture was diluted with dichloromethane (65 ml) and was washed with 2% aqueous sodium carbonate then water and dried ($MgSO_4$). The solvent was removed under reduced pressure and the resulting oil was dissolved in dry dichloromethane (10 ml) and added dropwise to a suspension, at 0°C, of calcium carbonate (1.3 g) in dichloromethane (3.3 ml) containing the Vilsmeier complex [obtained from phosphoryl chloride (1.3 ml) and dimethylformamide (1.0 ml, 12.9 mmol)]. After complete addition the mixture was stirred at 20°C for 1.5 h and then aqueous sodium acetate was added (to pH 7). The mixture was then stirred vigorously overnight. Aqueous sodium acetate was added (to pH 8) and after standing for 10 min the mixture was filtered to remove calcium carbonate. The organic phase was evaporated, washed with water (2 x 4 ml) and then dried with magnesium sulphate and evaporated under reduced pressure. Chromatography on silica gel [eluant ethyl acetate-hexane 3:7)] gave t-*butyl* 3-(2-*chloroethyl*)-9-([9^1-^{13}*C*]*formyl*)-8-(2-*methoxycarbonylethyl*)-2,7-*dimethyldipyrromethane*-1-*carboxylate* (**9a**) (391 mg, 62%), m.p. 148-150°C ; δ 1.50 (9H, s,

t-Bu), 2.06 and 2.22 (each 3H, s, 2- and 7-Me) 2.58 and 3.03 (each 2H, t, *J* 7.8 Hz, 3^1- and 3^2-CH_2), 2.88 and 3.40 (each 2H, t, *J* 7.7 Hz, 8^1- and 8^2-CH_2), 3.67 (3H, s, OMe), 3.95 (2H, s, 5-CH_2), 9.55 (1H, d, *J* 170.2 Hz, 9^1-CHO), and 9.93 and 10.88 (each 1H, br s, NH); (Found: M^+, 451. $C_{22}{}^{13}CH_{31}ClN_2O_5$ requires *M* 451).

(b) The formyl dipyrrylmethane (**9b**) was similarly prepared from the dipyrromethane carboxylic acid (**8**) (60 mg, 0.13 mmol) by treatment with *p*-toluenesulphonic acid hydrate (60 mg, 0.32 mmol) in dry methanol (1.2 ml). The decarboxylated dipyrromethane was then formylated using phosphoryl chloride (0.12 ml) and dimethylformamide (92 µl, 1.19 mmol). The product was recrystallised from dichloromethane-hexane to give the dipyrromethane (**9b**) (32.8 mg, 56%), m.p. 147-148°C (lit.,[8] m.p. 148-149°C); δ 1.47 (9H, s, *t*-Bu), 2.02 and 2.19 (each 3H, s, 2- and 7-Me), 2.54 and 3.0 (each 2H, t, *J* 7.8 Hz, 8^1- and 8^2-CH_2), 2.84 and 3.37 (each 2H, t, *J* 7.7 Hz, 3^1- and 3^2-CH_2), 3.63 (3H, s, OMe), 3.91 (2H, s, 5-CH_2), 9.51 (1H, s, 9^1-CHO), and 9.79 and 10.68 (each 1H, br s, NH); (Found: M^+, 450. $C_{23}H_{31}ClN_2O_5$ requires *M* 450).

3,18-Bis-(2-chloroethyl)-8,12-bis-(2-methoxycarbonylethyl)-2,7,13,17-tetramethyl-*b*-bilene-1,19-dicarboxylic acid hydrobromide (10) (a) The formyl dipyrromethane (**9a**) (74.5 mg, 0.165 mmol) and the dipyrromethane carboxylic acid (**4**) (76.5 mg, 0.164 mmol) in dichloromethane (3.8 ml) were treated with *p*-toluenesulphonic acid hydrate (168 mg) in methanol (6.9 ml) and stirred for 18 h at 19 °C under nitrogen. Dichloromethane was added, the mixture was washed with 0.2 M sodium carbonate (18 ml) and then water. The organic phase was dried ($MgSO_4$) and evaporated to dryness. The residue was dissolved in dichloromethane (6.5 ml) and hydrogen bromide gas (generated from bromine and 1,2,3,4-tetrahydro naphthalene) was bubbled through the solution for 5 sec (colour: amber to red) before the solvent was rapidly evaporated, the residue was dissolved in dry benzene and evaporated to dryness (twice). The [*10-*^{13}*C*]3,18-*bis*-(2-*chloroethyl*)-8,12-*bis*-(2-*methoxycarbonylethyl*)-2,7,13,17-*tetramethyl*-b-*bilene*-1,19-*dicarboxylic acid hydrobromide* (**10a**) was carried through in this state to the oxidation reaction. δ 2.02, 2.06, 2.08, and 2.25 (each 3H, s, 2-, 7-, 13- and 17-Me), 2.48-3.52 (16H, m, 3^1-, 3^2-, 8^1-, 8^2-, 12^1-, 12^2-, 18^1- and 18^1-CH_2), 3.62 (6H, s, 2 x OMe), 4.31 and 4.35 (each 2H, s, 5- and 15-CH_2), 7.60 (1H, d, *J* 160.4 Hz, 10-H), 10.2, 10.31, 13.19, and 13.32 (each 1H, br s, NH).

(b) Condensation of the formyl dipyrromethane (**9b**) (79.3 mg, 0.176 mmol) and the dipyrromethane dicarboxylic acid (**4**) (81.6 mg, 0.175 mmol) followed by treatment with hydrogen bromide gas using the same methodology as that described above gave of the crude hydrobromide (**10b**) (170 mg). δ 2.03-2.23 (12H, s, 2-, 7-, 13- and 17-Me), 2.45-3.50 (16H, m, 3^1-, 3^2-, 8^1-, 8^2-, 12^1-, 12^2-, 18^1- and 18^1-CH), 3.60 (6H, s, 2 x OMe), 4.30 and 4.34 (each 2H, s, 5- and 15-CH_2), 7.60 (1H, s, 10-H), 10.24, 10.36, 12.99, and 13.15 (each 1H, br s, NH).

3,18-Bis-(2-chloroethyl)-8,12-bis-(2-methoxycarbonylethyl)-2,7,13,17-tetramethylbilin-1,19(21*H*,24*H*)-dione (11) (a) b-Bilene hydrobromide (**10a**) (107.5 mg) was added to trifluoroacetic acid (degassed with nitrogen) (19 ml) and the mixture was stirred at 22°C for 15 min under nitrogen. The solution was cooled to 0-5°C before the addition of bromine (28

μl). After stirring for a further 1.5 h at 0-5°C the solution was poured over solid sodium hydrogen carbonate (21.3 g) and dichloromethane (14 ml) was added. Water (14 ml) was added and the organic layer was separated, dried ($MgSO_4$) and evaporated to give a blue residue which was chromatographed on silica gel thick layer plates [elution with chloroform-acetone (95:5)]. The major blue band was scraped off and the bis-(2-chloroethyl)-biliverdin (**11a**) was extracted into chloroform. Crystallisation from dichloromethane-hexane gave the *[10-^{13}C]*-3,18-*bis*-(2-*chloroethyl*)-8,12-*bis*-(2-*methoxycarbonylethyl*)-2,7,13,17-*tetramethylbilin*-1,19(21H,24H)-*dione* (**11a**) [64.6 mg, 57% with respect to the formyl dipyrromethane (**3**)], m.p.232-234°C; λ_{max}/nm 372 (ε 44 900) and 655 (12 200); δ 1.77, 2.08, 2.09, and 2.13 (each 3H, s, 2-, 7-, 13- and 17-Me), 2.50-3.64 (16H, m, 3^1-, 3^2-, 8^1-, 8^2-, 12^1-, 12^2-, 18^1- and 18^1-CH_2), 3.70 (6H, s, 2 x OMe), 5.87, 5.90 (each 1H, s, 5- and 15-CH), 6.75 (1H, d *J*155.53 Hz, 10-H), and 8.27 (3H, br s, NH); (Found: M^+, 683. $C_{34}{}^{13}CH_{40}Cl_2N_4O_6$ requires *M* 683)

(b) *b*-Bilene hydrobromide (**10b**) (107.5 mg) was treated with trifluoroacetic acid and bromine in the same manner as the preceding synthesis. The bis-chloroethyl biliverdin (**11b**) was crystallised from dichloromethane-hexane (97.2 mg, 54%), m.p. 233-235°C (lit., m.p.[8] 234-235°C); λ_{max}/nm 374 (ε 43 400) and 650 (11 200); δ 1.85, 2.07, 2.09, and 2.12 (each 3H, s, 2-, 7-, 13- and 17-Me), 2.48-3.65 (16H, m, 3^1-, 3^2-, 8^1-, 8^2-, 12^1-, 12^2-, 18^1- and 18^1-CH_2), 3.70 (6H, s, 2 x OMe), 5.87, 5.90, and 6.74 (each 1H, s, 5-, 15- and 10-H), and 8.50 (3H, br s, NH); (Found: M^+, 682. $C_{35}H_{40}Cl_2N_4O_6$ requires *M* 682).

8,12-Bis-(2-methoxycarbonylethyl)-2,7,13,17-tetramethyl-3,18-divinylbilin-1,19(21*H*,24*H*)-dione (biliverdin IXα dimethyl ester) (13) (a) The [10-^{13}C]3,18-bis-(2-chloroethyl) biliverdin (**11a**) (27 mg, 39.5 mmol) was heated at 115°C in degassed pyridine (21 ml) under argon for 15 min before addition of degassed aqueous sodium hydroxide (3%; 2.1 ml). The mixture was heated under reflux for a further 2 h and then cooled and treated with acetic acid (25 %; 3.6 ml). The solvent was evaporated using toluene to remove the residual traces of solvent by azeotropic distillation. Water (36 ml) was added and the precipitate was removed by filtration through Celite, washed with water and dried under reduced pressure. The blue-green residue together with the Celite was treated with 5% H_2SO_4 in methanol (14 ml) and stirred for 16 h. The Celite was removed by filtration and the filtrate was added to ice-cold aqueous sodium acetate, extracted with dichloromethane and the extract then washed with aqueous sodium hydrogen carbonate and water and dried ($MgSO_4$). The solvent was evaporated and the product was purified using preparative thick layer plates of silica gel (elution with methanol-chloroform [3:97]). The predominant band was rechromatographed using semi-preparative HPLC. The separation was performed at room temperature using a 10 μm Microporasil (125 Å) column (2.5 x 10 cm) and a RCM PrepPak 25 x 10 cartridge holder. The flow rate was 10 ml/min. The absorbance was measured at 375 nm. An isocratic solvent system of toluene-acetone-pyridine (95:4:1) was used. The compound which eluted first was not characterised due to insufficient material. The next fraction eluted was concentrated under reduced pressure to give *[10-^{13}C]*-8,12-*bis*-(2-*methoxycarbonylethyl*)-2,7,13,17-*tetramethyl*-3,18-*divinylbilin*-1,19(21H,24H)-*dione* (**13a**) (5 mg, 21 %), m.p.207-208°C from dichloromethane-hexane; λ_{max}/nm 380 (ε 48 200)

and 655 (14 900); δ (400 MHz) 1.86, 2,07, 2.10, and 2.17 (each 3H, s, 2-, 7-, 13- and 17-Me), 2.56 (4H, t, 12^2- and 18^2-CH_2) 3.93 (4H, t, 12^1- and 18^1-CH_2), 3.67 (6H, s, 2 x OMe), 6.00 and 6.06 (each 1H, s, 5- and 15-H), 6.78 (1*H*, d, *J* 155.64 Hz, 10-H), 5.62, 6.10, and 6.48 (3H, AMX, J_{AB} 1.5 Hz, J_{AX} 17.6 Hz, J_{BX} 11.6 Hz, 18-vinyl), 5.42, 5.66, and 6.61 (3H, ABX, J_{AB} 1.7 Hz, J_{AX} 17.6, J_{BX} 11.7 Hz, 3-vinyl), and 8.2 (1H, br s, NH); (Found: M^+, 611. $C_{34}{}^{13}CH_{38}N_4O_6$ requires *M*, 611) followed by [10-*[13]C*]-2,18-*propano*-8,12-*bis*-(2-*methoxycarbonylethyl*)-7,13,17-*trimethyl*-3-*vinylbilin*-1,19-*(*21H, 24H)-*dione* (**12a**) (6 mg, 24 %), m.p. 227-229°C (from dichloromethane-hexane); (λ_{max}/nm 310 (ε 28 000), 381 (37 000), and 642 (15 300); δ_H (300 MHz) 2.36, 2.39, and 2.40 (each 3H, s, 7-, 13- and 17-Me), 2.52, 2.78 and 2.93 (6H, m, 18^1-, 18^2- and 18^3-CH_2), 2.71 (4H, t, *J* 7.6 Hz, 12^1 and 18^1-CH_2), 3.23 and 3.24 (each 4H, t, *J* 7.6 Hz, 12^2 and 18^2-CH_2), 3.69 (6H, s, 2 x OMe), 5.11 (1H, s, NH), 5.36 (1H, s, NH), 5.74, 5.78, and 6.88 (3H, ABX, J_{AB} 1.6 Hz, J_{AX} 17.8 Hz, J_{BX} 11.6 Hz), 6.75 (1H, s, 15- H), 6.88 (1H, s, 5-H), 7.54 (1H, d, *J* 156.91 Hz, 10-H), 9.98 (1H, br s, NH); δ_C (300 MHz) 9.71, 9.74, and 10.43 (7-CH_3, 13-CH_3, and 17-CH_3), 20.37 (C-8^1 and C-12^1) 23.08, 23.46 and 30.15 (C-18^1, C-18^2, and C-18^3), 35.63 (C-8^2 and C-12^2), 51.77 (8^3 and 12^3 -OCH_3), 97.96 (C-5), 99.64 (C-15), 112.15 (C-10), 122.83 (C-3^2), 127.21 (C-3^1) 165.95 (C-1 and C-19), and 166.49 (8^3-C and 12^3-C)(Found: M^+ 611.284. $C_{34}{}^{13}CH_{38}N_4O_6$ requires *M*, 611.287).

(b) The-3,18-bis-(2-chloroethyl) biliverdin (**13b**) (10.5 mg, 15 mmol) was likewise treated with sodium hydroxide in pyridine. The product was purified using preparative thick layer plates [elution with dichloromethane-methanol (95:5)]. the biliverdin (**13b**) (4 mg, 44%), m.p.204-207°C (lit.,[31] m.p. 208-209°C); λ_{max}/nm 381 (ε 49 300) and 658 (15 100); δ 2.04, 2,07, 2.15, and 2.17 (each 3H, s, 2-, 7-, 13- and 17-Me), 2.56 and 3.93 (each 4H, t, 8^1-, 8^2-, 12^1-, 12^2-CH_2), 3.67 (6H, s, 2 x OMe), 5.97, 6.03, and 6.77 (each 1H, s, 5-, 10- and 15-H), 5.31-5.72 and 5.98-6.17 (4H, m, 3^2- and 18^2-CH_2), and 6.44-6.72 (2H, m, 3^1- and 18^1-H); (Found: M^+, 610. $C_{35}H_{38}N_4O_6$ requires *M* 610).

Dehydrochlorination using DBU/DBN The 3,18-bis-(2-chloroethyl) biliverdin (**11**) (8.7 mg, 12 mmol) was dissolved in deoxygenated dimethlformamide (3.5 ml). (DBU) (320 ml) was added and the reaction mixture was stirred at 25°C under nitrogen for 2 h. The reaction was quenched by the addition of dichloromethane (20 ml) and the organic phase was washed with water (3 x 15 ml), to remove the dimethylformamide, dried ($MgSO_4$) and evaporated to dryness. The products were chromatographed using HPLC. The separation was performed at room temperature using a 5 mm Nucleosil column (0.4 x 20 cm). The flow rate was 1.5 ml/min and the absorbance was measured at 375 nm. An isocratic solvent system of toluene-acetone-pyridine (97:2:1) was used. Incomplete resolution of the reaction products obviated further purification and characterisation.

8,12-Bis-(2-carboxyethyl)-2,7,13,17-tetramethyl-3,18-divinylbiladiene-*ac*-1,19(21*H*,24*H*)-dione (bilirubin IXα) (14) (a) Biliverdin IXα dimethyl ester (**13a**) (7.9 mg, 13 μmol) in methanol (160 ml) was treated with 1M sodium hydroxide containing 1mM etylenediaminetetraacetic acid (EDTA), disodium salt dihydrate (80 ml) for 30 min at 37°C. Glycine/hydrochloric acid buffer (80 ml) (0.4 M hydrochloric acid was adjusted to pH 1.8 with solid glycine) was added followed by 1 M hydrochloric acid (80 ml). The reaction

mixture was cooled to 5°C and 1.6 M sodium borohydride in ethanol (0.4 ml) was added dropwise. The mixture was stirred at 5-10°C for 30 min under argon. Successful transformation is indicated by a colour change from green to yellow. All solutions were flushed with argon for 10 min, prior to use. The bilirubin was extracted with chloroform and the extract washed with water and dried (Na_2SO_4). The solvent was evaporated under reduced pressure and the product applied to acid-washed TLC plates (elution with methanol-chloroform (2:98). The pigment was eluted from the silica gel with methanol-chloroform (5:95) and the filtrate evaporated to give [*10-^{13}C*]-8,12-*bis*-(2-*carboxyethyl*)-2,7,13,17-*tetramethyl*-3,18-*divinylbiladiene*-ac-1,19(21H,24H)-*dione* (**14a**) [6.1 mg, 80%, m.p. 238 °C (decomp.)] λ_{max}/nm 452 (ε 61 500); δ_H (400 MHz, $CDCl_3$) 1.98, 2.15, 2.16, and 2.17 (each 3H, s, 2-, 7-, 13- and 17-Me), 2.54-3.06 (8H, m, 8^1-, 8^2-, 12^1-, 12^2-CH_2), 4.09 (2H, d, *J* 151.1 Hz, 10-CH_2), 5.37, 6.18, and 6.49 (3H, AMX, J_{AM} 2 Hz, J_{AX} 17.6 Hz, J_{MX} 11.5 Hz, 18-vinyl), 5.59, 5.61, and 6.62 (3H, ABX, J_{AB} 1.8 Hz, J_{AX} 17.4 Hz, J_{BX} 11.8 Hz, 3-vinyl), 6.13 (1H, s, 15H), 6.20 (1H, s, 5-H), 9.26, 9.29, 10.68, and 10.79 (each 1H, s, 22-, 23-, 24-, and 21-H)(Found: M^+ 585.264. $C_{35}{}^{13}CH_{39}N_4O_6$ requires *M*, 585.263).

(b) Hydrolysis and reduction of [10-^{13}C]biliverdin IXα (**13b**) (5 mg, 8.2 μmol) was carried out using the same experimental procedure as described above. The bilirubin IXα (**14b**) was crystallised from methanol-chloroform [3.8 mg, 81%, m.p 235°C (decomp.) (lit.,[24] m.p. 234-275°C)]; λ_{max}/nm 452 (ε 61 200); δ [200 MHz, $(CD_3)_2SO$] 1.93, 2.0, 2.03 and 2.18 (each 3H, s, 2-, 7-, 13- and 17-Me), 1.85-2.53 (8H, m, 8^1-, 8^2-, 12^1-, and 12^2-CH_2), 3.98 (2H, s, 10-CH_2), 5.30, 6.21, and 6.59 (3H, AMX, 18-vinyl), 5.65, and 6.63 (3H, ABX, 3-vinyl), 6.10 (2H, s, 5- and 15-H), 9.94, 10.05, 10.47, and 10.50 (each 1H, s, 22-, 23-, 24-, and 21-H), 11.93 (2H, br s, COOH).

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