SYNTHESIS OF A [$^{13}CO_2H$]-LABELLED BILIRUBIN

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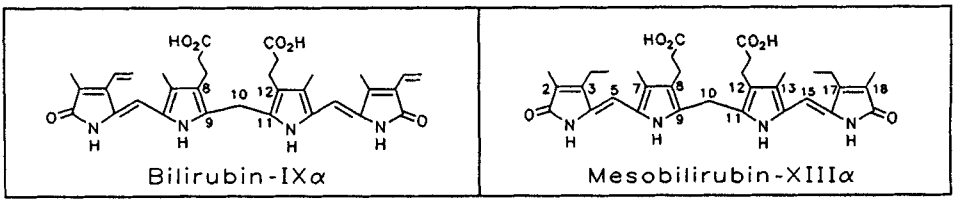
Summary

Bis-[$^{13}CO_2H$]-mesobilirubin-XIIIα was synthesized in 11% overall yield in 10 steps from 99% enriched $K^{13}CN$. The synthetic methods are adapted to a scale of a few grams to a few milligrams.

Key Words: Carbon-13, [$^{13}CO_2H$]-mesobilirubin-XIIIα, small scale synthesis

Introduction

Bilirubin-IXα, the yellow-orange, neurotoxic pigment of jaundice, is formed in normal metabolism in mammals by the turnover of hemoglobin and other heme proteins.[1] The symmetric analog, mesobilirubin-XIIIα, is not found in nature but can be prepared synthetically.[2] It shares many of the same solution conformational and spectroscopic properties as the natural pigment; and it is metabolized, transported and excreted by the same elimination pathways found *in vivo*.[3] It has, thus, served as an invaluable model for bilirubin in many studies. Yet, despite its implication in a diverse array of protein binding situations involved in transport and metabolism, the conformation of bilirubin in solution or when bound to proteins or in membranes is incompletely understood.



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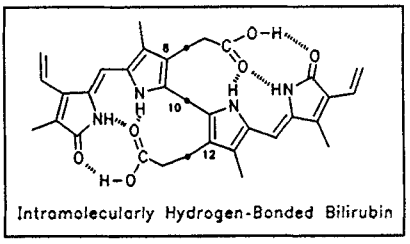
It has been recognized that many different conformations of the pigment are accessible by rotations of the dipyrrinone chromophores about the C_9-C_{10} and C_{10}-C_{11} single bonds.[4] But one unique conformation (shown at the right) brings the propionic acid CO_2H groups into a juxtaposition with the dipyrrinone pyrrole and lactam N-H and C=O groups favorable for conformation-stabilizing intramolecular hydrogen bonding. It is apparently in this preorganized structure that bilirubin is able to cross several selective physiologic barriers: the placenta (which is important in the transfer of fetal bilirubin to the maternal circulation) and the blood-brain barrier (which leads to irreversible neurologic damage. But it cannot cross others: the liver and the kidneys (which are normal selective barriers for bilirubin elimination and heme detoxification).[5] Even after excretion across the liver into bile, bilirubin exerts its influence as the source of nucleation of gallstones, which account for over 0.5 million cholecystectomies each year.



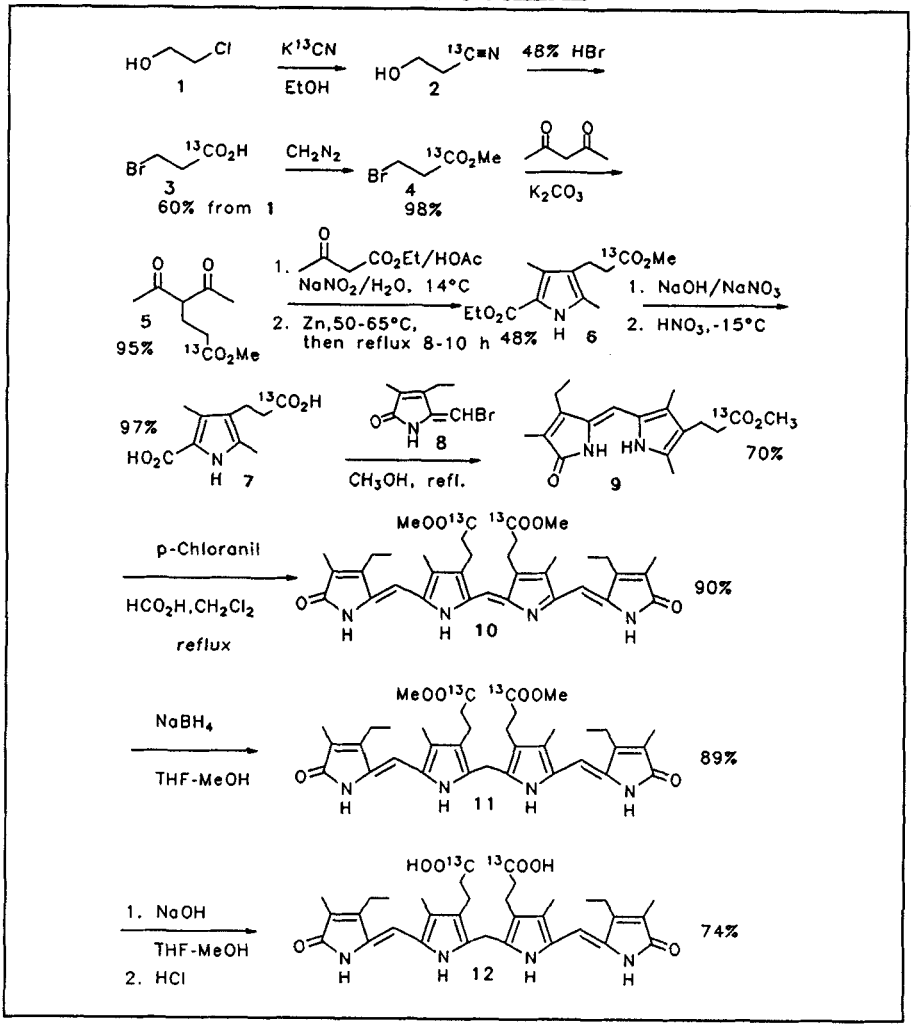
Intramolecularly Hydrogen-Bonded Bilirubin

In bilirubin metabolism and elimination, its propionic acid CO_2H groups appear to play an important role. Many of the metabolic events cited above and their dependence on bilirubin molecular structure might be examined in detail using ^{13}C-NMR spectroscopy by focussing on $^{13}CO_2H$ chemical shift changes that accompany ionization[6] during protein binding or in breaking hydrogen bonds. In order to prepare for such studies, a bilirubin analog, mesobilirubin-XIIIα, was prepared with 99% ^{13}C enriched CO_2H groups in ten steps and 11% overall yield.

Results and Discussion

Taking advantage of a procedure for the preparation of 3-chloropropionic-1-^{14}C acid from K^{14}CN described earlier in this journal,[7] we converted epichlorohydrin (**1**) to 3-bromopropanoic-1-^{13}C-acid (**3**), which becomes the propionic groups of mesobilirubin-XIIIα (Synthetic Scheme). At first, 3-bromopropanoic-1-^{13}C-acid was converted to the key monopyrrole intermediate (**6**) by a Fischer-Knorr type condensation of nitrosated ethyl acetoacetate with the C-alkylation product of pentane-2,4-dione and **4**. Previous syntheses[8] of unlabelled **5** were achieved *via* the Michael reaction of pentane-2,4-dione with acrylate esters, and it is likely here that methyl acrylate-1-^{13}C is the probable alkylating agent rather than **4**. When the alkylation reaction is followed by GC-MS, **4** is found to disappear at a faster rate than **5** is formed.

SYNTHETIC SCHEME



Saponification of monopyrrole diester **6** generates diacid **7**, which is unstable (toward α-decarboxylation). Sodium nitrate ($NaNO_3$) is added to the saponification solution in order to prevent freezing at the neutralization step, and pure diacid **7** can be isolated from the reaction in high yield by cooling to -15° to -8°C during the cautious addition of conc. nitric acid. (In earlier work, it was erroneously reported that sodium nitrite ($NaNO_2$) was used.)[8] Diacid **7** is an intermediate suitable for coupling with the known bromomethylenepyrrolinone (**8**) to afford methyl xanthobilirubinate-[$^{13}CO_2CH_3$] (**9**) in good yield. This key, bright yellow dipyrrinone intermediate was converted to the intensely blue tetrapyrrole, mesobiliverdin-XIIIα dimethyl ester (**10**) in excellent yield by oxidative coupling using *p*-chloranil.[2] Conversion of **10** to the

desired mesobilirubin-XIIIα-bis-[$^{13}CO_2H$] (**12**) proceeded straightforwardly in two steps, first by smooth reduction of the blue verdin (**10**) to the yellow rubin diester (**11**) using sodium borohydride, then by saponification.

Conclusion

A total synthesis is described for a bilirubin analog, mesobilirubin-XIIIα, with 99% ^{13}C labels in the two propionic acid carboxyl groups. The new pigment is expected to be useful in determining the pK_a values of the CO_2H groups by ^{13}C-NMR[6] and in elucidating intramolecular hydrogen bonding by heteronuclear ^{13}C-^1H NMR. These studies are underway in our laboratory.

Experimental

3-Bromopropanoic-1-^{13}C acid (3):

A solution of $K^{13}CN$ (2 g, 30.8 mmoles) (Isotec, Inc.) in water (5 mL) was added dropwise to a boiling solution of freshly distilled 2-chloroethanol (5 g, 62.6 mmoles) in 95% ethanol (30 mL). The mixture was heated at reflux for 5 hours, and examined for completion. (A drop of the reaction solution was tested with a 1 M solution of *p*-nitrobenzaldehyde in $(CH_3)_2SO$. If a red-violet color is observed, there is unreacted CN^- ion left in the reaction, and reflux should be continued until a negative color test is observed.) The reaction solution was added to acetone (100 mL) and dried with a large amount of magnesium sulfate. The salts were removed by filtration and washed with additional acetone. The solvents were removed using a rotary evaporator with the water bath at room temperature to prevent loss of product. Excess 2-chloroethanol was removed by aspirator distillation, and the resulting residue was distilled (b.p. 102-120°C at 9 mbar) to give a colorless oil. In a 100 mL round-bottom flask the 3-hydroxypropionic-1-^{13}C-nitrile was combined with aqueous 48% hydrobromic acid (50 mL) and heated at reflux for 3 hours. The solution was cooled and continuously extracted with diethyl ether (75 mL) overnight. The ether was dried with magnesium sulfate, filtered and removed (roto-vap) giving a light brown solid. The solid was sublimed at 0.7 mbar, 30°C. The remaining oil after sublimation was converted to product by heating at reflux in aqueous 48% hydrobromic acid (30 mL), continuous extraction, and sublimation as above in a total yield: 2.75 g (60%) as a white solid with mp 59-60°C (Lit.[9] 61-63°C). It had IR (film) ν: 2980, 1719, 1430, 1042, 817, 646, 597 cm^{-1}; ^1H-NMR ($CDCl_3$/TMS) δ: 3.00 (dt, 2H at C_2, $^3J_{HH}$=6.9 Hz, $^2J_{CH}$=7.2 Hz),

3.58 (dt, 2H at C_3, $^3J_{HH}$=6.9 Hz, $^3J_{CH}$=5.1 Hz), 11.2 (bs, 1H, COO*H*) ppm; ^{13}C-NMR ($CDCl_3$/TMS) δ: 174.80 ppm (8 scans); GC-MS *m/z*: 155 (M^+), 153 (M^+), 138, 136, 109, 107, 74, 43, 27.

3-Bromopropanoic-1-^{13}C acid methyl ester (4):

3-Bromopropanoic-1-^{13}C acid (2 g, 13 mmoles) was dissolved in ethyl ether (20 mL), stirred magnetically and cooled to 0°C. Using a 125 mL separatory funnel with a teflon stopcock, ethereal diazomethane was added dropwise until the solution was slightly yellow. The diethyl ether was removed (roto-vap); yield: 2.13 g, (98%) as a clear oil. It had IR (film) ν: 2953, 1741, 1438, 1218 cm^{-1}; ^{1}H-NMR ($CDCl_3$/TMS) δ: 2.13 (dt, 2H at C_2, $^3J_{HH}$=6.9 Hz, $^2J_{CH}$= 7.2 Hz), 3.52 (dt, 2H at C_3, $^3J_{HH}$=6.9 Hz, $^3J_{CH}$=5.1 Hz), 3.72 (d, 3H, OCH_3, $^3J_{CH}$=3.9 Hz) ppm; ^{13}C-NMR ($CDCl_3$/ TMS) δ: 170.96 ppm (8 scans); GC-MS *m/z*: 169 (M^+), 167 (M^+), 138, 136, 109, 107, 88, 79, 60.

4-Acetyl-5-oxohexanoic-1-^{13}C-acid methyl ester (5):

In a 50 mL round-bottom flask equipped with a reflux condenser and a heating mantle, 3-bromopropanoic-1-^{13}C-acid methyl ester (2.13 g, 12.7 mmoles) with 2,4-pentanedione (5 g, 50 mmoles) and potassium carbonate (2.63 g, 19 mmoles) were combined. The mixture was heated to 40°C for 15 hours, and then cooled to room temperature. Dichloromethane (20 mL) was added to the solution, and then the salts were removed by filtration and washed with additional dichloromethane (20 mL). The mixture was washed with water (2 x 50 mL), dried with magnesium sulfate and the solvent was removed (roto-vap). The excess pentanedione was removed by aspirator distillation using a short path distillation apparatus. The remaining liquid was distilled at 90-100°C/0.7 mbar (Lit.[10] 150-151°C/13 mbar) to yield 2.27 g (95%). It had IR (film) ν: 2983, 1732, 1702, 1361, 1187 cm^{-1}; ^{1}H-NMR ($CDCl_3$/TMS) δ: 2.16 (s, 4H), 2.20 (s, 2H), ~2.30 (m, 1H), ~2.4 (m, 1H), ~2.6 (m, 2H), 3.67 (d, 3H, OCH_3, $^3J_{CH}$=3.9 Hz), 3.73 (t, 1H at C_4, $^3J_{HH}$=6.9 Hz) ppm; ^{13}C-NMR ($CDCl_3$/TMS) δ: 172.94 ppm (8 scans, keto), 172.99 (8 scans, enol); GC-MS *m/z*: 187 (M^+), 156, 145, 114, 113, 84, 56, 55.

2-Carboethoxy-3,5-dimethyl-4-propyl (3-^{13}C-carbomethoxy)-1*H*-pyrrole (6):

In a 100 mL three-neck round-bottom flask ethyl acetoacetate (1.63 g, 12.5 mmole) was dissolved in 5 mL of acetic acid. The flask was equipped with a magnetic stirrer, thermometer

and an addition funnel. The stirred solution was cooled with an ice bath to 5°C and sodium nitrite (1.2 g in 10 mL of water) was added dropwise at a rate to keep the temperature of the solution below 14°C. When the addition was complete the solution was stirred at room temperature overnight (20 hours). Then, 4-acetyl-5-oxohexanoic-1-^{13}C-acid methyl ester (2.17 g, 11.6 mmoles) was added to the reaction all at once. Zinc dust (2 g, 30.8 mmoles) was added in small portions at a rate to keep the reaction temperature between 50°C and 65°C. The reaction took about 1 hour. The flask was equipped with a condenser and the reaction was heated at reflux overnight. The reaction was cooled and poured into ice water (200 mL) and the precipitate was collected by filtration (saving the mother liquor for later workup). The solid product was dissolved in dichloromethane (150 mL) and filtered to remove excess zinc, dried over magnesium sulfate and the dichloromethane was removed (roto-vap) giving the desired pyrrole as a brown solid. The mother liquor from above was extracted with dichloromethane (100 mL), washed with water (3 x 50 mL) and dried over magnesium sulfate. The dichloromethane was removed (roto-vap) leaving a brown oil. The oil was taken up in 95% ethanol (75 mL), and water was added until the solution became turbid. 95% ethanol (35 mL) was added and the solution was cooled to 5°C in an ice bath. Scratching the flask with a glass rod facilitates the precipitation of the brown product. The combined solids were dried overnight in a desiccator over P_2O_5 at 0.7 mbar to give the pure diester in 48% yield (1.42 g) with mp 70-72°C (Lit.[11] mp 73°C). It had IR (film) ν: 3301, 2976, 1733, 1662, 1272, 1094 cm^{-1}; ^{1}H-NMR ($CDCl_3$/TMS) δ: 1.34 (t, 3H, -CH_2-CH_3, J=6.9 Hz), 2.21 (s, 3H, CH_3), 2.25 (s, 3H, CH_3), 2.42 (dt, 2H, -CH_2-C$H_2CO_2CH_3$, $^3J_{HH}$=7.0 Hz, $^2J_{CH}$=7.2 Hz), 2.71 (dt, 2H, -CH_2-$CH_2CO_2CH_3$, $^3J_{HH}$=7.0 Hz, $^3J_{CH}$=5.0 Hz), 3.66 (d, 3H, OCH_3, $^3J_{CH}$=3.5 Hz), 4.30 (q, 2H, -CH_2-CH_3, J=6.9 Hz), 8.7 (bs, 1H, NH) ppm; ^{13}C-NMR ($CDCl_3$/TMS) δ: 174.81 ppm (8 scans); GC-MS *m/z*: 254 (M^+), 209, 180, 134, 106, 77, 65.

2-Carboxy-3,5-dimethyl-4-propyl(3-^{13}C-carboxy)-1*H*-pyrrole (7):

The 2-carboethoxy-3,5-dimethyl-4-propyl(3-^{13}C-carbomethoxy)-1H-pyrrole (1.42 g, 5.6 mmole) was dissolved in ethanol (20 mL) and treated with sodium hydroxide (3 g in 2.5 mL water with 2.5 g sodium nitrate) in a 100 mL round-bottom flask. The solution was heated at reflux for 4 hours, and then cooled to room temperature. The ethanol was removed (roto-vap) and any remaining salts were redissolved with minimal water. The solution was cooled to -15°C with

a dry ice/acetone bath and stirred rapidly. Nitric acid (8 mL, conc.) was added dropwise to the solution at a rate to keep the temperature below -8°C. The light brown solid was collected by filtration, washed with water (30 mL) and immediately placed into a desiccator and dried under vacuum overnight. The unstable product was used immediately in the next step; yield: 1.15 g (97%); mp 110°C (dec) (Lit.[8] mp 114°C).

Methyl [$^{13}CO_2CH_3$]-Xanthobilirubinate (9):

In a 100 mL round-bottom flask 2-carboxy-3,5-dimethyl-4-propyl(3-^{13}C-carboxy)-1*H*-pyrrole (1.15 g, 5.5 mmole) and 5-bromoethylene-4-ethyl-3-methyl-2-oxo-1*H*-pyrrole (**8**)[8] (1.8 g, 8.3 mmole) were dissolved in methanol (60 mL). The flask was equipped with a reflux condenser and a heating mantle and heated at reflux for 10 hours. The reaction was cooled to room temperature and the yellow precipitate was collected by filtration. The yellow solid was recrystallized using dichloromethane (just enough to dissolve) and methanol (3 x the volume of dichloromethane) and cooling to -30°C to yield 1.2 g (70%) yellow needles, mp 212-214°C (Lit.[8] mp 217-218°C). It had IR (film) ν: 3346, 2962, 1732, 1673, 1632, 1174 cm^{-1}; ^{1}H-NMR ($CDCl_3$/TMS) δ: 1.18 (t, 3H, -CH_2CH_3, $^3J_{HH}$=7.5 Hz), 1.94 (s, 3H, CH_3), 2.14 (s, 3H, CH_3), 2.41 (s, 3H, CH_3), 2.45 (dt, 2H, -$CH_2CH_2CO_2CH_3$, $^3J_{HH}$=6.9 Hz, $^2J_{CH}$=7.0 Hz), 2.55 (q, 2H, -CH_2CH_3, $^3J_{HH}$=7.5 Hz), ~2.75 (m, 2H, -$CH_2CH_2CO_2CH_3$), 3.68 (d, 3H, OCH_3, $^3J_{CH}$=3.6 Hz), 6.13 (s, 1H, =CH), 10.32 (bs, 1H, pyrrole N*H*), 11.22 (bs, 1H, lactam N*H*) ppm; ^{13}C-NMR ($CDCl_3$/TMS) δ: 174.34 ppm (8 scans).

Bis-[^{13}COOH]-Mesobiliverdin-XIIIα Dimethyl Ester (10):

In a 250 mL round-bottom flask **9** (500 mg, 1.58 mmoles) was dissolved in dichloromethane (100 mL) and stirred magnetically. *p*-Chloranil (600 mg, 2.43 mmoles) was dissolved in dichloromethane (75 mL) and added to the reaction flask. Formic acid (98%, 16 mL, 0.45 moles) was added and the reaction was heated at reflux in the dark for 18 hours. The solution was cooled to -30°C and the white precipitate was filtered off. The green-blue solution was transferred to a 500 mL beaker and cooled in an ice bath. The solution was neutralized with saturated aqueous sodium bicarbonate (100 mL), slowly at first. The resulting blue solution was washed with 5% (wt.) aq. sodium bicarbonate (3 x 50 mL) until the aqueous layer was colorless. The organic layer was washed with aq. saturated NaCl solution and dried with magnesium sulfate. The magnesium sulfate was removed by filtration and the dichloromethane was removed by rotary

evaporator. The crude verdin was purified by flash chromatography (Woelm TLC silica gel deactivated with 10% water; solvent: dichloromethane/methanol 99:1) to yield 435 mg (90%) of blue needles with mp 230-231°C (Lit.[2] mp 232-234°C). It had IR (film) ν: 3355, 2967, 1694, 1677, 1591, 1151 cm^{-1}; ^{1}H-NMR ($CDCl_3$/TMS) δ: 1.22 (t, 6H, -CH_2CH_3, $^3J_{HH}$=7.5 Hz), 1.83 (s, 6H, 2 x CH_3), 2.09 (s, 6H, 2 x CH_3), ~2.53 (m, 8H, 2 x -CH_2CH_2-CO_2CH_3), ~2.90 (m, 4H, 2 x CH_2CH_3), 3.68 (d, 6H, 2 x OCH_3, $^3J_{CH}$=3.9 Hz), 5.93 (s, 2H, 2 x =CH), 6.75 (s, 1H, pyrrole N*H*), 8.10 (bs, 2H, lactam N*H*) ppm; ^{13}C-NMR ($CDCl_3$/TMS) δ: 173.73 ppm (8 scans).

Mesobilirubin-XIIIα-bis-[^{13}COOH] Dimethyl Ester (11):

In a 250 mL Erlenmeyer flask **10** (300 mg, 0.49 mmoles) was dissolved in a minimal amount of hot tetrahydrofuran (about 100 mL). Methanol (50 mL) was added, and while the solution was sonicated, sodium borohydride (60 mg, 1.6 mmoles) was added in small portions over a period of 10 minutes. With continued sonication, the reaction was kept under a flow of nitrogen in the dark while it was carried to completion. After 15 minutes the solution turned from blue to green, and after 2 hours the solution was yellow. Hydrochloric acid (6*N*) was added dropwise until a pH of 5 was obtained. The mixture was taken up in dichloromethane (100 mL) and extracted with water (5 x 100 mL) and then with saturated aq. NaCl (100 mL), giving a bright yellow solution. The organic layer was dried with magnesium sulfate, filtered and the solvent was removed (roto-vap). The rubin was purified by flash chromatography (Woelm TLC silica gel deactivated with 10% water) using dichloromethane/ethanol (99:1) as eluent to yield 270 mg (89%) with mp 229-232°C (Lit.[2] mp 234-236°C). It had IR (film) ν: 3344, 2925, 1694, 1660, 1455, 1152 cm^{-1}; ^{1}H-NMR ($CDCl_3$/TMS) δ: 1.00 (t, 6H, 2 x -CH_2CH_3, J=7.5 Hz), 1.47 (s, 6H, 2 x CH_3), 2.10 (s, 6H, 2 x CH_3), 2.25 (q, 4H, 2 x CH_2CH_3, $^3J_{HH}$=7.5 Hz), 2.45 (dt, 4H, 2 x -$CH_2CH_2CO_2CH_3$, $^3J_{HH}$=7.0 Hz, $^2J_{CH}$=8.5 Hz), 2.83 (dt, 4H, 2 x -$CH_2CH_2CO_2CH_3$, $^3J_{HH}$=7.0 Hz, $^3J_{CH}$=2.8 Hz), 3.68 (d, 6H, 2 x OCH_3, $^3J_{CH}$=3.6 Hz), 4.13 (s, 2H, -CH_2-), 5.90 (s, 2H, 2 x =CH), 10.28 (bs, 2H, 2 x pyrrole N*H*), 10.58 (bs, 2H, 2 x lactam N*H*) ppm; ^{13}C-NMR ($CDCl_3$/TMS) δ: 174.29 ppm (8 scans).

Mesobilirubin-XIIIα-bis-[^{13}COOH] (12):

In a 100 mL round-bottom flask equipped with a reflux condenser and a heating mantle **11** (250 mg, 0.40 mmoles) was dissolved in tetrahydrofuran (20 mL) with methanol (20 mL). The solution was stirred magnetically while an aqueous solution of 2M sodium hydroxide (8 mL, 16

mmoles) was added. The solution was heated at reflux under nitrogen in the dark for 4 hours. The reaction was cooled to room temperature and the methanol was removed (roto-vap). The mixture was cooled with an ice bath to 5°C and hydrochloric acid (10%) was added to adjust the pH to 5. The resulting yellow precipitate was recovered by filtration. The rubin was purified by flash chromatography (Woelm TLC silica gel deactivated with 10% water using dichloromethane/methanol 99:1 as solvent) then recrystallized from dichloromethane/methanol to yield 175 mg (74%) of bright yellow solid, mp 308-309°C (Lit.[2] mp 312-315°C). It had IR (KBr) ν: 3415, 3264, 2445, 1689, 1473, 1255, 1095 cm^{-1}; ^{1}H-NMR ($CDCl_3$/TMS) δ (ppm): 1.12 (t, 6H, 2 x -CH_2CH_3, $^3J_{HH}$=7.5 Hz), 1.86 (s, 6H, 2 x CH_3), 2.16 (s, 6H, 2 x CH_3), 2.48 (q, 4H, 2 x -CH_2CH_3, $^3J_{HH}$=7.5 Hz), 2.56 (dddd, 1H, -$CH_XHCH_2CO_2H$, $^3J_{HH}$=2.6 Hz, $^3J_{HH}$= 4.7 Hz, $^2J_{HH}$=14.9 Hz, $^3J_{CH}$=3.7 Hz), 2.79 (dddd, 1H, -$CH_2CH_CHCO_2H$, $^3J_{HH}$=2.8 Hz, $^3J_{HH}$=4.7 Hz, $^2J_{HH}$=18.8 Hz, $^2J_{CH}$=6.7 Hz), 2.86 (dddd, 1H, -$CH_2CH_BCO_2H$, $^3J_{HH}$=13.4 Hz, $^3J_{HH}$=2.6 Hz, $^2J_{HH}$=18.8 Hz, $^2J_{CH}$=6.7 Hz), 2.99 (dddd, 1H, -$CH_ACH_2CO_2H$, $^3J_{HH}$= 13.4 Hz, ^3J=2.8 Hz, $^2J_{HH}$=14.9 Hz, $^3J_{CH}$=3.7 Hz), 4.07 (s, 2H, -CH_2-), 6.05 (s, 2H, 2 x =CH), 9.15 (bs, 2H, 2 x lactam N*H*), 10.58 (bs, 2H, 2 x pyrrole NH), 12.5 (bs, 2H, 2 x CO_2H); ^{13}C-NMR ($CDCl_3$/TMS) δ: 179.50 ($^{13}CO_2H$) ppm (128 scans); 7.95 (C_2/C_{18} CH_3), 10.08 (C_7/C_{13}, CH_3), 14.87 (C_3^2/C_{17}^2, CH_3), 17.84 (C_3^1/C_{17}^1, CH_2), 18.55 (C_8^1/C_{12}^1, CH_2), 22.23 (C_{10} CH_2), 32.27 (C_3^2/C_{12}^2 CH_2), 100.49 (C_5/C_{15} CH), 123.35 (C_6/C_{14}, s), 123.64 (C_2/C_{18}, s), 124.15 (C_7/C_{13}, s), 128.38 (C_4/C_{16}, s), 133.16 (C_9/C_{11}, s), 148.35 (C_3/C_{17}, s), 174.90 (C_1/C_{19} C=O), 179.48 (C_8^3/C_{12}^3 COOH) ppm (51,200 scans); HR-MS: exact mass calculated for$^{13}C_2C_{31}H_{40}O_6N_4$, 590.30412; found, 590.30412.[12]

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