Monatsh Chem (2009) 140:111–119
DOI 10.1007/s00706-008-0050-1

ORIGINAL PAPER

^{13}C-labeled bilirubin: synthesis of 3^1(3^2),17^1(17^2)-di-[^{13}C]-mesobilirubin-XIIIα

Stefan E. Boiadjiev · David A. Lightner

Received: 17 July 2008 / Accepted: 23 July 2008 / Published online: 25 September 2008

Abstract The title compound, labeled with ^{13}C in the ethyl groups was synthesized from K^{13}CN and low-molecular-weight components. The synthetic relay compound was 3^1(3^2)[^{13}C]-xanthobilirubinic acid methyl ester in a synthetic route that leads to a label in the ethyl β-substituent of a dipyrrinone model for bilirubin. This labeled dipyrrinone was oxidatively coupled to the dimethyl ester of mesobiliverdin-XIIIα, thereby providing a route to a ^{13}C-labeled mesobiliverdin and mesobilirubin, with one carbon of each ethyl being 98% ^{13}C-enriched.

Keywords Pyrrole · Synthesis · ^{13}C-isotope

Introduction

Bilirubin (Fig. 1a), the yellow-orange, neurotoxic pigment of jaundice [1–4] and the end product of heme metabolism in mammals is a lipophilic linear tetrapyrrole [2–5]. It circulates through the body as a tightly-bound complex with serum albumin and is disposed of by hepatic uptake, conjugation to glucuronic acid and excretion into bile [1, 6, 7]. Although much is becoming known of its structure, e.g., bilirubin has been shown to adopt a folded ridge-tile-like conformation in the crystal [8–13] and in solution [14–16], the details of its metabolism remain sketchy. And although it is thought that bilirubin binds to albumin enantio-specificially in a ridge-tile conformation (Fig. 1b) [17–20], its binding site is open to conjecture. To address the question of bilirubin's binding site on serum albumin, especially human serum albumin (HSA), some years ago we synthesized the first ^{13}C-labeled bilirubin, di-[^{13}CO$_2$H]-mesobilirubin-XIIIα [21–23]. It proved to be most useful in assessing the conformation of bilirubin in solution and its pK_a, but it has proven to be not quite adequate for determining the pigment's binding site on HSA. In order to continue our quest to learn more of the binding of bilirubins to HSA, it was becoming clear that the ^{13}C label should be located in an alkyl group. Thus, in the following we report the synthesis of a new ^{13}C-labeled analog, labeled in the ethyl groups, which is only the second example of a highly-enriched bilirubinoid (**1**, Fig. 1c) with 98% ^{13}C-enrichment on carbons of each of the two ethyl groups of mesobilirubin-XIIIα (**1**).

S. E. Boiadjiev · D. A. Lightner (✉)
Department of Chemistry,
University of Nevada, Reno, NV 89557-0216, USA
e-mail: lightner@scs.unr.edu

Results and discussion

Synthesis aspects

The synthesis of ^{13}C-labeled mesobilirubin-XIIIα is outlined in Scheme 1. The later steps (**8** → **1**) in the synthesis are known from earlier work [24–26] but were not usually carried out on a small scale or with the care necessary for such. The early stages of the synthesis were designed to incorporate the ^{13}C label using either ^{13}CH$_3$I or K^{13}CN (98% ^{13}C) as the source of the label. Our thoughts were of using the former, as its *Grignard* reagent to react with the aldehyde group of ethyl 3,5-dimethyl-4-formyl-1*H*-pyrrole-2-carboxylate, a reaction reported by Fischer and Zeile [27] to afford an 80% yield of the 1-hydroxyethyl addition product, a solid and presumable stable secondary alcohol. Despite its attraction, we found that by following the literature procedure—pouring a slurry of the pyrrole aldehyde in ether into one equiv. of an ethereal solution of

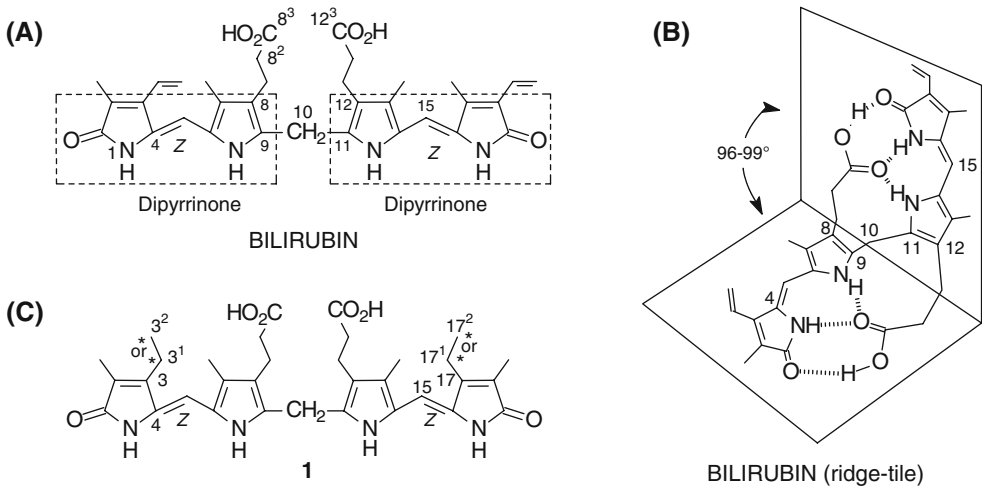


Fig. 1 (**a**) Linear representation of bilirubin, an unstable conformation. (**b**) The most stable conformation of bilirubin is not linear (or porphyrin-like) but folded into a half-opened book or ridge-tile shape that is stabilized by six intramolecular hydrogen bonds. (Only one enantiomer is shown.) (**c**) Mesobilirubin-XIIIα, a bilirubin analog, showing the locations (*asterisks*) of the ^{13}C labels in the ethyl groups

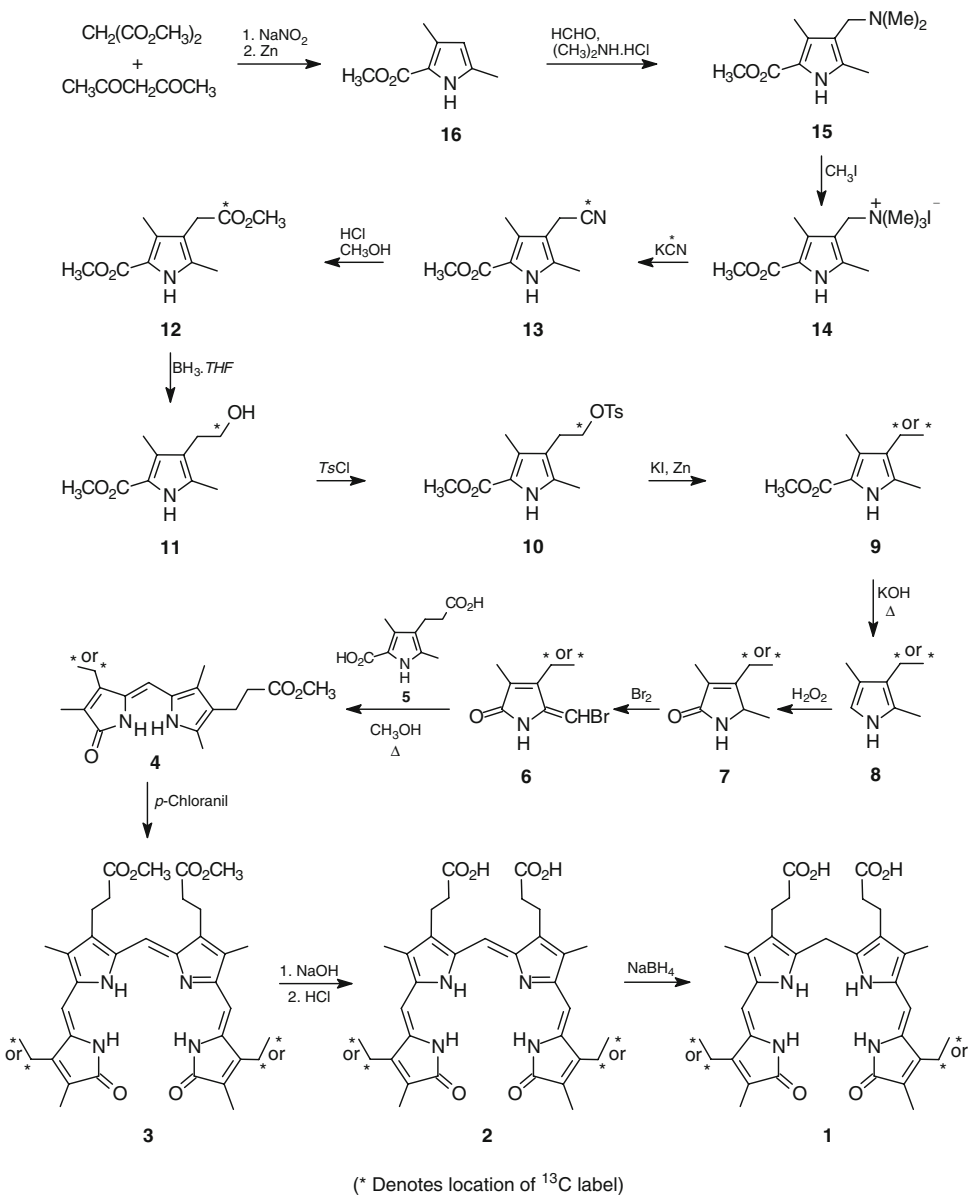
CH_3MgI (freshly made), the alcohol product was isolable only after a very difficult chromatographic separation, in 39% yield, from the only slightly less polar starting aldehyde. The remainder was mostly unreacted starting material. Repeating the reaction with a change of solvent from ether to THF, to generate a homogeneous reaction, gave a complex mixture of product along with unreacted starting material, and this modification was not pursued further. Protecting the pyrrole NH of the starting aldehyde as a *t-Boc* derivative using di-*tert*-butylcarbonate resulted in a thick suspension when it came into contact with CH_3MgI. After work-up, the crude product contained at least five new products and unreacted starting material. Thus, the "aldehyde + Grignard" approach was abandoned in favor of exploring reactions from KCN as the source of $K^{13}CN$ (Scheme 1).

The required monopyrrole starting material (**16**) [28] was synthesized and sufficient material was accumulated for the entire reaction sequence and for trial scale reactions with unlabeled intermediates. After some preliminary experiments with the ethyl ester of **16**, we adopted the methyl ester rather than the more familiar ethyl ester in order to promote crystallinity in the early steps of the overall synthesis. It was determined that ~75 mmol quantities of $K^{13}CN$ (~5 g) could be used as the limiting reagent with convenient-size glassware for subsequent transformations. Optimizations at each step were not limited only to maximizing the yields of isolated pure compounds but also included proper glassware set-ups and, especially, gauging the convenient scale for using expensive $K^{13}CN$. Among the more attention-diverting scale-downs (to 100 mmol scale) were those associated with the synthesis of kryptopyrrole **8** and its oxidation and bromination to pyrrolinone **7** and bromomethylene-pyrrolinone **6**, steps that heretofore had been typically conducted on the 1-mol scale.

The synthesis of **1** thus began with monopyrrole **16**, from which we envisioned a *Mannich* reaction to introduce the cyano group, as reported by Treibs [29]. The readily-obtained piperidine analog of **15**, obtained in 93% yield, turned out to be sluggish to quaternize and so **16** was reacted four times at a 125 mmol scale with aq. formaldehyde and dimethylamine to give a 70% yield of pure crystalline **15** (or 77% yield after reprocessing the mother liquors). Here, quaternization with CH_3I smoothly gave a 99% yield of **14**. The amount of CH_3I used in this *Menschutkin* reaction was optimized at 3 equiv. The salt (**14**) was reacted within 18 h in refluxing *THF* with 1 equiv. KCN to afford an 87% yield of pure crystalline nitrile **13**. (The alternative procedure via the piperidine analog afforded only a 78% yield.) Using a 20% excess of **14** ensured that all of the $K^{13}CN$ was converted to nitrile **13**. Any residual **15** was removed by an acid wash; residual **14** was removed by extensive neutral (H_2O) washing.

Conversion of the acetonitrile side chain of **13** to the acetic ester group of **12** was achieved by treatment with dry methanol saturated with HCl gas over 5 days. This reaction was carried out in a round-bottomed flask, filled so as to leave only a very small head volume, that was tightly stoppered and wired closed, to give a 97% yield of **12**. The acetic ester group of **12** was reduced directly to the hydroxyethyl derivative **11** in 91% yield using diborane generated from BF_3 etherate and KBH_4. Direct reduction thereby by-passed additional steps involving selective saponification of the acetic ester and diborane reduction of the acetic acid group, a procedure which we had used earlier in a synthesis of di-[$^{13}CO_2H$]-mesobilirubin-XIIIα [15, 22]. It was necessary to overcome some potential

Scheme 1



problems with the use of diester **13** instead of its acetic acid analog: Conversion of **15** to **11** was achieved in four steps, without purifications, a procedure that necessitated high yields at each step, with no accumulation of impurities, and working around the fact that diester **12** has a foam-like character when wet and is very difficult and tedious to filter. (Also, when dry, **12** is prone to disperse in a direction opposite to that intended when transferring it, due to static electricity.) Despite all this, ^{13}C-labeled nitrile **13** was converted in three separate runs to **12** (in 91–97% yield) by applying a simple improvement in the work-up. This involved dissolving the wet crude product in $CHCl_3$–CH_2Cl_2, drying the solution and, after evaporation of all solvents, triturating the solid residue with hexane to promote filtration of **12** without much loss due to manipulations.

Reduction of **12** to **11**, also accomplished in three separate runs, led to the desired product in 87–91% yield. Tosylation of **11** to **10** consistently gave an 85% yield. Unreacted **11** was not detected (TLC) but a small amount of a significantly less polar product was identified as the corresponding chloro analog (methyl 3,5-dimethyl-4-(2-chloroethyl)-1*H*-pyrrole-2-carboxylate). This by-product is thought to occur by adventitious displacement of tosylate from **10** by chloride ion (chloride ion is a by-product of the tosylation). Although the ^{1}H NMR spectrum of **10** appeared to be very clean, its ^{13}C NMR spectrum showed ^{13}C enrichment in three minor impurities: a tosylate (**10**) with ~5% enrichment at the β-carbon (relative to the tosylate group), and the chloro analog with 50% of the molecules being 98% ^{13}C-enriched at the α carbon and 50% at the β carbon of the chloroethyl group. These data

provided an early indication that, as was expected from earlier work [30], scrambling of the ^{13}C label in the two carbons of the ethyl group might be expected when **10** undergoes nucleophilic displacement of tosylate by iodide in its conversion of **10** to **9** (Scheme 1). The presence of ~5% scrambled label in tosylate **10** indicates that the initially-formed tosylate is capable of rearrangement via formation of an anchimerically-assisted ion pair (Fig. 2a).

The skeletal rearrangement inferred from scrambling of the ^{13}C label in the (2-tosylethyl) pyrrole β-substituent confirms an earlier observation by Smith et al. [30] in the benzyl ester analog of **11**, with no ^{13}C label but with deuterium substitution—benzyl 3,5-dimethyl-4-(1,1-dideuterio-2-hydroxyethyl)-1*H*-pyrrole-2-carboxylate. Smith et al. found that treatment of that pyrrole with $SOCl_2$ in pyridine led to the expected replacement of the OH group by Cl but with a ~1:1 ratio of 4-(1,1-dideuterio-2-chloroethyl). A similar rearrangement was found by bromination of the alcohol using CBr_4–$(C_6H_5)_3P$ and 4-(2,2-dideuterio-2-chloroethyl) groups. They cited the rearrangements as the first examples of anchimeric assistance by monocyclic pyrroles. Subsequent to this report, we found that even with pyrrole nitrogen protected by a *t*-butoxycarbonyl group, which should make the nitrogen lone pair of electrons less able to participate, rearrangement still occurred. Smith et al. also showed that a ^{13}C label in the hydroxyethyl side chain (at C(4)) was scrambled, which is what we reconfirmed in the current studies.

The tosyl group of **10** was removed by reaction with KI and Zn. This reduction, consistently affording **9** in 87% yield, goes first via formation of the alkyl iodide (Fig. 2b), probably by S_N2 displacement on the already label-scrambled **10**. But whether the label is completely scrambled in **10** during the reaction conditions, or whether the largely unrearranged tosylate is displaced by iodide, and rearrangement occurs at the alkyl iodide state (Fig. 2b) is a matter of relative rates, which were not explored in this interesting example of anchimeric assistance. The net result is that 50% of **9** has 98% ^{13}C in the CH_3 group of the ethyl, and 50% of **9** has 98% ^{13}C in the CH_2.

All of the available pyrrole ester (**9**) was converted to kryptopyrrole **8** (52% yield). This step and the next experienced low yields, which we were unable to raise. Kryptopyrrole **8** was immediately oxidized to pyrrolinone **7**. Due to their unstable nature and the small quantities of precious ^{13}C-labeled material available at this state of the synthesis, neither **8** nor **7** were characterized by NMR but were converted in rapid sequence to bromomethylene-pyrrolinone **6**, which we obtained unfortunately in only 29% yield. (In preliminary trial runs using natural isotopic abundant **9** on the same small reaction scale, the bromination step yields were usually >60%). The entire amount of **6** was converted by heating in CH_3OH to methyl xanthobilirubinate (**4**) in 87% yield using a 1:2 molar ratio of **6** to pyrrole diacid **5**. Thus, from rearranged **9**, we now had dipyrrinone **4**, of which 50% of its molecules had 98% ^{13}C at the 3^1 CH_2 group and 50% at the 3^2 CH_3 group of the ethyl group located at C(3).

Standard oxidative self-coupling of **4** using chloranil in hot CH_2Cl_2 in the presence of formic acid gave an 87% yield of the expected mesobiliverdin-XIIIα dimethyl ester, of which (statistically) 50% had 98% ^{13}C in positions 3^1 and 17^2 or 3^2 and 17^1, 25% in positions 3^1 and 17^1, and 25% in positions 3^2 and 17^2—all due to the rearrangement encountered in going from tosylate **10** to ester **9**. Mesobiliverdin-XIIIα dimethyl ester **3** was saponified under mild conditions in THF–CH_3OH using NaOH and the isolated verdin (**2**) reduced immediately to afford a 67% yield of bright yellow, pure mesobilirubin-XIIIα (**1**) after radial chromatography and crystallization.

Structural and NMR spectroscopic aspects

Most of the compounds synthesized were known previously with only the natural isotopic abundance (~1.1%) of ^{13}C present, and correlations of the site-specific 98% ^{13}C-enriched analogs nicely matched the ^1H NMR spectra of the comparison standards. Clearly, the ^{13}C NMR spectra could not be identical in every aspect, and the presence of the unusually high enrichment presented new and interesting

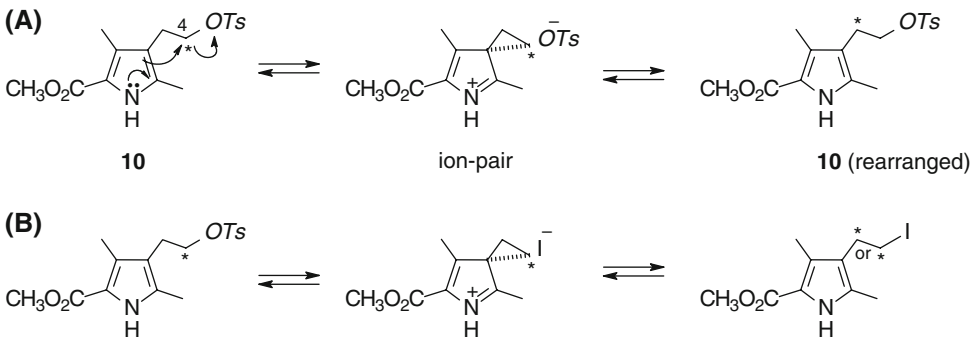


Fig. 2 (**a**) Rearrangement of ^{13}C label in **10** via ion-pair formation. (**b**) Rearrangement of ^{13}C label during the conversion of **10** to **9** via the ion-pair with I^- or via the already rearranged tosylate in (**a**), assuming the rate of ion-pair formation in the latter and collapse to rearranged **10** is faster than attack by I^- on **10**

data. One aspect, the ~5% scrambling of the label in tosylation of **11** could not have been detected by ordinary ^{1}H NMR. This has a bearing on mechanism, but as early as the preparation of nitrile **13**, interesting data were already presented in the ^{13}C NMR spectrum: The ^{13}C label of the CN group dominates the spectrum ($\delta_{CN} = 117.7$ ppm) in a single transient. The adjacent methylene protons (natural isotopic abundance signal near $\delta \sim 3.4$ ppm) are found as a doublet with $^{2}J_{CH} = 10.4$ Hz; the adjacent methylene carbon at $\delta = 12.8$ ppm as a doublet with $^{1}J_{CC} = 56.8$ Hz. The satellites of the ^{13}CN peak at $\delta = 117.7$ ppm were clearly visible ($^{1}J_{CC} = 56.8$ Hz), but the expected $^{2}J_{CC}$ to pyrrole ring carbon-4 could not be determined, because C(4) has a chemical shift ($\delta = 117.5$ ppm) very close to that of the CN.

Dimethyl ester **12** also has an interesting ^{13}C NMR spectrum, with both the adjacent methylene hydrogens at $\delta = 3.38$ ppm and the OCH_3 hydrogens at $\delta = 3.66$ ppm appearing as doublets: $^{2}J_{CH} = 7.6$ Hz and $^{2}J_{CH} = 4.0$ Hz, respectively. Similarly, in the ^{13}C NMR spectrum, the methylene group adjacent to the labeled ester at $\delta = 29.9$ ppm and the OCH_3 group at $\delta = 51.8$ ppm appeared as doublets: $^{1}J_{CC} = 58.2$ Hz, $^{2}J_{CC} = 2.8$ Hz, respectively. In contrast to nitrile **13**, **12** does not have an AB carbon spin system, and C(4) of the pyrrole is clearly seen as a doublet at $\delta = 114.4$ ppm ($^{2}J_{CC} = 2.8$ Hz). The labeled ester carbon at $\delta = 172.2$ ppm clearly showed the (~1.1%) natural isotopic abundance satellites (58.2 Hz apart) from coupling to the adjacent CH_2 carbon. The coupling ($^{3}J_{CC} = 1.6$ Hz) between C(5) and the labeled carbonyl carbon was determined by a special processing technique involving no exponential multiplication but multiplication with a shifted sine bell function.

In alcohol **11** the ^{13}C NMR spectrum clearly showed the presence of the isotope label only at $\delta = 62.6$ ppm (the α-carbon of the hydroxyethyl side chain) and its influence on the β-CH_2 ($\delta = 27.5$ ppm, d, $^{1}J_{CC} = 36.3$ Hz) and on pyrrole carbon **4** ($\delta = 117.4$ ppm, d, $^{2}J_{CC} = 1.9$ Hz). In the ^{1}H NMR spectrum the β-CH_2 appeared as an apparent six-line multiplet; whereas, the α-CH_2 is like a dtd ($^{1}J_{CH} = 142.8$ Hz). Spectral data for the tosylate (**10**) again conformed to expectations, although they hinted at rearrangement to a small extent and to a 1:1 extent in the (2-chloroethyl) by-product.

In contrast to the relative simplicity of labeled nitrile, ester, alcohol, and tosylate, **9** gave a much more interesting and revealing NMR spectrum because it clearly indicated scrambling of the label in the –CH_2–CH_3 chain, i.e., that **9** was a mixture of two regio-isomers, each with one ^{13}C label. Thus two CH_3 signals were found, each a dt: one with $^{1}J_{CH} = 126.0$ Hz and $^{3}J_{HH} = 7.6$ Hz (for –$CH_2{}^{13}CH_3$) and a second with $^{2}J_{CH} = 4.6$ Hz and $^{3}J_{HH} = 7.6$ Hz (for –$^{13}CH_2CH_3$). In correspondence, the two CH_2 signals were each a dq: one ($\delta = 2.37$ ppm) with $^{1}J_{CH} = 125.4$ Hz and $^{3}J_{HH} = 7.6$ Hz, and a second ($\delta = 2.38$ ppm) with $^{2}J_{CH} = 4.9$ Hz and $^{3}J_{HH} = 7.6$ Hz. Pyrrole carbon C(4) was found as two doublets: one with $^{1}J_{CC} = 48.8$ Hz and a second with $^{2}J_{CC} = 2.3$ Hz. Taken collectively, the NMR data prove that in **9** the ^{13}C label is 50:50 dispersed into the two carbons of the β-ethyl group.

The "scrambled" label, of course, also appears in methyl xanthobilirubinate (**4**). In the ^{1}H NMR spectrum, two methyl triplets ($^{1}J_{CH} = 127.6$ Hz, $^{3}J_{HH} = 7.6$ Hz and $^{2}J_{CH} = 4.3$ Hz, $^{3}J_{HH} = 7.6$ Hz) and two methylene quartets ($^{1}J_{CH} = 127.6$ Hz, $^{3}J_{HH} = 7.6$ Hz and $^{2}J_{CH} = 5.3$ Hz, $^{3}J_{HH} = 7.6$ Hz) were observed. Three ^{13}C NMR spectra of **4** were acquired with various experimental parameters. Waiting for 10 s between the observation pulses gave a 47:53 integral ratio between $^{13}CH_2$ (at 17.93 ppm) and $^{13}CH_3$* (at 15.04 ppm). Besides the extensive splitting of the dipyrrinone C(3) signal, expected from observations in **6** and **9**, the C(1) lactam carbonyl definitely also showed two doublets. By resolution enhancement of one of the ^{13}C NMR spectra, the C(1) carbonyl was found to be spin–spin coupled with both labels of the CH_2CH_3 through three ($^{3}J_{CC} = 4.7$ Hz) and four ($^{4}J_{CC} = 0.7$ Hz) bonds. The presence of this coupling pattern was confirmed also in mesobiliverdin-XIIIα dimethyl ester (**3**) and mesobilirubin-XIIIα (**1**). Similar coupling via $^{2}J_{CC}$ and $^{3}J_{CC}$ was not found to C(4) of **4**, but was seen in **1**. The ^{1}H and ^{13}C NMR spectra of **1** and **3** were no more complex than the spectra of **4**. Careful integration of the ^{13}C signals from the ethyl groups gave a 51:49 ratio; thus with certainty it can be said that label scrambling at the **10** → **9** conversion step is statistically 1:1, and no preference of the label for one position or the other in the ethyls is found.

Concluding comments

The synthesis of mesobilirubin-XIIIα, mesobiliverdin-XIIIα dimethyl ester and methyl xanthobilirubinate with ^{13}C labels in the ethyl groups has been demonstrated successfully from K^{13}CN (98% enriched) as the source of ^{13}C.

Experimental

NMR spectra were acquired on a Varian Unity Plus spectrometer at 11.75 T magnetic field strength operating at ^{1}H frequency of 500 MHz and ^{13}C frequency of 125 MHz in solutions of $CDCl_3$ (referenced at 7.26 ppm for ^{1}H and 77.00 ppm for ^{13}C) or $(CD_3)_2SO$ (referenced at 2.49 ppm for ^{1}H and 39.50 ppm for ^{13}C). J-modulated spin-echo (*A*ttached *P*roton *T*est) and *gHMBC* experiments were used to assign the ^{13}C NMR spectra. UV–visible spectra were

recorded on a Perkin–Elmer Lambda 12 spectrophotometer. Radial chromatography was carried out on Merck silica gel PF_{254} with $CaSO_4$ binder, preparative layer grade, using a Chromatotron (Harrison Research, Palo Alto, CA, USA) with 1, 2, or 4 mm thick rotors, and analytical thin-layer chromatography was carried out on J.T. Baker silica gel IB-F plates (125 μm layer). Melting points were determined on a Mel-Temp capillary apparatus and are corrected. Combustion analyses were carried out by Desert Analytics, Tucson, AZ, USA, and found to be within ±0.3% of theoretical values.

The spectral data were obtained in spectral grade solvents (Aldrich or Fisher) which were distilled under Ar stream just prior to use. Before the distillation $CHCl_3$ was passed through a basic alumina column. Distillation of $(CH_3)_2SO$ solvent was carried out at 0.5 mmHg vacuum collecting the solvent at 0°C and thawing it under Ar. The starting monopyrroles, methyl 3,5-dimethyl-1*H*-pyrrole-2-carboxylate (**16**) and ethyl 3,5-dimethyl-4-(2-carboethoxyethyl)-1*H*-pyrrole-2-carboxylate were prepared as described in the literature [28].

Methyl 3,5-Dimethyl-4-dimethylaminomethyl-1H-pyrrole-2-carboxylate (**15**; $C_{11}H_{18}N_2O_2$)

To a solution of 1.53 g (10 mmol) *β*-free pyrrole (**16**) in 60 cm^3 CH_3OH was added a solution of 20.3 g (250 mmol) $(CH_3)_2\overset{+}{N}H_2Cl^-$ in 16 cm^3 H_2O, 25 cm^3 CH_3OH, and 12 cm^3 37% aq. HCHO, and the mixture was heated at 60–65°C. After 4 h, more *Mannich* reagent made from 4.8 g $(CH_3)_2\overset{+}{N}H_2Cl^-$/3.7 cm^3 H_2O/6 cm^3 CH_3OH/3 cm^3 HCHO was added, and stirring and heating continued for a total of 7 h. After cooling the reaction mixture was poured into 200 cm^3 sat. aq. $NaHCO_3$, then 30 cm^3 2 mol L^{-1} aq. NaOH was added. The product was extracted into $CHCl_3$ (4 × 30 cm^3). The combined organic extracts were washed with H_2O (3 × 100 cm^3), dried over anh. Na_2SO_4, and filtered. The solvent was evaporated under vacuum (roto-vap), and the residue was purified by radial chromatography. Crystallization from ethyl acetate–hexane gave pure **15**. Yield: 1.4 g (70%); mp 106–107°C; ^{1}H NMR ($CDCl_3$): $\delta = 2.18$ (6H,d, $N(CH_3)_2$), 2.23 (3H, s, 3-CH_3), 2.29 (3H, s, 5-CH_3), 3.17 (2H, s, CH_2), 3.82 (3H, s, OCH_3), 8.98 (1H, brs) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 10.7$ (3-CH_3), 11.6 (5-CH_3), 45.1 ($N(CH_3)_2$), 50.8 (OCH_3), 116.7 (C-5), 118.8 (C-5), 128.6 (C-4), 131.9 (C-2), 162.2 (C=O) ppm.

Methyl 3,5-dimethyl-4-(trimethylammoniummethyl iodide)-1H-pyrrole-2-carboxylate (**14**; $C_{12}H_{21}N_2O_2I$)

To a solution of 420 mg (2.0 mmol) tertiary amine **15** in 20 cm^3 abs. ethanol was added 1.0 cm^3 (16.0 mmol) CH_3I, and the mixture was stirred for 3 h at room temperature. The volatiles were evaporated under aspirator vacuum (roto-vap), and the solid residue was kept under vacuum at 45°C/0.1 mmHg for 2 h to afford the quaternary salt (**14**). Yield: 697 mg (99%); mp 203–206°C (decomp); ^{1}H NMR ($(CD_3)_2SO$): $\delta = 2.28$ (6H, s, 3- and 5-CH_3), 2.97 (9H, s, $(NCH_3)_3$), 3.75 (3H, s, OCH_3), 4.31 (2H, s), 11.82 (1H, brs) ppm; ^{13}C NMR ($(CD_3)_2SO$): $\delta = 11.3$ (3-CH_3), 11.82 (5-CH_3), 50.8 (OCH_3), 51.0 (3 × $N(CH_3)_3$), 59.1 (CH_2), 109.1 (C-5), 117.5 (C-3), 128.8 (C-4), 156.3 (C-2), 160.9 (C=O) ppm.

Methyl 3,5-dimethyl-4-[^{13}CN]cyanomethyl-1H-pyrrole-2-carboxylate (**13**; $C_9{}^{13}CH_{12}N_2O_2$)

The crude methiodide (**14**) obtained from 4.0 mmol 4-dimethylaminomethyl pyrrole **15** was added to 15 cm^3 THF followed by a solution of 228 mg (3.5 mmol) $K^{13}CN$ in 3 cm^3 H_2O, and the mixture was heated at reflux for 4 h. After cooling, it was diluted with 100 cm^3 H_2O, and the product was extracted with $CHCl_3$ (3 × 50 cm^3). The combined extracts were washed with H_2O (3 × 50 cm^3), dried (Na_2SO_4), and filtered. The solvent was removed under vacuum, and the residue was triturated with ethyl acetate–hexane to give pure cyanomethyl pyrrole **13** (>95% pure by NMR). Yield: 630 mg (87%). Sample for analysis was recrystallized from ethyl acetate–hexane: mp 174–175°C; ^{1}H NMR ($CDCl_3$): $\delta = 2.38$ (3H, s, 3-CH_3), 2.31 (3H, s, 5-CH_3), 3.42 (2H, s), 3.84 (3H, s, OCH_3), 9.10 (1H, brs) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 10.4$ (3-CH_3), 11.4 (5-CH_3), 12.8 (CH_2), 51.2 (OCH_3), 110.1 (C-2), 117.5 (CN), 117.7 (C-4), 126.7 (C-3), 130.5 (C-5), 162.0 (C=O) ppm.

Methyl 3,5-dimethyl-4-[^{13}C=O]-carbomethoxymethyl-1H-pyrrole-2-carboxylate (**12**; $C_{10}{}^{13}CH_{14}NO_4$)

Nitrile **13** (13.37 g, 69.2 mmol) was stirred in 380 cm^3 HCl-saturated CH_3OH for 110 h. After evaporation to dryness, ice-cold H_2O was added (150 cm^3), and the mixture was stirred for 20 h. The product was separated by filtration, washed with water (3 × 50 cm^3), and dissolved in $CHCl_3$–CH_2Cl_2 (2 × 200 cm^3). The organic solution was washed with H_2O (3 × 100 cm^3), dried over Na_2SO_4, and filtered. The solvents were evaporated under vacuum (roto-vap) and the residue was triturated with ethyl acetate (~15 cm^3) and hexane (~100 cm^3), and the pyrrole product (**12**) was filtered and dried. Yield: 15.14 g (97%); 138–139°C; ^{1}H NMR ($CDCl_3$): $\delta = 2.23$ (3H, s, 3-CH_3), 2.27 (3H, s, 5-CH_3), 3.38 (2H, d, $^2J_{CH} = 7.6$ Hz, CH_2), 3.66 (3H, d, $^3J_{CH} = 4.0$ Hz, acetate OCH_3), 3.82 (3H, s, α-ester OCH_3), 9.00 (1H, brs) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 10.5$ (3-CH_3), 11.5 (5-CH_3), 29.9 (4-CH_2, d, $^1J_{CC} = 58.2$ Hz), 50.9 (α-ester OCH_3), 51.8 (acetate OCH_3, $^2J_{CC} = 2.8$ Hz), 114.4 (C-4, d, $^2J_{CC} = 2.8$ Hz), 116.9 (C-2), $^2J_{CC} = 2.8$ Hz), 127.6 (C-3), 131.1 (C-5, d, $^3J_{CC} = 1.6$ Hz), 162.1 (α-C=O), 172.2 (acetate C=O) ppm.

Methyl 3,5-dimethyl-4-([2-^{13}C]-2-hydroxyethyl)-1H-pyrrole-2-carboxylate (**11**; $C_9{}^{13}CH_{15}NO_3$)

A slow stream of diborane was passed through a solution of 15.84 g (70 mmol) dimethyl ester **12** in 230 cm^3 anh. THF during 75 min. The mixture was then stirred for an additional 2 h. The reaction was quenched by addition of CH_3OH (50 cm^3), then water (100 cm^3). The organic solvents were evaporated under vacuum (roto-vap). The residue was dissolved in 300 cm^3 $CHCl_3$, washed with 1 mol L^{-1} aq. NaOH (200 cm^3), 2% HCl (200 cm^3), H_2O (3 × 150 cm^3), dried (Na_2SO_4), and filtered. The solvent was evaporated under vacuum (roto-vap), and the residue was recrystallized from ethyl acetate–hexane to afford alcohol **11**, sufficiently pure to be carried forward to the next step. Yield: 12.68 g (91%); mp 131–132°C; ^{1}H NMR ($CDCl_3$): δ = 2.22 (3H, s, 3-CH_3), 2.26 (3H, s, 5-CH_3), 2.63 (2H, m, $^3J_{HH}$ = 6.8 Hz, 4^2-CH_2O), 3.65 (2H, m, $^3J_{HH}$ = 6.8 Hz, 4^1-CH_2), 3.82 (3H, s, OCH_3), 9.14 (1H, brs) ppm; ^{13}C NMR ($CDCl_3$): δ = 10.6 (3-CH_3), 11.4 (5-CH_3), 27.5 (d, $^1J_{CC}$ = 36.3 Hz, 3^1-CH_2), 50.9 (OCH_3), 62.61 (CH_2–O), 116.9 (C-2), 117.4 (d, $^2J_{CC}$ = 1.9 Hz, C-4), 127.5 (C-3), 131.1 (C-5), 162.3 (C=O) ppm.

Methyl 3,5-dimethyl-4-(2[^{13}C]-2-Tosyloxyethyl)-1H-pyrrole-2-carboxylate (**10**; $C_{16}{}^{13}CH_{21}NO_4S$)

To a solution of 12.68 g (63.97 mmol) alcohol **11** in 160 cm^3 anh. CH_2Cl_2 and 19.2 cm^3 triethylamine kept at 0°C was added *p*-toluenesulfonyl chloride (18.3 g, 95.95 mmol) during 1 h. The mixture was stirred for 16 h, allowing the temperature to reach ambient. After dilution with 5% aq. HCl (300 cm^3) the mixture was washed with H_2O (3 × 200 cm^3), dried ($MgSO_4$), and filtered. The solvent was evaporated under vacuum (roto-vap), and the residue was recrystallized from ethyl acetate–hexane tosylate **10** that was sufficiently pure to be carried on to the next step. Yield: 19.10 g (85%); mp 141–143°C; ^{1}H NMR ($CDCl_3$): δ = 2.10 (3H, s, 3-CH_3), 2.14 (3H, s, 5-CH_3), 2.41 (3H, s, CH_3-Arom), 2.70 (2H, m, $^3J_{HH}$ = 7.0 Hz, 4^1-CH_2), 3.82 (3H, s, OCH_3), 3.99 (2H, dt, $^1J_{CH}$ = 250.8 Hz, $^3J_{HH}$ = 7.0 Hz, 4^1-CH_2O), 7.26 (2H, d, $^3J_{HH}$ = 8.4 Hz, *m*-H), 7.66 (2H, d, $^3J_{HH}$ = 8.4 Hz, *o*-H), 8.72 (1H, brs) ppm; ^{13}C NMR ($CDCl_3$): δ = 10.3 (3-CH_3), 11.3 (5-CH_3), 21.5 (CH_3-Arom), 24.0 (4^1-CH_2), 50.88 (OCH_3), 69.74 (4^2-CH_2O), 115.6 (C-2), 116.8 (C-4), 127.1 (C-3), 127.6 (*m*-CH), 129.6 (*o*-CH), 131.0 (5-CH_3), 132.9 (*p*-C), 144.5 (*i*-C), 162.0 (2-C=O) ppm.

Methyl 3,5-dimethyl-4-[2-^{13}C]-ethyl-1H-pyrrole-2-carboxylate and methyl 3,5-dimethyl-4-[1-^{13}C]-ethyl-1H$_1$-pyrrole-2-carboxylate (**9**; $C_9{}^{13}CH_{15}NO_2$)

To a solution of 18.95 g (53.77 mmol) tosylate **10** in 450 cm^3 1,2-dimethoxyethane was added 45.5 g (700 mg A) Zn dust, 40.4 g (269 mmol) NaI, and 22 cm^3 H_2O. The mixture was heated at reflux for 5 h. It was then filtered hot, and the solids were washed with $CHCl_3$. The filtrate was evaporated until only H_2O distilled. The residue was dissolved in $CHCl_3$–CH_2Cl_2 (300 cm^3), washed with 3% $Na_2S_2O_3$ (150 cm^3), 2% HCl (150 cm^3), H_2O (3 × 150 cm^3), dried ($MgSO_4$), and filtered. The solvents were evaporated under vacuum (roto-vap) and recrystallized from ethyl acetate–hexane to give pure ethyl pyrrole **9**. Yield: 8.04 g (82%); mp 114–116°C; ^{1}H NMR ($CDCl_3$): δ = 1.05 (1.5H, dt, $^1J_{CH}$ = 126.0 Hz, $^3J_{HH}$ = 7.6 Hz, 4^2-$^{13}CH_3$), 1.05 (1.5H, dt, $^2J_{CH}$ = 4.6 Hz, $^3J_{HH}$ = 7.6 Hz, 4^1-$^{13}CH_2$–), 2.20 (3H, s, 3-CH_3), 2.27 (3H, s, 5-CH_3), 2.37 (1H, dq, $^1J_{CH}$ = 125.4 Hz, $^3J_{HH}$ = 7.6 Hz, 4^1-$^{13}CH_2$–), 2.38 (1H, dq, $^2J_{CH}$ = 4.9 Hz, $^3J_{HH}$ = 7.6 Hz, 4^2-$^{13}CH_3$), 3.82 (3H, s, OCH_3), 8.79 (1H, brs) ppm; ^{13}C NMR ($CDCl_3$): δ = 10.4 (3-CH_3), 11.2 (5-CH_3), 15.3 (4^2-13CH_3), 17.2 (4^1-CH_2-), 50.8 (OCH_3), 116.4 (C-2), 123.77 (d, $^1J_{CC}$ = 48.8 Hz, ethyl), 123.77 (d, $^2J_{CC}$ = 2.3 Hz, ethyl), 126.8 (C-3), 129.4 (C-5), 162.3 (2-C=O) ppm.

5-Bromomethylene-4-([2-^{13}C]-ethyl)-3-methyl-3-pyrrolin-2-one and 5-bromomethylene-4-([1-^{13}C]-ethyl)-3-methyl-3-pyrrolin-2-one (**6**; $C_7{}^{13}CH_{10}BrNO$)

The synthesis is carried out from solid **9** without isolating relatively unstable liquid or oily intermediates **8** and **7**, which are well-known from earlier work and fully characterized in our laboratory [24–26]. Thus:

(A) Pyrrole ester **9** (24.68 g, 135.4 mmol) from combining three separate reductions of tosylate pyrrole **10**, 115 cm^3 diethylene glycol, 28 cm^3 H_2O, and 22.8 g (407.0 mmol) KOH were combined, and the mixture was heated slowly under N_2 until the head temperature of a small distillation apparatus reached 100°C (45 min). The distillate was collected until the temperature reached 220°C during an additional 35 min. The distillate was diluted with CH_2Cl_2 (150 cm^3), washed with H_2O (2 × 100 cm^3), and dried over Na_2SO_4. The solvent was evaporated under vacuum (roto-vap), and the residue was distilled under vacuum to give 8.69 g (52%) of kryptopyrrole **8**, bp 62–64°C/0.7 mmHg.

(B) A solution of 8.69 g (70 mmol) kryptopyrrole **8** in 25 cm^3 CH_3OH and 9.5 cm^3 H_2O was purged with N_2 for 1 h. Then 30% aqueous hydrogen peroxide (9.3 cm^3, ~82 mmol) was added during 6 h at 50°C internal temperature. The mixture was kept one hour more at 50°C, then heated at reflux for 2 h. After cooling, a solution of 1.9 g K_2CO_3 in 4.2 cm^3 H_2O was added, and the mixture was stirred overnight at ambient temperature. Water (25 cm^3) was added, and the product was extracted with CH_2Cl_2 (5 × 20 cm^3). The aqueous layer was acidified to pH ~ 4 and extracted with an additional portion of 50 cm^3 CH_2Cl_2. The combined organic extracts were washed with brine (20 cm^3), dried over $MgSO_4$, and filtered. The solvent was removed under vacuum (roto-vap), and the residue was

distilled under vacuum to afford 7.0 g (72%) of pyrrolinone **7**, bp 114–128°C/0.7 mmHg, which was used without delay in the bromination step.

(C) To a solution of 7.09 g (50.6 mmol) pyrrolinone **7** in 77 cm^3 anh. ethyl acetate, warmed to 45°C, was added Br_2 (5.2 cm^3, 16.3 g, 101.2 mmol) during 50 min. Then the mixture was heated (15 min) to reflux for 15 min. After cooling in an ice bath and dilution with 95 cm^3 more ethyl acetate, it was neutralized with sat. aqueous $NaHCO_3$ (pH > 7). The organic layer was washed with 5% aqueous $NaHCO_3$ (100 cm^3), brine (100 cm^3) and dried ($MgSO_4$). After filtration and evaporation of the solvent, the residue was recrystallized from ethyl acetate–hexane to give bromomethylene pyrrolinone **6**. Yield: 3.13 g (29%); mp 139–140°C; ^{1}H NMR ($CDCl_3$): $\delta = 1.12$ (1.5H, dt, $^1J_{CH} = 128.2$ Hz, $^3J_{HH} = 7.6$ Hz, 4^2-$^{13}CH_3$), 1.12 (1.5H, dt, $^2J_{CH} = 4.3$ Hz, $^3J_{HH} = 7.6$ Hz, 4^1-$^{13}CH_2$), 1.85 (3H, s, 3-CH_3), 2.40 (1H, dq, $^1J_{CH} = 128.2$ Hz, $^3J_{HH} = 7.6$ Hz, 4^1-CH_2), 2.40 (1H, dq, $^2J_{CH} = 5.3$ Hz, $^3J_{HH} = 7.6$ Hz, $4^2 = {}^{13}CH_3$), 5.90 (1H, s, =CHBr), 7.55 (1H, brs) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 8.33$ (3-CH_3), 14.2 (4^2-$^{13}CH_3$), 17.8 (4^1-$^{13}CH_2$), 86.56 (d, $^2J = 1.4$ Hz, C-3), 141.3 (br, C-5), 145.1 (2xd, $^1J = 46.5$ Hz, $^2J = 3.3$ Hz, C-4), 171.3 (~t, C-2) ppm.

[3^1-^{13}C] and [3^2-^{13}C]-Methyl xanthobilirubinate (**3**; $C_{17}{}^{13}CH_{24}N_2O_3$)

(A) Saponification of pyrrole diester. A mixture of 7.75 g (29 mmol) ethyl 3,5-dimethyl-4-(2-carbethoxymethyl)-1*H*-pyrrole-2-carboxylate [24–26] diethyl ester, 5.8 g (145 mmol) NaOH, 60 cm^3 95% ethanol, and 24 cm^3 50% aqueous $NaNO_3$ was heated at reflux for 3 h. All of the ethanol solvent was evaporated under vacuum (roto-vap). The residue was mixed with 10 cm^3 more 50% $NaNO_3$, chilled to −15°C and slowly acidified with a mixture of conc. HNO_3–50% aqueous $NaNO_3$ ~ 1:5. The diacid product (**5**) was separated by filtration, washed with H_2O (2 × 20 cm^3) and dried overnight under vacuum.

(B) Condensation. Diacid **5** was mixed with 3.13 g (14.4 mmol) bromomethylene pyrrolinone **6**, 75 cm^3 CH_3OH, and one drop 48% HBr, and the mixture was heated at reflux for 6 h. Then it was kept overnight at −15°C. The product was separated by filtration. It was dissolved in 500 cm^3 $CHCl_3$, washed with H_2O (3 × 100 cm^3), dried (Na_2SO_4), and filtered. The solvent was evaporated under vacuum (roto-vap), and the residue was recrystallized from CH_3OH–CH_2Cl_2 to give xanthobilirubic acid methyl ester (**3**). Yield: 3.99 g (87%); mp 221–224°C; ^{1}H NMR ($CDCl_3$): $\delta = 1.17$ (1.5H, dt, $^1J_{CH} = 127.6$ Hz, $^3J_{HH} = 7.6$ Hz, 3^2-$^{13}CH_3$), 1.17 (1.5H, dt, $^2J_{CH} = 4.3$ Hz, $^3J_{HH} = 7.67$ Hz, 3^2-CH_3), 1.94 (3H, s, 2-CH_3), 2.14 (3H, s, 7-CH_3), 2.41 (3H, s, 9-CH_3), 2.45 (2H, t, $J = 7.3$, 8.4 Hz, 8^2-CH_2), 2.54 (1H, dq, $^1J_{CH} = 127.6$ Hz, $^3J_{HH} = 7.6$ Hz, 3^2-$^{13}CH_2$), 2.55 (1H, dq, $^2J_{CH} = 5.3$ Hz, $^3J_{HH} = 7.6$ Hz, 3^2-CH_2), 2.74 (2H, t, $J = 8.4$, 7.3 Hz, 8^1-CH_2), 3.68 (3H, s, OCH_3), 6.13 (1H, s, 5-CH), 10.37 (1H, brs), 11.32 (1H, brs) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 8.5$ (2-CH_3), 9.6 (7-CH_3), 11.5 (9-CH_3), 15.0 (3^2-$^{13}CH_3$), 17.9 (3^1-$^{13}CH_2$), 19.8 (8^1-CH_2), 35.1 (8^2-CH_2), 51.6 (OCH_3), 101.0 (5-CH=), 119.0 (C-8), 122.37 (C-6), 122.40 (d, $^2J = 1.9$ Hz, C-2), 124.6 (C-7), 127.1 (C-4), 131.6 (C-9), 148.30 (d, $^1J = 46.5$ Hz, 3^1-$^{13}CH_2$), 148.31 (d, $^2J = 2.8$ Hz, 3^2-$^{13}CH_3$), 173.7 (8^3-C=O), 174.09 (d, $^3J = 4.7$ Hz, C-2 in $^{13}CH_2CH_3$ isomer), 174.09 (d, $^4J = 0.7$ Hz, C-2 in $CH_2{}^{13}CH_3$ isomer) ppm.

Bis-[^{13}C]-mesobiliverdin-XIIIα dimethyl ester (**3**; $C_{33}{}^{13}C_2H_{42}N_4O_6$)

In the usual way [28] but on a much reduced scale, ^{13}C-labeled methyl xanthobilirubinate (**4**) was converted to ^{13}C-labeled mesobiliverdin-XIIIα dimethyl ester (**3**), which is a mixture of ^{13}C isomers: 25% [3^1-^{13}C, 17^1-^{13}C], 50% [3^1-^{13}C, 17^2-^{13}C] and 25% [3^2-^{13}C, 17^2-^{13}C] as follows.

A mixture of 1.29 g (4 mmol) dipyrrinone (**4**), 880 cm^3 CH_2Cl_2, 44 cm^3 HCO_2H, and 2.46 (10 mmol) *p*-chloranil was heated at reflux for 24 h. Then the volume was reduced to one half by distillation, and reflux was continued for 6 h more. The mixture was chilled overnight at −15°C, and the separated solid was removed by filtration. The cold filtrate was neutralized with sat. aqueous $NaHCO_3$, then quickly washed with 4% aqueous NaOH (2 × 150 cm^3), dried (Na_2SO_4), and filtered. The solvent was evaporated under vacuum (roto-vap), and the residue was purified by radial chromatography on silica. The pure fractions were recrystallized from ethyl acetate–hexane to give pure mesobiliverdin-XIIIα dimethyl ester **3**. Yield: 1.07 g (87%); mp 244–246°C; ^{1}H NMR ($CDCl_3$): $\delta = 1.20$ (3H, dt, $^1J_{CH} = 4.3$ Hz, $^3J_{HH} = 7.6$ Hz, 3^2,17^2-$^{13}CH_3$), 1.20 (3H, dt, $^2J_{CH} = 4.3$ Hz, $^3J_{HH} = 7.6$ Hz, 3^1,17^2-CH_3), 1.79 (6H, s, 2,18-CH_3), 2.08 (6H, s, 7, 13-CH_3), 2.50 (2H, dq, $^2J_{CH} = 127.9$ Hz, $^3J_{HH} = 7.6$ Hz, 3^1,17^1-$^{13}CH_2$), 2.50 (2H, dq, $^2J_{CH} = 5.5$ Hz, $^3J_{HH} = 7.6$ Hz, 3^1,17^1-CH_2), 2.55 (4H, t, $J = 7.6$ Hz, 8^2,12^2-CH_2), 2.91 (4H, 5, $J = 7.6$ Hz, 8^1,12^1-CH_2), 3.67 (6H, s, OCH_3), 5.90 (2H, s, 5,15-CH), 6.73 (1H, s, 10-CH=), 7.7-8.4 (br, N(22)H), 8.26 (2H, brs, N(21), N(24)-H) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 8.3$ (2, 18-CH_3), 9.5 (7,13-CH_3), 14.4 (3^2,17^2-$^{13}CH_3$), 17.8 (3^1,17^1-$^{13}CH_2$), 19.8 (8^1,12^1-CH_2), 35.21 (8^2,12^2-CH_2), 51.7 (OCH_3), 96.1 (5.15-CH=), 114.2 (10-CH=), 128.35 (7,13), 128.37 (d, $J = 1.3$ Hz, C(2), C(18)), 137.5 (8, 12), 139.9 (4, 16), 140.9 (9, 11), 146.64 (d, $^1J = 4.6$ Hz, 3^1,17^1-$^{13}CH_2$), 146.65 (d, $^2J = 3.0$ Hz, 3^2,17^2-$^{13}CH_3$), 150.0 (6, 14), 172.44 (d, $^3J = 4.7$ Hz, 1,19), 172.44 (d, $^4J = 0.6$ Hz, 1,19), 173.1 (8^3,12^3-C=O) ppm.

Bis-[^{13}C]-mesobilirubin-XIIIα (**1**; $C_{31}{}^{13}C_2H_{40}N_4O_6$)
The synthetic rubin consists of three regio-isomers, based on the locations of the ^{13}C label in the ethyl groups, as noted for the verdin precursor (**3**).

A solution of 308 mg (0.5 mmol) verdin in 170 cm^3 THF–CH_3OH and 180 cm^3 0.2 mol L^{-1} aqueous NaOH plus 150 mg ascorbic acid was degassed with Ar for 0.5 h and then was stirred at 50°C for 4 h. After cooling, it was diluted with 50 cm^3 0.1 mol L^{-1} NaOH, washed with 100 cm^3 $CHCl_3$ (which was discarded), and the aqueous solution was acidified with ice-cold 10% aqueous HCl to pH < 4. The product was extracted with CH_2Cl_2 (4 × 80 cm^3). The combined extracts were washed with H_2O (1 × 100 cm^3), dried over Na_2SO_4, and filtered. The solvent was evaporated under vacuum (roto-vap) to give crude verdin diacid (**2**), which was carried forward to the rubin.

Verdin diacid **2** was dissolved in 125 cm^3 CH_3OH, and the solution was purged with Ar for 20 min. Then (at ~5°C) $NaBH_4$ was added in small portions until a yellow solution was obtained (~1.4 g, 37 mmol) during 20 min. The mixture was diluted with 150 cm^3 H_2O and acidified with 6 cm^3 acetic acid then with 5 cm^3 10% aqueous HCl, and the product was extracted with $CHCl_3$–CH_2Cl_2 1:1 (4 × 100 cm^3). The extracts were washed with H_2O (3 × 80 cm^3), dried (Na_2SO_4), and filtered. The solvents were evaporated under vacuum (roto-vap) and the residue was purified by radial chromatography (1–2% CH_3OH–CH_2Cl_2) and recrystallized from CH_2Cl_2–CH_3OH to give bright yellow mesobilirubin-XIIIα (**1**). Yield: 197 mg (67%); mp 297–299°C (decomp.); ^{1}H NMR ($CDCl_3$): $\delta = 1.12$ (3H, dt, $^1J_{CH} = 127.6$ Hz, $^3J_{HH} = 7.6$ Hz; $3^2,17^2$-$^{13}CH_3$), 1.12 (3H, dt, $^2J_{CH} = 4.3$ Hz, $^3J_{HH} = 7.6$ Hz; $3^1,17^1$-1^3CH_2), 1.86 (6H, s; 2,18-CH_3), 2.15 (6H, s; 7,13-CH_3), 2.48 (2H, dq, $^1J_{CH} = 127.6$ Hz, $^3J_{HH} = 7.6$ Hz; $3^1,17^1$-$^{13}CH_2$), 2.48 (2H, dq, $^2J_{CH} = 5.4$ Hz, $^3J_{HH} = 7.6$ Hz; $3^1,17^1$-CH_2), 2.55 (2H, m; $8^1,12^1$-CH), 2.81 (2H, m; $8^2,12^2$-CH), 2.88 (2H, m; 8^2, 12^2-CH), 3.01 (2H, m; $8^1,12^1$-CH), 4.06 (2H, s; 10-CH_2), 6.05 (2H, s; 5,15-CH=), 9.15 (2H, s; N(22)-H, N(23)-H), 10.58 (2H, s; N(21)-H, N(24)-H), 13.65 (2H, brs, CO_2H) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 8.0$ ($2^1,18^1$-CH_3), 10.1 ($7^1,13^1$-CH_3), 14.9 ($3^2,17^2$-$^{13}CH_3$), 17.8 ($3^1,17^1$-$^{13}CH_2$), 18.5 ($8^1,12^1$-CH_2), 22.2 (10-CH_2), 32.6 ($8^2,12^2$-CH_2), 100.5 (5,15-CH=), 119.4 (7,13), 123.3 (d, $J = 1.6$ Hz, C(2), C(18)), 123.7 (6,14), 124.1 (8,13), 128.4 (2 × d, $^2J = 3.5$ Hz, $^3J = 0.8$ Hz, C(4), C(16)), 133.2 (9,11), 148.4 (2 × d, $^1J = 46.1$ Hz, $^2J = 3.0$ Hz, C(3), C(17)), 174.9 (2 × d, $^3J = 4.9$ Hz, $^4J = 0.7$ Hz; 1,19), 179.5 (CO_2H) ppm.

Acknowledgments We thank the US National Institutes of Health (HD 17779) for generous support of this research. Dr *Stefan E. Boiadjiev* is on leave from the Institute of Organic Chemistry, Sofia, Bulgaria. We thank the US National Science Foundation (CHE-0521191) for matching funds to acquire a 400 MHz NMR spectrophotometer and upgrade our 500 MHz NMR.

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