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ORIGINAL PAPER

Synthesis and properties of mesobilirubins XIIγ and XIIIγ and their mesobiliverdins

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Abstract The title pigments, with propionic acid groups displaced to the lactam end rings, were synthesized for the first time by the "1 + 2 + 1" approach, coupling two equivalents of a monopyrrole to a dipyrrylmethane (to give XIIγ), or the "2 + 2" approach, self-coupling two equivalents of a dipyrrinone (to give XIIIγ). Using the "1 + 2 + 1" approach, mesobilirubin IIIα was also prepared. Mesobilirubins XIIγ and XIIIγ are more polar than mesobilirubin IIIα and unlike IIIα cannot effectively engage the propionic acid groups in intramolecular hydrogen bonding to the dipyrrinone components. The new mesobilirubins give exciton coupled circular dichroism spectra in the presence of human serum albumin or quinine, with the XIIγ isomer exhibiting Cotton effect intensities nearly as strong as those from the IIIα isomer; whereas, the XIIIγ isomer exhibits far weaker intensities. Mesobilirubin IIIα requires glucuronidation for hepatobiliary elimination; whereas, XIIγ and XIIIγ do not, and they are excreted intact across the liver into bile. The corresponding biliverdins XIIγ and XIIIγ are reduced only slowly by biliverdin IXα reductase, in contrast to the fast reduction of the natural IXα isomer.

Keywords Dipyrrole · Tetrapyrrole · Hydrogen bonds · Circular dichroism

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Introduction

In his successful quest to determine the chemical structure of heme, for which he was awarded the Nobel Prize in Chemistry in 1930, Hans Fischer recognized 15 possible position isomers for two vinyl (V), four methyl (M), and two propionic acid (P) groups distributed around the porphyrin macrocyclic periphery and located at the pyrrole β-carbons. In 1927, he labeled these isomers I–XV in a long paper published with Stangler [1] and showed 11 nonsymmetric and the four symmetric structures (in Fig. 1) of mesoporphyrins, wherein the vinyl (V) groups are reduced to ethyl (E). Two years later together with Zeile [2] he proved by synthesis that the heme of nature possesses the nonsymmetric structure called protoporphyrin IX. In the current work, we are interested in bilirubin analogues with a symmetric ordering of the β-substituents as found in three of the four symmetric mesoporphyrins: III, IV, XII, and XIII (Fig. 1).

By 1929, it was becoming recognized that the yellow pigment of jaundice arose in nature from heme catabolism (Fig. 2). Yet its structure as bilirubin IXα was not established until 13 years later by Fischer and Plieninger [3], again by total synthesis. Along the twisting road to its structure determination, Fischer and co-workers [4] prepared two different symmetric derivatives of bilirubin: mesobilirubin IIIα (**1**) and XIIIα (Fig. 1). The latter was synthesized again 54 years later [5, 6]. Although neither pigment was prepared from macrocyclic ring opening of two of the four symmetric mesoporphyrins, III and XIII, they might be viewed as having been derived from them by cleaving the porphyrin macrocycle at the α-carbon on the symmetry plane orthogonal to the plane of the macrocycle (Fig. 1), with loss of the α-carbon. These two mesobilirubins exhibit many of the solubility and solution properties

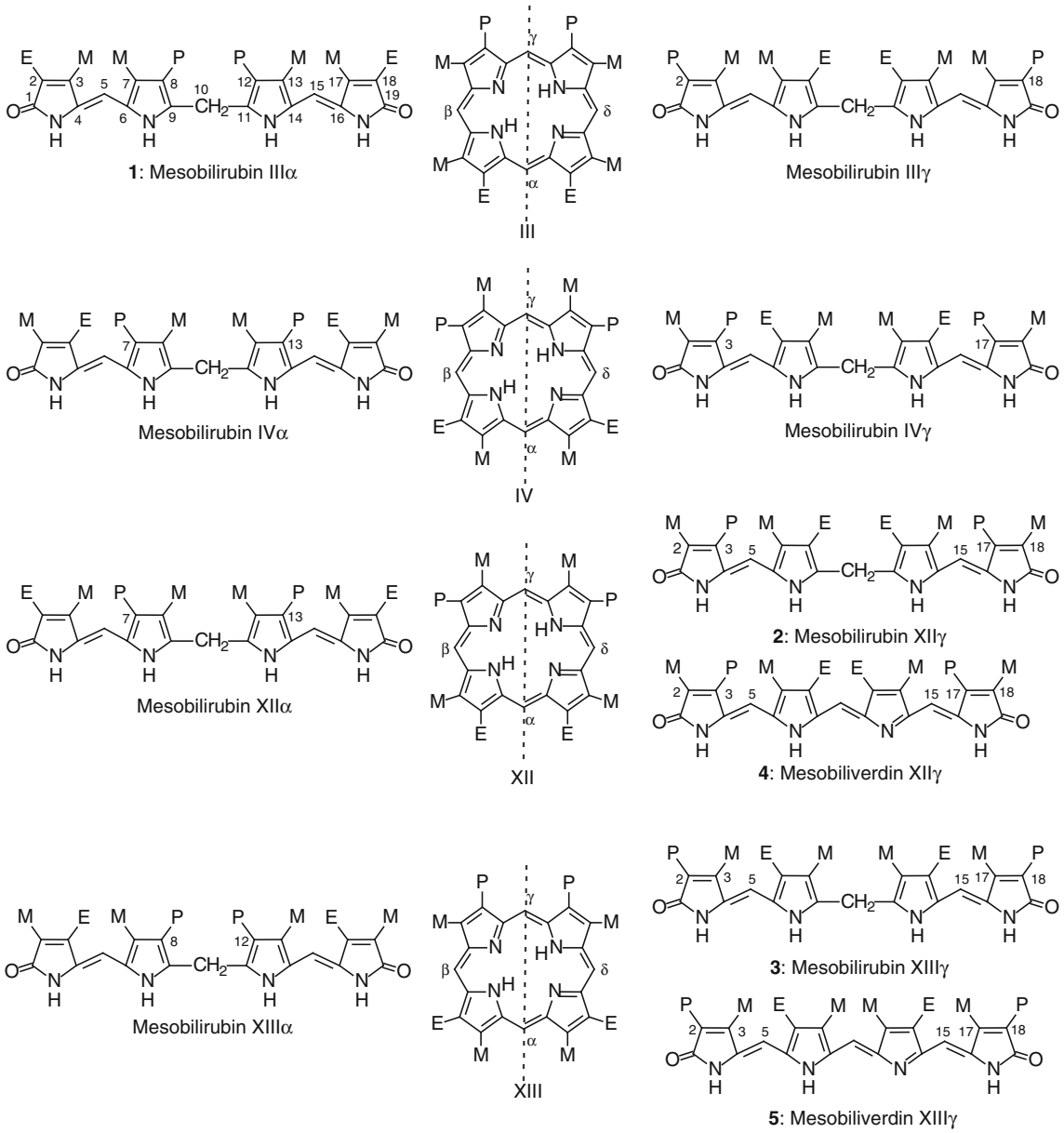


Fig. 1 *Center* the four symmetric mesoporphyrins (III, IV, XII, and XIII) and the corresponding (*left*) mesobilirubins α and mesobilirubins γ due to opening of the macrocyclic ring at and with loss of the α- or γ-carbon. A *dashed line* through the α and γ carbons lies at the intersection of the plane of the macrocycle and an orthogonal plane of symmetry represented by the *dashed vertical line*. *E* ethyl, *M* methyl, *P* propionic acid

of bilirubin IXα and have been used as bilirubin IXα analogues in studies of metabolism, photochemistry, spectroscopy, and pK_a. The key structural element relating these three pigments is the location of their propionic acid groups at carbons 8 and 12, which permits tight intramolecular hydrogen bonding (Fig. 2b). Although elision of the α-carbon of mesoporphyrins III and XIII leads to mesobilirubins fully capable of intramolecular hydrogen bonding, elision of the α-carbon of mesoporphyrins IV and XII does not, because the derived mesobilirubins (IVα and XIIα) have propionic acids located away from C(8) and C(12), at C(7) and C(13). In fact, although mesobilirubin XIIα (Fig. 1) is not known, the properties of synthetic mesobilirubin IVα [5] (Fig. 1) support the contention that the location of the acid groups at C(8) and C(12) is essential for intramolecular hydrogen bonding. It was found to have very different solubility properties and metabolism from bilirubin IXα [7].

Similarly, one can imagine macrocyclic ring opening of symmetric mesoporphyrins III, IV, XII, and XIII at the γ-carbon lying on the out-of-plane symmetry plane to produce the corresponding mesobilirubins IIIγ, IVγ, XIIγ,

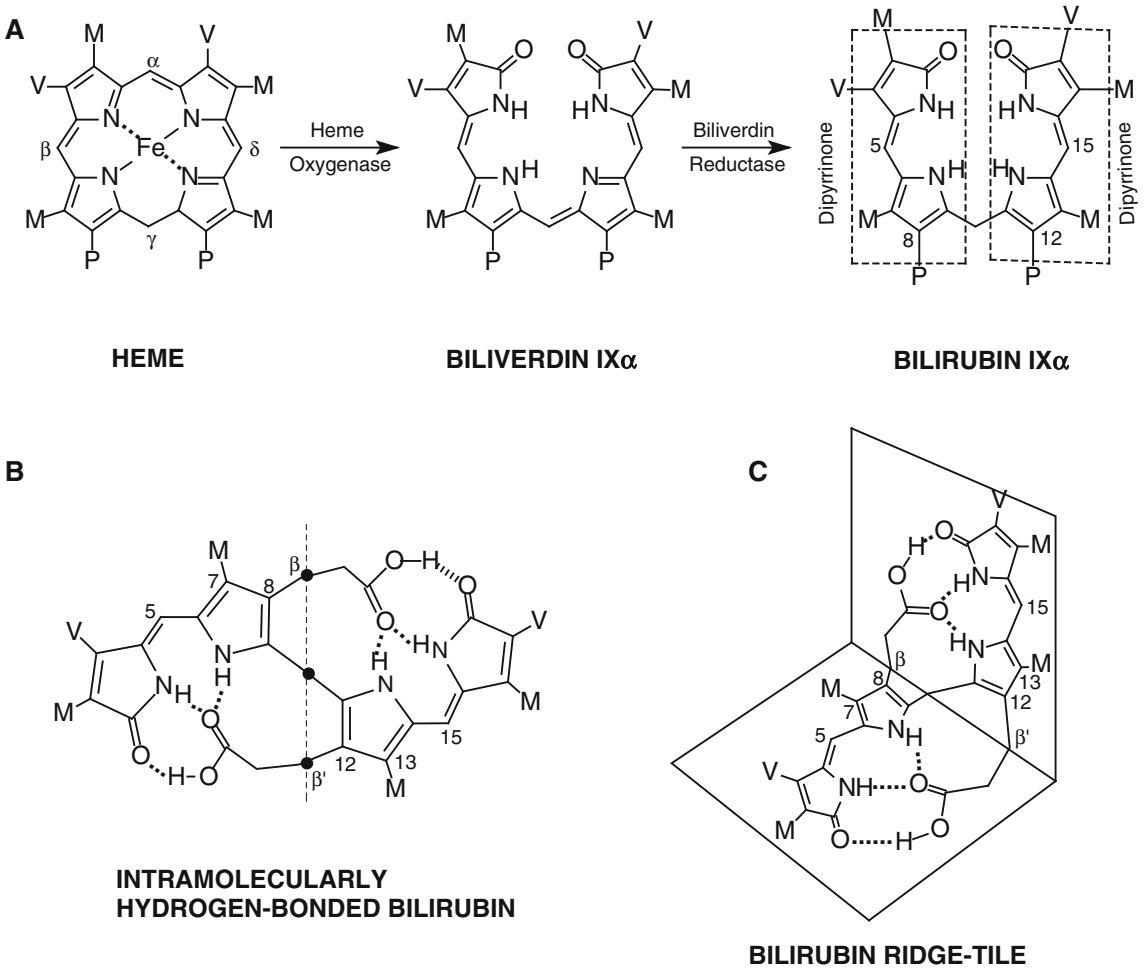


Fig. 2 **a** Biogenetic sequence from heme to 4(*Z*),15(*Z*)-bilirubin shown in a porphyrin-like shape. Rotating the bilirubin dipyrrinones about the C(9)–C(10) and C(10)–C(11) bonds brings them into juxtaposition with the C(8) and C(12) propionic acid groups so as to enable intramolecular hydrogen bonding, as in planar projection (**b**). When the latter is folded along the *vertical dashed line*, the 3D structure of bilirubin seen in the crystal (**c**) emerges with a half-opened book or ridge-tile shape with the seam along the line from the propionic acid β and β′ carbons through C(10). The *dotted lines* in **b** and **c** represent hydrogen bonds. *V* vinyl, *M* methyl, *P* propionic acid

and XIIIγ—all of which would have their propionic acids located on the lactam end rings at C(2)/C(18) or C(3)/C(17). And, like mesobilirubin IVα, neither is expected to engage in intramolecular hydrogen bonding. Note that all of the mesobilirubins γ of Fig. 1 have propionic acid groups located on the end rings; whereas, in the mesobilirubins α they are located on the central two pyrrole rings. Symmetric mesobilirubins with a propionic acid group located on each lactam end ring (IIIγ, XIIIγ; IVγ, XIIγ) had not heretofore been synthesized.

In the following, we describe the syntheses and properties of two new mesobilirubins, XIIγ (**2**) and XIIIγ (**3**), and their mesobiliverdins. Their syntheses provided samples for investigating the importance of hydrogen bonding in hepatic elimination and for the preparation of mesobiliverdins XIIγ and IIIγ (**4** and **5**, respectively), which along with previously prepared mesobiliverdins (XIIIα, IVα) were examined as substrates for biliverdin reductases by Prof. Timothy Mantle at Trinity College, Dublin [8]. We also describe a new, efficient synthesis of mesobilirubin IIIα (**1**), which was first prepared by Siedel and Fischer in 1933 [4] and more recently prepared by catalytic hydrogenation of bilirubin IIIα, which had been isolated following acid-catalyzed constitutional isomerization of bilirubin IXα to a mixture of IIIα, IXα, and XIIIα [9–11].

Results and discussion

Synthetic aspects

Two general strategies for bile pigment synthesis [12] were considered for the preparation of the symmetrical mesobilirubins IIIα, XIIγ, and XIIIγ (**1**–**3**, Fig. 1). The syntheses

of **1** and **2** followed the "1 + 2 + 1" approach, where two equivalents of a monopyrrole were condensed with one dipyrrylmethane. The synthesis of **3** followed the "2 + 2" approach where two equivalents of a dipyrrinone were self-condensed. As indicated above, we had synthesized the XIIIα and IVα isomers earlier [5, 6], and we considered synthesizing mesobilirubin IIIγ and IVγ but our approaches were either unsuccessful or the starting materials for **2** and **3** were more readily obtained.

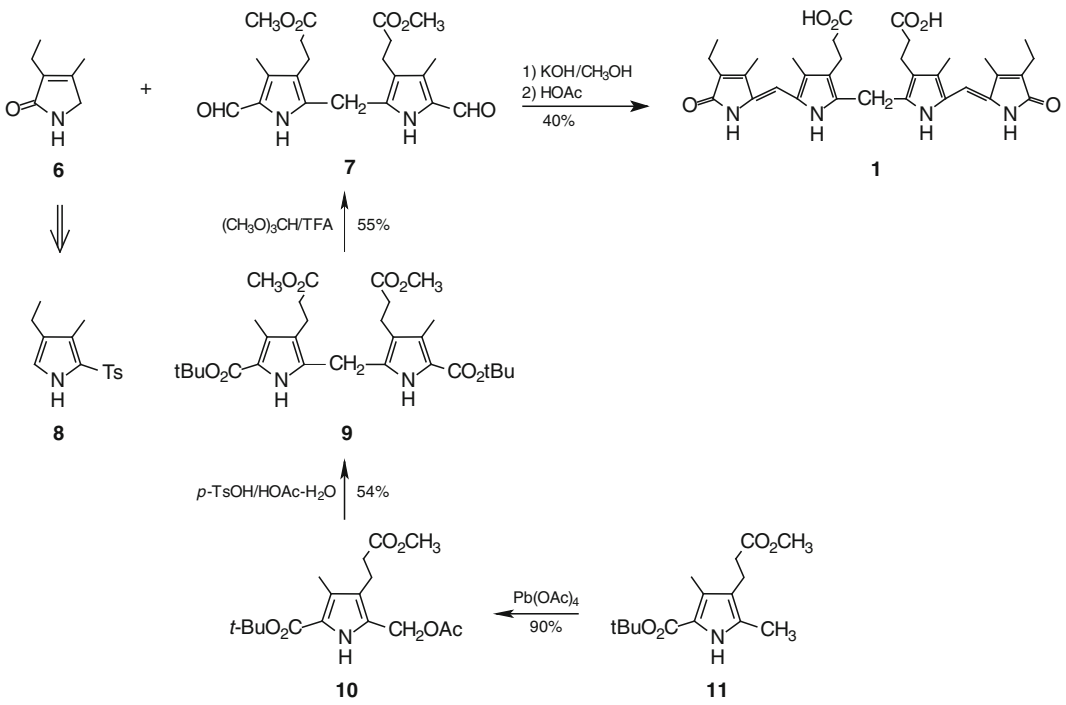
Thus, as outlined in Scheme 1, the synthesis of **1** required pyrrolinone **6** and dipyrrylmethane-dialdehyde **7**. The former was known [13] and had been prepared previously by the Barton–Zard route [14] by first reacting 2-acetoxy-3-nitropropane [14], obtained by acetylating the condensation product of nitroethane with propionaldehyde, then reacting the acetate with *p*-toluenesulfonylmethylisocyanide, prepared by a high yield procedure [15]. The resultant 4-ethyl-3-methyl-2-tosyl-1*H*-pyrrole (**8**) [13] was brominated in CH_2Cl_2 at the α-free carbon using *N*,*N*,*N*-trimethylanilinium bromide perbromide to give the 2-bromo-5-tosylpyrrole [16] in 86 % yield. The latter was hydrolyzed to the 5-tosylpyrrolin-2-one [13] (70 % yield) in H_2O–trifluoroacetic acid and then reduced to pyrrolinone **6** [17] (76 %) using $NaBH_4$.

Dialdehyde **7** was prepared in three steps from the known monopyrrole **11** [18], first by mono-acetoxylation of the α-CH_3 to **10** using $Pb(OAc)_4$ [18] then by solvolysis of **10** in aqueous acetic acid using *p*-toluenesulfonic acid (*p*-TsOH) as catalyst. The resultant dipyrrylmethane tetracarboxylate ester **9** [19] was treated with trifluoroacetic acid to deprotect the *t*-butyl ester groups, decarboxylate the resultant acids, and react with trimethylorthoformate to give dialdehyde **7** [20]. Mesobilirubin IIIα (**1**) was obtained directly from condensation of **7** with **6** in methanolic KOH.

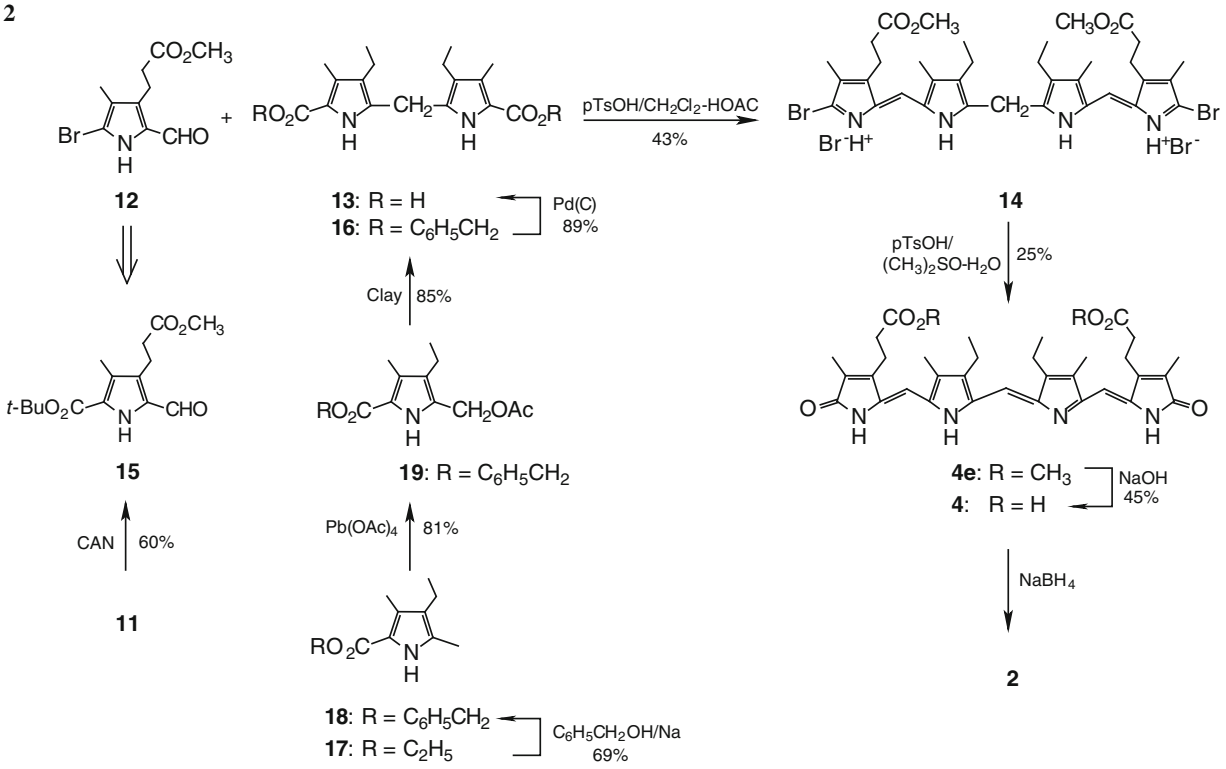
The synthesis of mesobilirubin XIIγ (**2**) also followed the "1 + 2 + 1" approach and involved making a bis(bromodipyrrylmethane) dihydrobromide **14** following the general procedure of Pandey et al. [21] as outlined in Scheme 2. Thus, the known bromopyrrole aldehyde **12** [21] was condensed with the known symmetric dipyrrylmethane dicarboxylic acid **13** [22] under conditions (*p*-TsOH) where the latter underwent double decarboxylation to form a reactive (bis-α-H) dipyrrylmethane intermediate. The resulting dibromotetrapyrrole dihydrobromide **14** was then converted to mesobiliverdin XIIγ dimethyl ester (**4e**) in $(CH_3)_2SO$ + *p*-TsOH + H_2O. The corresponding verdin diacid **4** was obtained by saponification of **4e** and reduced with $NaBH_4$ to yield mesobilirubin XIIγ (**2**).

For preparation of starting monopyrrole **12**, the α-CH_3 of *t*-butyl 3,5-dimethyl-4-(2-methoxycarbonylethyl)-1*H*-pyrrole-2-carboxylate (**11**) [18] of Scheme 1 was oxidized to the α-CHO using ceric ammonium nitrate (CAN) to give **15** [23], and bromine was introduced to yield **12** following the literature procedure [21].

Scheme 1



Scheme 2

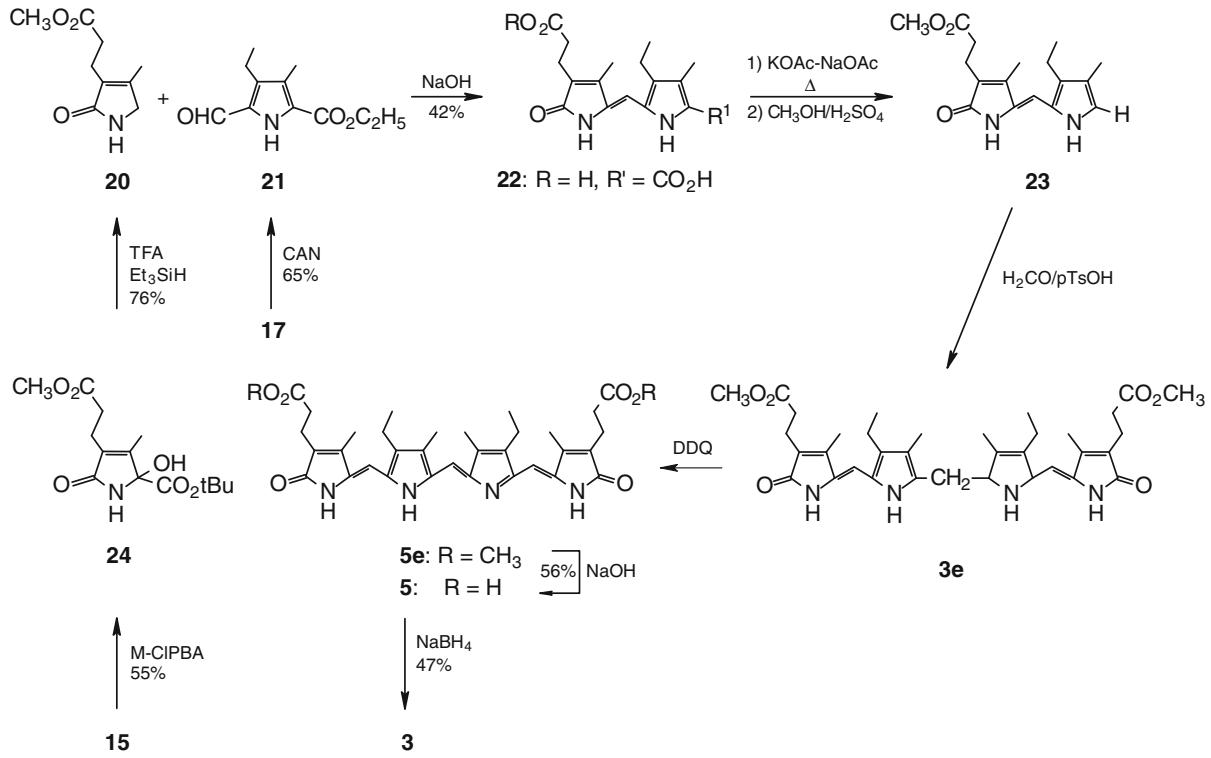


For the preparation of **13**, we found it convenient to debenzylate dibenzyl ester **16** [24], a conversion that proceeded in higher yield than saponifying the corresponding diethyl ester, which we had converted from **17** in a similar way. Dipyrrylmethane dibenzyl ester **16** was obtained following solvolysis of the 5-acetoxymethyl derivative **19** [22, 24] of benzyl 3,5-dimethyl-4-ethyl-1*H*-pyrrole-2-carboxylate (**18**) [24]. Thus, ethyl 3,5-dimethyl-4-ethyl-1*H*-pyrrole-2-carboxylate (**17**) [5, 6, 25] was transesterified to produce the corresponding benzyl ester **18** [26], which was converted to the 5-acetoxymethyl derivative **19** [24] using $Pb(OAc)_4$ in acetic acid. Solvolysis [24, 27–29] of **19** on Montmorillonite clay afforded dibenzyl ester **16** [24] in 85 % yield, which was debenzylated [28] using H_2/Pd(C)/triethylamine in tetrahydrofuran (THF) to yield **13** in 89 % yield.

The synthesis of mesobilirubin XIIIγ (**3**) followed the "2 + 2" approach, as outlined in Scheme 3. Thus, the known pyrrolinone **20** [30, 31] was reacted with pyrrole aldehyde **21** [23] to provide dipyrrinone diacid **22**. The last compound was converted to the α-H dipyrrinone methyl ester **23** in 52 % yield over two steps: (1) α-decarboxylation in molten sodium acetate–potassium acetate [32, 33] to give the mono-acid, then (2) Fischer esterification. Direct self-coupling of α-H dipyrrinone acid of **23** using formaldehyde plus *p*-TsOH or HCl [34, 35] gave the polar, air-sensitive desired product **3**, but it was contaminated with unreacted starting material and other difficult-to-remove impurities. Attempts to purify tetrapyrrole **3** were complicated by the facile formation of its verdin due to adventitious air oxidation. In order to avoid problems in isolating pure **3**, dipyrrinone methyl ester **23** was self-coupled with formaldehyde to produce the dimethyl ester **3e** of mesobilirubin XIIIγ, which was oxidized directly by DDQ to the corresponding verdin dimethyl ester **5e** in 62 % yield over two steps. The more easily purified verdin dimethyl ester **5e** was then saponified to verdin diacid **5**, and the latter was reduced to rubin **3** with $NaBH_4$. This route to **3**, similar to that developed for the synthesis of mesobilirubin IVα (Fig. 1) [36], gave purer final product than direct saponification of **3e**.

Starting material **20** for the synthesis of **3** was prepared from pyrrole diester **11** [18] (of Scheme 1), which was synthesized by condensing the C-alkylation adduct of pentane-2,4-dione and methyl acrylate [37] with the oxime of *tert*-butyl acetoacetate. Elaboration of **11** to **20** proceeded via oxidation using CAN [23] to the 5-formyl derivative **15** [18] of Scheme 2, which underwent a Baeyer–Villiger oxidation using *m*-chloroperoxybenzoic acid to afford *t*-butyl 2,5-dihydro-2-hydroxy-4-(2-methoxycarbonylethyl)-3-methyl-5-oxo-1*H*-pyrrole-2-carboxylate (**24**). The last compound was converted to **20** using $(C_2H_5)_3SiH$

Scheme 3



in TFA. The overall yield from **11** was 16 %. An even higher yield procedure to **20** (28 % overall) has been achieved starting from acetaldehyde and methyl γ-nitrobutyrate [31]. The monopyrrole coupling partner **21** was obtained simply by CAN oxidation [23] of the well-known ethyl 3,5-dimethyl-4-ethyl-1*H*-pyrrole-2-carboxylate (**17**) [25, 35].

Structural aspects and NMR

The constitutional structures of **1**, **2**, and **3** were confirmed by their ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra in $(CD_3)_2SO$ solvent. The data for these pigments are presented in Table 1, along with data for the well-studied pigment mesobilirubin XIIIα [5, 6, 38]. The data show the correct total number of protons and carbons expected for symmetrical molecules. Clear differences are evident among these isomers despite the fact that they all have the same functional groups. The assignments of the methyl groups in the ^{1}H NMR are based on results from homonuclear Overhauser effect (NOE) experiments. Thus, in **1** a strong NOE between the hydrogens at C(5/15) and methyls at C(7/13) distinguishes the latter from the C(3/17)–CH_3. Similarly, in **3** and **5** weak NOEs were found between C(5/15)–H and C(3/17)–CH_3. In **2** and **6**, observation of a weak NOE between the C(5/15)–H CH_3 and the C(3/17)–CH_3 allowed distinction between the C(2/18)–CH_3 and C(7/13)–CH_3. In addition to distinguishing between methyl groups, the geometric structural assignment, particularly the (*Z*)-configuration of the C(4/15) exocyclic double bond of the dipyrrinone moieties has also been confirmed from these experiments. Signals for the methylene protons of the propionic acid chain often overlapped with the methylene signal of the ethyl group. To distinguish one from the other, homonuclear decoupling experiments were performed to clarify the region. The assignment of the ^{13}C spectrum involved ^{1}H-detected heteronuclear multiple-quantum coherence (HMQC) and ^{1}H detected multiple-bond heteronuclear multiple-quantum coherence (HMBC). The former was used to determine the connectivity of groups in the molecule, while the latter was used for long-range coupling.

Hydrogen bonding aspects and NMR

Nuclear magnetic resonance spectroscopy, especially ^{1}H NMR, is also a powerful way of establishing whether a molecule engages in conformations stabilized by intramolecular hydrogen bonds. Analysis of the ^{1}H NMR chemical shifts (δ) of the pyrrole and lactam N–H signals is a direct way of verifying intramolecular hydrogen bonding. Spectra in both $(CD_3)_2SO$ and $CDCl_3$ were analyzed.

Table 1 Carbon and proton NMR chemical shift/ppm assignments in $(CD_3)_2SO$ for mesobilirubins

Atom no.	MBR XIIIα		MBR IIIα (**1**)		MBR XIIγ (**2**)		MBR XIIIγ (**3**)	
Carbon no.[a]	Carbon	Proton	Carbon	Proton	Carbon	Proton	Carbon	Proton
1, 19	171.9	–	171.6	–	171.7	–	171.4	–
2, 18	122.9	–	122.5	–	122.8	–	126.2	–
2^1, 18^1	14.8	2.00(s)	16.3	2.23(q)	8.2	1.7 (s)	19.1	2.44(t)
2^2, 18^2	–	–	13.5	0.99(t)	–	–	32.5	2.38(t)
2^3, 18^3	–	–	–	–	–	–	173.7	12.1(bs)
3, 17	147.1	–	140.9	–	144.2	–	142.1	–
3^1, 17^1	17.2	2.49(q)	9.1	2.05(s)	19.4	2.75(t)	9.4	2.06(s)
3^2, 17^2	8.1	1.08(t)	–	–	34.3	2.41(t)	–	–
3^3, 17^3	–	–	–	–	173.5	12.2(bs)	–	–
4, 16	127.8	–	129.4	–	127.3	–	130.0	–
5, 15	97.7	5.93(s)	98.0	5.93(s)	98.3	6.00(s)	98.3	5.96(s)
6, 14	122.4	–	121.9	–	122.3	–	121.0	–
7, 13	122.0	–	119.2	–	121.8	–	114.9	–
7^1, 13^1	9.2	1.77(s)	9.3	1.99(s)	9.1	2.00(s)	17.1	2.45(q)
7^2, 13^2	–	–	–	–	–	–	61.1	0.99(t)
7^3, 13^3	–	–	–	–	–	–	–	–
8, 12	119.3	–	192.1	–	124.0	–	128.7	–
8^1, 12^1	19.2	2.40(t)	19.2	2.40(t)	16.7	2.20(q)	8.2	1.71(s)
8^2, 12^2	34.6	1.99(t)	34.3	1.95(t)	15.1	0.75(t)	–	–
8^3, 12^3	174.1	11.9(bs)	174.0	11.9(bs)	–	–	–	–
9, 11	130.4	–	130.3	–	130.0	–	130.4	–
10	23.3	3.94(s)	23.5	3.95(s)	23.6	3.92(s)	23.7	3.91(s)
21, 24	–	9.83	–	9.75	–	9.87	–	9.80
22, 23	–	10.34	–	10.32	–	10.31	–	10.30

Run in $(CD_3)_2SO$ at approximately 2×10^{-2} M concentration at 25 °C. The multiplicities of the proton signals are designated in the usual way

[a] The numbering system for the mesobilirubins may be found in **1** of Fig. 1

Typically, in the former solvent, the lactam NH appears between 9.7 and 9.9 ppm, while the pyrrole NH appears between approximately 10.2 and 10.4 ppm—irrespective of whether the pigment is a diacid or diester, or a dipyrrinone [39]. In contrast, in $CDCl_3$, where hydrogen bonding can prevail, the lactam NH chemical shifts of rubin diacids typically lie between approximately 10.6 and 10.8, and the pyrrole NH resonances lie between approximately 9.2 and 9.3 ppm. This pattern generally signifies intramolecular hydrogen bonding of the type in Fig. 2. In contrast, rubin diesters, which typically engage in dipyrrinone to dipyrrinone intermolecular hydrogen bonding, exhibit NH chemical shifts that fall between approximately 10.2 and 10.5 ppm. The ^{1}H NMR data summarized in Table 2 show little distinction between mesobilirubins and their esters in $(CD_3)_2SO$ but large differences in $CDCl_3$. In $CDCl_3$, the dimethyl esters all exhibit similar pyrrole NH and lactam N–H chemical shifts consistent with intermolecular hydrogen bonding. The acids in $CDCl_3$, however, present a rather difference picture. Mesobilirubins XIIIα and IIIα (**1**) exhibit very nearly identical lactam NH chemical shifts and likewise for the pyrrole N–H chemical shifts. The data for both compounds indicate intramolecular hydrogen bonding of the type seen in Fig. 1c and d. Mesobilirubins IVα, XIIγ (**2**), and

Table 2 Comparison of N–H chemical shifts/ppm of mesobilirubins and their dimethyl esters (**e**) in $CDCl_3$ and $(CD_3)_2SO$

Mesobilirubins[a] and esters (**e**)	Chemical shift in $CDCl_3$		Chemical shift in $(CD_3)_2SO$	
	Lactam N–*H*	Pyrrole N–*H*	Lactam N–*H*	Pyrrole N–*H*
IIIα (**1**)	10.59	9.16	9.74	10.31
XIIIα	10.58	9.15	9.77	10.31
IVα	10.85	9.65	9.82	10.36
XIIγ (**2**)	11.72	11.62	9.87	10.31
XIIIγ (**3**)	12.12	12.04	9.80	10.30
XIIIα (**e**)	10.54	10.27	9.74	10.40
IVα (**e**)	10.47	10.27	9.78	10.32
XIIγ (**2e**)	10.64	10.27	9.88	10.30
XIIIγ (**3e**)	10.26	10.26	9.84	10.30

[a] For structures, consult Fig. 1

XIIIγ (**3**) differ significantly. While the predominant conformation of IVα might be folded, but not intramolecularly hydrogen bonded so as to effect diamagnetic anisotropic shielding of the pyrrole NHs each lying above the opposing pyrrole "aromatic" ring, the strong deshielding of both the lactam and pyrrole NHs of **2** and **3** suggest rather strong intermolecular hydrogen bonding, stronger than that seen in their dimethyl esters, and promoted perhaps by their poor solubility in $CDCl_3$.

Solubility aspects

Like mesobilirubin IVα (Fig. 1), both the XIIγ (**2**) and XIIIγ (**3**) pigments are extremely sensitive to light and air oxidation. Hence, the solvents used to prepare the solutions were saturated with nitrogen prior to use and work-up is one under dim light. (It is due to this sensitivity to oxidation that all purifications of the XIIγ and XIIIγ pigments were done at the verdin ester stage.) Once the rubins were obtained, minimum handling of the compounds was necessary since the longer they remain in solution, the more likely they are to become oxidized. The compounds once isolated were sealed under argon and stored in the dark and cold. Though they slowly acquire a greenish tinge, spectral analysis (NMR) of the greenish compounds showed that they were still quite pure. As expected, and in marked contrast, the IIIα pigment (**1**) was much less air-sensitive and behaved more like the XIIIα isomer and bilirubin IXα.

The solubility properties of bilirubin IXα and its various analogues are known to depend considerably on the ability of the molecule to form intramolecular hydrogen bonds [38–41]. It is through engaging in such interactions that the polar propionic acid groups of the molecule are tied up with the dipyrrinone's lactam and pyrrole groups (Fig. 2). This sequestering of the polar groups renders the molecule much less polar and more lipophilic, a property demonstrated by the natural pigment as well as the synthetic analogues, mesobilirubins XIIα and IIIα (**1**) [5, 6, 38, 42]. In contrast **2** and **3**, like mesobilirubin IVα [5], with propionic acid groups located on sites removed from C(8) and C(12), cannot participate in intramolecular hydrogen bonding. Consequently, they are much more polar than **1**, mesobilirubin XIIIα, and bilirubin IXα. The polarity of the pigments may be recognized by their solubility properties. Most simple non-hydrogen-bonded carboxylic acids (e.g., benzoic and naphthoic acids) are soluble in weak bases, e.g., 5 % aq. sodium bicarbonate. Thus, mesobilirubin IV was found to be soluble in 5 % aq. sodium bicarbonate, while bilirubin IXα and mesobilirubins XIIIα and IIIα (**1**) are insoluble. In contrast, bilirubin IXα and mesobilirubins XIIIα and IIIα (**1**) are soluble at 1×10^{-3} M in the nonpolar solvent chloroform; whereas, mesobilirubins IVα, XIIγ (**2**), and XIIIγ (**3**) are insoluble.

These properties are in keeping with intramolecular hydrogen-bonded structures for bilirubin IXα and mesobilirubins XIIIα and IIIα (**1**) but not mesobilirubin IVα. Consistent with the behavior of the last compound and the expectations from an inability of their propionic acid groups to become involved in intramolecular hydrogen bonding with the dipyrrinone lactam and pyrrole moieties, mesobilirubins XIIγ (**2**) and XIIIγ (**3**) were expected and found to dissolve in aq. bicarbonate and be insoluble in $CHCl_3$.

Chromatographic aspects

The relative polarities of bile pigments can also be assessed by their chromatographic properties. In a silica thin-layer chromatography (TLC) system using as eluent $CHCl_3$–CH_3OH (10:1 by vol), mesobilirubin XIIIα has a larger retention factor ($R_f = 0.9$) than the more polar isomer, mesobilirubin IVα ($R_f = 0.3$). Like mesobilirubin XIIIα, its IIIα isomer (**1**) and natural bilirubin are also very fast moving on this TLC system. In contrast, as seen in Table 3, the R_f values of mesobilirubins XIIγ (**2**) and XIIIγ (**3**) all move slower, like mesobilirubin IVα, as reflects their more polar nature. And when converted to their less polar dimethyl esters **2e** and **3e**, they move much faster.

The TLC behavior was consistent with that from the reversed-phase high-performance liquid chromatography (HPLC) system developed by McDonagh et al. [42]. On this reversed-phase HPLC system, more polar pigments tend to elute faster than the less polar. As reference HPLC standards, intramolecularly hydrogen-bonded and thus "non-polar" bilirubin and mesobilirubin XIIIα exhibit approximately 18-min retention times, much longer than mesobilirubin IVα, which because it is incapable of intramolecular hydrogen bonding is more polar and faster moving (Table 3). In contrast, rubin dimethyl esters, which are also incapable of

Table 3 Comparison of TLC retention factors/R_f and HPLC retention times for mesobilirubins

TLC[a] R_f value	Mesobilirubin	HPLC[b] Ret. time/min
1.0	Bilirubin IXα	18.4
0.9	Mesobilirubin XIIIα	17.7
0.3	Mesobilirubin IVα	7.4
1.0	Mesobilirubin IIIα (**1**)	18.8
0.3	Mesobilirubin XIIγ (**2**)	7.2
0.3	Mesobilirubin XIIIγ (**3**)	15.5
0.8	**2e**	5.4
0.9	**3e**	7.2

[a] TLC were run on silica gel TLC plates with $CHCl_3/CH_3OH$, 10:1 (by vol irrigant) from [5]. [b] Eluent: 0.1 *M* di-*n*-octylamine acetate in CH_3OH containing 5 % water, 1.0 cm^3/min flow rate, as per Ref. [42]

intramolecular hydrogen bonding, run faster (like mesobilirubin IVα) and in this HPLC system may be considered to be more polar than the corresponding diacids, as has been observed for the dimethyl esters of bilirubin and mesobilirubin XIIIα. As expected, the HPLC data for **1** (18.8 min) correlates well with that of its XIIIα isomer; whereas, the more polar XIIγ isomer (**2**) (7.19 min) behaves like IVα and the dimethyl esters—consistent with the absence of intramolecular hydrogen bonding. Surprisingly, the XIIIγ isomer (**3**), which was predicted by TLC to behave on HPLC like the IVα and XIIγ isomers, ran slower (15.5 min). This might suggest that while **3** is more polar than the XIIIα and IIIα (**1**) isomers, as expected, it is less polar than the IVα and XIIγ (**2**) isomers—despite the indications from the TLC data. It is unclear whether the divergent HPLC behavior of **2** and **3** is an artifact of this particular reversed-phase solvent system or whether it indicates some type of intramolecular hydrogen bonding in **3**.

UV–Vis absorption and circular dichroism (CD) aspects

Ultraviolet–visible spectroscopy (UV–Vis) can provide valuable information about the conformation of bilirubin analogues in solution and can provide insight into possible intramolecular hydrogen bonding. Because rubin pigments are bichromophores, formed from two dipyrrinone chromophores conjoined to a CH_2 group with minimal or no π-orbital overlap, they constitute molecular excitons. Exciton coupling theory can be a versatile adjunct for the interpretation of their UV–Vis data [43, 44]. Thus, as revealed in Table 4, where it is clear that mesobilirubins XIIIα and IIIα (**1**) have nearly identical long wavelength UV–Vis absorption characteristics across the range of solvents, and their XIIγ (**2**) and XIIIγ (**3**) isomers also display characteristics nearly identical to each other, the two sets of pigments display only a partial qualitative similarity. In $CHCl_3$, a solvent that promotes hydrogen bonding, mesobilirubins XIIIα and IIIα exhibit an intense absorption with λ^{max} near 431 nm. These pigments are known to adopt an intramolecularly hydrogen-bonded ridge-tile conformation (as in Fig. 1c) in $CHCl_3$ [44–47]. Rather differently, **2** and **3** exhibit two well-defined λ^{max}, one near 420 nm and a more intense absorption at $\lambda^{max} \approx 390$ nm (Table 4). The data for **2** and **3** are reminiscent of rubin dimethyl esters, which are known to be intermolecularly hydrogen-bonded dimers in $CHCl_3$ [47] (for leading reference, see [48]). The data from CD studies are even more telling.

Circular dichroism [43] is a powerful adjunct for providing insight into the conformations of bilirubin and its analogues [44, 49]. The exciton coupling model is once again suitable for interpreting the CD data of the bichromophoric rubins. Owing to the isotropic (unpolarized) nature of the light beam in UV–Vis spectroscopy, the two exciton transitions exhibit the same sign. When the splittings are not widely separated, the substantial overlap often gives rise to broad, non-symmetrical UV–Vis bands. With polarized light, however, the exciton splitting leads to two transitions with oppositely signed Cotton effects (CEs), as seen in Table 5. According to exciton chirality theory [43, 44, 49], a negative dihedral angle formed by the electric transition moments lying along the long axis of the dipyrrinone chromophores (see Fig. 3) predicts a negative first (longer-wavelength) CE and a positive (shorter-wavelength) second CE, i.e., a (−) exciton chirality. The signals are predicted to be most intense when the dihedral angle is close to 100°, as seen in folded conformations such as the *M*-helical ridge-tile bilirubin shown Fig. 3. The mirror image *P*-helical conformation of ridge-tile bilirubin would be expected to express a (+) exciton chirality.

The (−) exciton chirality of mesobilirubins XIIIα and IIIα (**1**) in $CHCl_3$ in the presence of quinine (Table 5) is

Table 4 Solvent dependence of UV–Vis absorption data for mesobilirubins (pigment concentration = 1.5×10^{-5} M)

Solvent	XIIIα		IIIα (**1**)		XIIγ (**2**)		XIIIγ (**3**)	
	λ^{max}	ε^{max}	λ^{max}	ε^{max}	λ^{max}	ε^{max}	λ^{max}	ε^{max}
Benzene	435	58,800	435	55,300	427	31,700	424	26,990
	417sh	54,700	417sh	51,500	396	36,850	393	38,800
$CHCl_3$	431	57,800	432	58,000	420	24,600	424	32,400
					390	41,800	394	51,350
CH_3CN	426	56,500	424	54,200	418	20,700	420	18,900
	390sh	46,300			375	38,200	386	45,100
CH_3OH	426	50,700	426	55,000	431	35,300	426	34,600
	401sh	43,100			340	37,600	397	38,950
$(CH_3)_2SO$	426	57,000	426	58,150	432	44,200	429	40,000
	397sh	49,100	395sh	48,250	398	35,100	396	34,400

λ^{max}/nm, ε^{max}/dm^3 mol^{-1} cm^{-1}

Table 5 Induced circular dichroism data for mesobilirubins in $CHCl_3$ solvent + quinine (1:200 pigment/quinine molar ratio) and in 0.1 M phosphate buffer + human serum albumin (1:2 pigment/HSA molar ratio)

Mesobilirubin	CD in $CHCl_3$ in quinine			CD on HSA		
	$\Delta\varepsilon_1$ (λ_1)	λ at $\Delta\varepsilon = 0$	$\Delta\varepsilon_2$ (λ_2)	$\Delta\varepsilon_1$ (λ_1)	λ at $\Delta\varepsilon = 0$	$\Delta\varepsilon_2$ (λ_2)
XIIIα	−61 (433)	405	+42 (389)	+57 (442)	410	−62 (391
IIIα (**1**)	−69 (431)	404	+40 (386)	+89 (423)	396	−61 (388)
IVα	+8 (438)	402	−7 (380)	+18 (439)	–	+9 (365)
XIIγ (**2**)	−45 (439)	395	+70 (371)	+105 (437)	406	−65 (387)
XIIIγ (**3**)	+10 (438)	418	−15 (388)	+16 (437)	416	−23 (394)

λ/nm, $\Delta\varepsilon$/dm^3 mol^{-1} cm^{-1}

As in Refs. [48, 50]

interpreted in terms of intramolecularly hydrogen-bonded conformers (Fig. 2) as *M*- and *P*-helical interconverting conformational enantiomers (*M* and *P*) with the *M*-helical predominating [44, 49]. In buffered HSA [49, 50], the signs invert, and the major conformation is *P*-helical. In the absence of chiral perturbation, the two isoenergetic conformations are in dynamic equilibrium, with equal amounts of the two enantiomers present, and the pigment is thus a racemic mixture of conformational enantiomers and does not exhibit optical activity.

In contrast, mesobilirubin IVα, which cannot adopt the intramolecularly hydrogen-bonded conformation of Fig. 2, gives only a weak monosignate curve in aqueous buffered HSA [49, 50] and a weaker (+) exciton chirality spectrum in $CHCl_3$ + quinine (Table 5) [49]. The isomeric mesobilirubins XIIγ (**2**) and XIIIγ (**3**), which likewise cannot adopt the intramolecularly hydrogen-bonded conformations of Fig. 3, were expected to behave similarly to IVα and exhibit weak CD Cotton effects. Although such behavior was seen for **3**, in contrast, **2** gave an intense bisignate CD Cotton effect with (−) exciton chirality in $CHCl_3$ + quinine and an even more intense (+) exciton chirality in aq. buffered HSA. Clearly the data suggest that the chiral complexation action of quinine that selects for the *M*-helical conformers of mesobilirubin XIIIα and IIIα (**1**) also selects for the same conformation of **2** even in the absence of intramolecular hydrogen bonding due to relocation of the propionic acids from C(8)/C(12) to C(3)/C(17), but not when they are relocated to the ends of the pigment at C(2)/C(18). Likewise, the binding pocket on HSA that selects for the *P*-helical conformations of XIIIα and IIIα (**1**) also selects strongly for the same (*P*)-helicity enantiomer of **3**. Clearly, chiral complexing agents exert much less *M*- vs. *P*-helical selectivity in IVα and XIIIγ (**3**), but why this is not the case for **2** is unexplained.

Hepatic metabolism

Bilirubin IXα, the cytotoxic yellow pigment of jaundice, is produced in mammals by catabolism of heme (Fig. 2). Insoluble in water, lipophilic, and protein-bound in plasma, its hepatobiliary elimination requires hepatic acyl glucuronidation by the phase 2 isozyme-specific glucuronosyl transferase (UGT1A1), which appears to select the pigment's intramolecularly hydrogen-bonded propionic acids for conjugation [51]. Earlier studies [7, 52] showed that bilirubin and mesobilirubin XIIIα are glucuronidated and eliminated in (normal) Sprague–Dawley (SD) rats, but are not glucuronidated and eliminated intact in the mutant (jaundiced) UGT1-deficient Gunn rat, which as in the rare, recessive Crigler–Najjar syndrome of humans, has a congenital deficiency of the glucuronosyl transferase enzyme. In contrast, mesobilirubin IVα, which is polar and not intramolecularly hydrogen bonded, is excreted intact in both types of rat. Consequently, we expected mesobilirubin IIIα to require glucuronidation for transhepatic excretion into bile; whereas, mesobilirubin XIIγ and XIIIγ were expected to be excreted intact in, e.g., a Gunn rate [53]. In rat metabolism studies conducted by the late Prof. Antony F. McDonagh (G.I. Unit, University of California, San Francisco Medical School) using methods described in Ref. [51], it was shown that mesobilirubin IIIα (**1**) was excreted into bile as a mixture of mono- and diglucuronides in the SD rat but not excreted in the Gunn rat. In contrast, mesobilirubin XIIγ (**2**) was excreted rapidly intact in both the SD and Gunn rat. Similarly, the XIIIγ isomer (**3**) was excreted intact in the Gunn rat, along with a minor metabolite having a verdin UV–Vis spectrum. Peculiarly, in the SD rat **3** was excreted partly unchanged and partly as a monoglucuronide and a trace of diglucuronide. The unexpected formation of glucuronides of **3** in the SD rat would seem to suggest partial intramolecular hydrogen bonding, as suggested by the reversed-phase HPLC retention time (Table 3).

Concluding comments

The synthesis of mesobilirubins IIIα, XIIγ, and XIIIγ gave evidence to their distinctive physical, spectroscopic, and

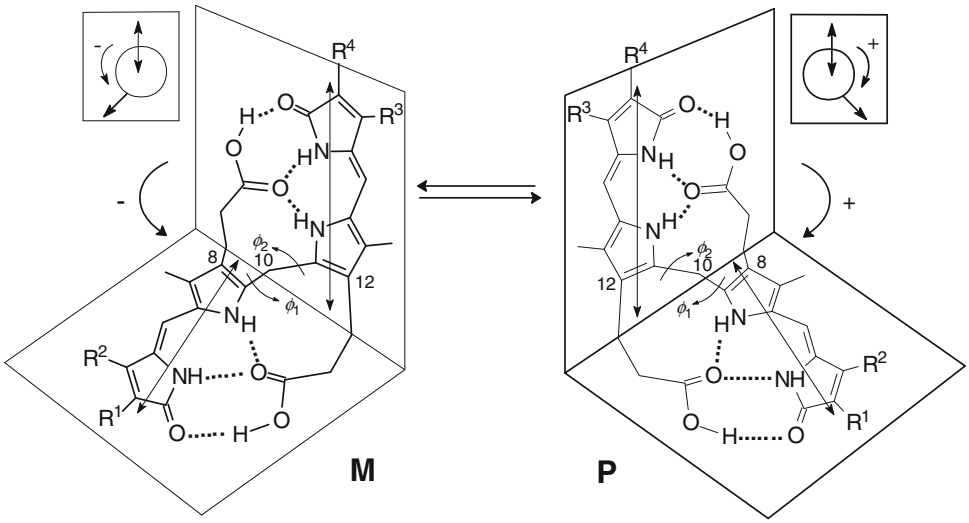


Fig. 3 Intramolecularly hydrogen-bonded enantiomeric conformations (**M** and **P**) of bilirubin ($R^1 = R^3 = M$, $R^2 = R^4 = V$), mesobilirubin IIIα **1** ($R^1 = R^4 = E$, $R^2 = R^3 = M$), and mesobilirubin XIIIα ($R^1 = R^4 = M$, $R^2 = R^3 = E$), folded into ridge-tile shapes. The (**M** ⇆ **P**) interconversion is accomplished by rotating the dipyrrinones about ϕ_1 and ϕ_2. The dipyrrinone chromophores are planar, and the angle of intersection of the two planar dipyrrinones is approximately 100° for $\phi_1 \approx \phi_2 \approx 60°$. The *double-headed arrows* represent the approximate direction and intensity of the dipyrrinone long-wavelength electric transition dipole moments. The relative orientations or helicities (*M* minus, *P* plus) of those vectors are shown (*inset*) for each enantiomer. The (−) dipole helicity correlates with *M*-molecular chirality and the (+) helicity with *P*-molecular chirality

metabolic properties based on the ability to form intramolecular hydrogen bonds. The corresponding verdins served as substrates for distinguishing and defining structural features conducive to reduction (to the corresponding rubins) by biliverdin IXα reductase and biliverdin IXβ reductase [8].

Experimental

All NMR spectra were obtained on a GE QE-300 MHz (^{1}H) or a Varian 500 MHz (^{1}H) and 125 MHz (^{13}C) spectrophotometer, respectively, in deuteriochloroform unless otherwise indicated. Chemical shifts were reported in ppm referenced to the residual chloroform proton signal at 7.26 ppm and ^{13}C signal at 77.23 ppm unless otherwise noted. Melting points were taken on a Mel-Temp capillary apparatus and are corrected. Elemental analyses, obtained from Desert Analytics, Tucson, Arizona, were with ±0.4 % of the calculated values. High resolution determinations of molecular ions were obtained from the University of Nebraska Mass Spectrometry Facility. All UV–Vis spectra were recorded on a Perkin-Elmer λ-12 spectrophotometer and CD spectra were recorded on a JASCO-600 instrument. Analytical TLC was carried out on J.T. Baker silica gel IB-F plates (125 μm layer). Flash column chromatography was carried out using 60–200 mesh silica gel (M. Woelm, Eschwege). For final purification, radial chromatography was carried out on 1 or 2 mm thick rotors of Merck silica gel PF_{254} with calcium sulfate binder, preparative layer grade, using a Chromatotron (Harrison Research, Inc., Palo Alto, CA). All solvents were reagent grade obtained from Fisher-Acros.

The spectral data were obtained in spectral-grade solvents (Aldrich or Fisher). Quinine was from Aldrich; HSA, fatty and free, was from Sigma; $Pb(OAc)_4$ was from Aldrich; CAN was from Fisher. Starting monopyrroles (**8**, **11**, **17**, **21**) were synthesized according to the literature methods [13, 18, 23, 25, respectively].

Mesobilirubin IIIa (**1**, $C_{33}H_{40}N_4O_6$)

In a 10 cm^3 round-bottom flask were placed 125 mg diformyldipyrrylmethane **7** (0.31 mmol) [20], 0.389 mg pyrrolinone **6** (3.1 mmol), and 6.2 cm^3 4 M methanolic potassium hydroxide solution. The solution was heated at reflux under N_2 for 6 h. Then 15 cm^3 H_2O was added to dilute the reaction mixture. After cooling to 0 °C, the solution was acidified using 10 % aq. HCl. The resultant precipitate was collected by filtration and triturated with 5 cm^3 CH_3OH. Purification by radial chromatography was repeated twice, each time using $CHCl_3$ as the eluent. The desired product **1** was collected in the first fraction. This was followed by a fraction containing 23 mg (0.05 mmol) of the monocoupled product in 16 % yield. Yield of **1**: 75 mg, 0.13 mmol (40 %); m.p.: 282–284 °C (dec); IR (KBr): $\bar{\nu}$ = 3,418, 3,247, 2,965, 2,915, 1,698, 1,648, 1,452, 1,250 cm^{-1}; ^{1}H and ^{13}C NMR in Table 1; UV–Vis in Table 4; CD in Table 5.

Mesobilirubin XIIγ (**2**, $C_{33}H_{40}N_4O_6$)
The predecessor verdin ester **4e** (0.15 g, 0.26 mmol) was dissolved in 25 cm^3 CH_3OH and 25 cm^3 THF under an atmosphere of N_2. The mixture was cooled in an ice bath at 0 °C before 0.7 g sodium borohydride (18.5 mmol) was added over a period of 30 min, after which the reaction was quenched with 50 cm^3 water. The aqueous solution was washed with CH_2Cl_2 (3 × 20 cm^3) to remove nonpolar impurities before acidifying the solution with 10 % aq. HCl to precipitate the product. Centrifugation followed by washings with water (3 × 20 cm^3), filtration, and drying in a vacuum desiccator afforded **2**. Yield: 56 mg, 0.10 mmol (37 %); m.p.: 162–163 °C (dec); IR (KBr): $\bar{\nu}$ = 3,421, 2,961, 1,654, 1,460 cm^{-1}; ^1H and ^{13}C NMR in Table 1; UV–Vis in Table 4; CD in Table 5. FAB-HRMS: *m/z* calcd for $C_{33}H_{40}N_4O_6$ 588.294785, found 588.2926; calcd for $C_{33}H_{40}N_4O_6Na$ 611.284555, found 611.2841.

Mesobilirubin XIIIγ (**3**, $C_{33}H_{40}N_4O_6$)
The precursor verdin **5** (0.150 g, 0.26 mmol) was dissolved in 15 cm^3 CH_3OH and 15 cm^3 THF under an atmosphere of N_2. The mixture was cooled in an ice bath at 0 °C before 0.7 g sodium borohydride (18.5 mmol) was added over a period of 30 min. As soon as the color turned to yellow-brown, the reaction was quenched with 30 cm^3 water. The aqueous solution was washed with CH_2Cl_2 (3 × 20 cm^3) to remove nonpolar impurities before acidifying the solution with 10 % aq. HCl to precipitate the product. Centrifugation followed by washings with water (3 × 30 cm^3), filtration, and drying in a vacuum desiccator afforded rubin **3**. Yield: 71.4 mg, 0.12 mmol (47 %); m.p.: 176–177 °C (dec); IR (KBr): $\bar{\nu}$ = 3,346, 2,964, 2,921, 1,671, 1,459 cm^{-1}; ^1H and ^{13}C NMR in Table 1; UV–Vis in Table 4; CD in Table 5. FAB-HRMS: *m/z* calcd for $C_{33}H_{40}N_4O_6$ 588.294785, found 588.2950; calcd for $C_{33}H_{40}N_4O_6Na$ 611.284555, found 611.2848.

Mesobiliverdin XIIγ (**4**, $C_{33}H_{38}N_4O_6$)
All solvents were purged with N_2 before use. In a 250 cm^3 round-bottom flask were placed 60 mg verdin dimethyl ester **4e** (0.098 mmol), 60 cm^3 of a 1:1 (by vol) THF–CH_3OH solution, 40 cm^3 0.2 M aq. NaOH solution, 10 mg ascorbic acid, and 10 mg EDTA. The dark blue solution was stirred at 37 °C for 4 h and diluted with 100 cm^3 water. The organic solvents were removed using a rotary evaporator. The aqueous solution was cooled to 0 °C before acidification using 10 % aq HCl. The resulting precipitate was collected by centrifugation, washed with water (2 × 50 cm^3), filtered, and again washed with water and CH_2Cl_2. After drying in a lyophilizer, verdin acid **4** was obtained. Yield: 26 mg, 0.044 mmol (45 %); m.p.: 170–175 °C (dec); IR (KBr): $\bar{\nu}$ = 3,222, 2,957, 2,921, 1,697, 1,594, 1,220 cm^{-1}; ^1H NMR ($(CD_3)_2SO$): δ = 12.15 (1H, bs, CO_2H), 9.86 (1H, bs, NH), 6.82 (1H, s, C(10)H), 6.01 (2H, s, C(5,15)H), 2.70 (4H, t, J = 6.3 Hz, $CH_2CH_2CO_2H$), 2.58 (4H, q, J = 6.8 Hz, CH_2CH_3), 2.43 (4H, t, J = 6.3 Hz, CH_2CH_2,CO_2H), 2.02 (6H, s, CH_3), 1.68 (6H, s, CH_3), 1.06 (6H, t, J = 6.8 Hz, CH_2CH_3) ppm; ^{13}C NMR ($(CD_3)_2SO$): δ = 173.5, 171.9 (C=O), 149.4, 143.4, 141.0, 139.8, 139.7, 128.8, 126.9 (Ar), 115.1 (C(10)), 96.3 (C(5,15)), 33.8 ($CH_2CH_2CO_2H$), 19.2 ($CH_2CH_2CO_2H$), 17.1 (CH_2CH_3), 16.2 (CH_2CH_3), 9.0, 8.3 (CH_3) ppm; FAB-HRMS: *m/z* calcd for $C_{33}H_{38}N_4O_6$ 586.279135, found 586.2766.

Mesobiliverdin XIIγ dimethyl ester (**4e**, $C_{35}H_{42}N_4O_6$)
In a 500 cm^3 round-bottom flask were placed 0.62 g 2-bromo-5-formyl-4-(2-methoxycarbonylethyl)-3-methylpyrrole (**12**, 1.95 mmol), 0.3 g 3,3′-diethyl-4,4′-dimethyl-2,2′-dipyrrylmethane-5,5′-dicarboxylic acid (**13**, 0.94 mmol), 60 cm^3 methanol, and 280 cm^3 CH_2Cl_2. As soon as the solutes were completely dissolved, a solution of 1.17 g *p*-toluenesulfonic acid (6.15 mmol) in 60 cm^3 CH_3OH was added. The solution was stirred at room temperature overnight and then washed with 570 cm^3 H_2O, 150 cm^3 saturated aq. $NaHCO_3$, and again with 280 cm^3 water. After drying over anhyd. Na_2SO_4, the solvent was removed using a rotary evaporator. The residue was dissolved in benzene, which was removed under vacuum. This process was repeated three more times before the residue was crystallized from ether to give dihydrobromide salt of **14**. Yield: 0.36 g, 0.4 mmol (43 %). It was used directly in the next step.

In a 1 dm^3 round-bottom flask were placed 0.36 g of the preceding tetrapyrrole **14** (0.4 mmol) and 800 cm^3 dimethyl sulfoxide. The solution was stirred at room temperature for 1.5 h, during which the color of the solution changed from red to black-blue. The reaction solution was poured into 6 dm^3 ice cold water. The aqueous solution was divided into four portions and each portion was extracted with diethyl ether (3 × 150 cm^3). The organic phases were combined and washed with water before drying over anhyd. Na_2SO_4. After separation, the solvent was removed by a rotary evaporator, and the black residue was purified by three successive flash chromatography runs first using 4 % (vol) CH_3OH in CH_2Cl_2, then three times using increasing amounts of ethyl acetate in CH_2Cl_2 (30, 50, 60 %). The dark blue band was collected. Removal of the solvent and recrystallization of the residue in CH_2Cl_2/hexane gave pure verdin diester **4e**. Yield: 60 mg, 0.098 mmol (25 %); m.p.: 201–202 °C; IR (KBr): $\bar{\nu}$ = 3,384, 2,960, 1,735, 1,690, 1,582, 1,212 cm^{-1}; ^1H NMR: δ = 8.20 (3H, bs, NH), 6.69 (1H, s, C(10)H), 5.96 (2H, s, C(5,15)H), 3.70 (6H, s, CO_2CH_3), 2.82 (4H, q, J = 7.6 Hz, CH_2CH_3), 2.59 (4H, t, J = 7.6 Hz, $CH_2CH_2CO_2CH_3$), 2.55 (4H, t, J = 7.46 Hz, $CH_2CH_2CO_2CH_3$), 2.08 (6H, s C_7–CH_3), 1.80 (6H, s, C_2–CH_3), 1.17 (6H, t, J = 7.6 Hz, CH_2CH_3) ppm; ^{13}C NMR:

δ = 172.9, 172.1 (C=O), 149.9, 142.7, 141.5, 141.0, 139.2, 129.7, 127.3 (Ar), 114.2 (C(10)H), 96.7 (C(5,15)H), 51.7 (CO_2CH_3), 33.6 ($CH_2CH_2CO_2CH_3$), 19.6 ($CH_2CH_2CO_2CH_3$), 17.7 (CH_2CH_3), 16.0 (CH_2CH_3), 9.2 (CH_3), 8.3 (CH_3) ppm.

Mesobiliverdin XIIIγ (**5**, $C_{33}H_{38}N_4O_6$)

Solvents were purged with nitrogen for 30 min before use. In a 100 cm^3 round bottom flask were placed 42.6 mg verdin dimethyl ester **5e** (0.07 mmol), 40 cm^3 THF/CH_3OH (1:1 by vol) solution, 30 cm^3 0.2 M aq. NaOH solution, and 10 mg ascorbic acid. The dark blue solution was stirred at 40–42 °C for 4 h and was washed with CH_2Cl_2 (2 × 100 cm^3). The aqueous layer was cooled to 0 °C before it was acidified using 10 % aq. HCl. The product precipitated and was collected by centrifugation and filtration. It was washed with large amounts of water and CH_2Cl_2 and lyophilized to give verdin **5**. Yield: 22.8 mg, 0.04 mmol (56 %); m.p.: 205–207 °C; IR (KBr): $\bar{\nu}$ = 3,387, 2,909, 1,738, 1,692, 1,586, 1,225 cm^{-1}; ^{1}H NMR: δ = 10.09 (2H, bs, lactam NH), 7.16 (1H, bs, C(10)H), 6.07 (2H, s, C(5,15)H), 3.60 (6H, s, CO_2CH_3), 2.59 (4H, q, J = 7.6 Hz, CH_2CH_3), 2.47 (8H, m, $CH_2CH_2CO_2CH_3$), 2.36 (6H, s, C(8,12)CH_3), 2.13 (6H, s C(3,17)CH_3), 1.15 (6H, t, J = 7.6 Hz, CH_2CH_3) ppm; ^{13}C NMR: δ = 173.1, 160.9 (C=O), 146.5, 143.7, 142.4, 135.7, 132.9, 131.8, 116.6 (Ar), 51.6 ($CH_2CH_2CO_2CH_3$), 31.8 ($CH_2CH_2CO_2CH_3$), 19.0 ($CH_2CH_2CO_2CH_3$), 17.7 (CH_2CH_3), 14.8 (CH_2CH_3), 10.2 (C(8,12)CH_3), 9.8 (C(3,17)CH_3) ppm; FAB-HRMS: *m/z* calcd. for $C_{33}H_{38}N_4O_6$ 586.279135, found 586.2778.

Mesobiliverdin XIIIγ dimethyl ester (**5e**, $C_{35}H_{42}N_4O_6$)

In a 25 cm^3 round bottom flask equipped with a magnetic stir bar were placed 15 cm^3 CH_2Cl_2, 50 mg paraformaldehyde (1.67 mmol), and 40 mg *p*-toluenesulfonic acid (0.21 mmol). Note: Paraformaldehyde is not very soluble in CH_2Cl_2; it was allowed to stir in the solvent with *p*-toluenesulfonic acid before adding in **23**. Thus, the mixture was allowed to stir for 1.5 h. Then, 400 mg α-H dipyrrinone methyl ester **23** (1.32 mmol) was added. The mixture was allowed to stir at room temperature in the absence of light for 16 h and an atmosphere of N_2. The course of the reaction was followed by TLC. Upon completion of the reaction, the mixture was diluted with 100 cm^3 CH_2Cl_2. Then it was washed successively with water (2 × 100 cm^3), saturated aq. $NaHCO_3$ (2 × 100 cm^3), and again water (1 × 100 cm^3). After drying over anhyd. Na_2SO_4, the solvent was removed. The residue of **3e** was dried in a vacuum desiccator and was used in the next step without purification.

In a 250 cm^3 round bottom flask were placed 246.4 mg of the crude **3e** (0.4 mmol) from above, 120 cm^3 dry THF, and 90.8 mg DDQ (0.4 mmol). The solution turned blue instantaneously. It was allowed to react for 30 min; then the deep blue solution was poured into a 1 dm^3 separatory funnel containing 400 cm^3 CH_2Cl_2 and 400 cm^3 2 % aq. ascorbic acid. The aqueous layer was extracted two more times with CH_2Cl_2 (2 × 100 cm^3). The organic fractions were combined and washed repeatedly with saturated aq. Na_2CO_3, until the basic solution ceased to turn yellow. This was followed by another washing with water, drying over anhyd. Na_2SO_4, and removal of the solvent. The black-blue precipitate was redissolved in the minimum amount of CH_2Cl_2 and was chromatographed to isolate the desired blue verdin product **5e**, which was recrystallized from CH_2Cl_2/hexane. Yield: 152 mg, 0.25 mmol (62 %); m.p.: 228–229 °C; IR (KBr): $\bar{\nu}$ = 3,387, 2,909, 1,738, 1,692, 1,586, 1,220 cm^{-1}; ^{1}H NMR: δ = 10.09 (2H, bs, lactam NH), 7.16 (1H, bs, C(10)H), 6.07 (2H, s, C(5,15)H), 3.60 (6H, s, CO_2CH_3), 2.59 (4H, q, J = 7.6 Hz, CH_2CH_3), 2.47 (8H, m, $CH_2CH_2CO_2CH_3$), 2.36 (6H, s, C(8,12)CH_3), 2.13 (6H, s, C(3,17)CH_3), 1.15 (6H, t, J = 7.6 Hz, CH_2CH_3) ppm; ^{13}C NMR: δ = 173.1, 160.9 (C=O), 146.5, 143.7, 142.4, 135.7, 132.9, 131.8, 116.6 (Ar), 51.6 ($CH_2CH_2CO_2CH_3$), 31.8 ($CH_2CH_2CO_2CH_3$), 19.0 ($CH_2CH_2CO_2CH_3$), 17.7 (CH_2CH_3), 14.8 (CH_2CH_3), 10.2 (C(8,12)CH_3), 9.8 (C(3,17)CH_3) ppm.

3,3′-Diethyl-4,4′-dimethyl-2,2′-dipyrrylmethane-5,5′-dicarboxylic acid (**13**, $C_{17}H_{22}N_4O_4$)

To 5 g benzyl 5-acetoxymethyl-4-ethyl-3-methyl-1*H*-pyrrole-2-carboxylate (**19**, 15.87 mmol) [24] in 110 cm^3 CH_2Cl_2, 22 g Montmorillonite K-10 clay was added, and the mixture was allowed to react for 30 min at room temperature. When TLC indicated completion of the reaction, the clay was removed by filtration, followed by washings with 100 cm^3 CH_2Cl_2. Evaporation of the solvent on a rotary evaporator gave a cream-colored residue. Flash chromatography and recrystallization from CH_3OH gave **16**. Yield: 3.37 g, 6.77 mmol (85 %); m.p.: 125–127 °C (Ref. [21] 126–127 °C).

Dibenzyl ester **16** (5 g, 10.04 mmol) was placed in a 2 dm^3 round-bottom flask together with 1 dm^3 THF, 0.28 cm^3 diethylamine, and 1.11 g 10 % palladium on carbon. The suspension was hydrogenated at room temperature and one atmosphere overnight while following the reaction by TLC. The catalyst was removed by filtration through a bed of Celite. The solvent was removed, and the reddish residue was recrystallized from THF–hexane. Yield: 2.85 g, 8.96 mmol (89.3 %); m.p.: 126–128 °C (dec); ^{1}H NMR ($(CD_3)_2SO$): δ = 11.0 (2H, s, NH), 3.73 (2H, s CH_2), 2.33 (4H, q, J = 7.3 Hz, CH_2CH_3), 2.14 (6H, s, CH_3), 0.87 (6H, t, J = 7.3 Hz, CH_2CH_3) ppm; ^{13}C NMR ($(CD_3)_2SO$): δ = 162.4 (C=O), 129.9, 124.7, 122.1, 117.3 (Ar), 21.7 (CH_2), 16.6 (CH_2CH_3), 15.2 (CH_2CH_3), 10.0 (CH_3) ppm.

1,10-Dihydro-2-(carboxyethyl)-7-ethyl-3,8-dimethyl-1-oxodipyrrin-9-carboxylic acid (**22**, $C_{17}H_{20}N_2O_5$)

In a 50 cm^3 round-bottom flask equipped with a magnetic stir bar were placed 0.6862 g pyrrolinone **20** (3.75 mmol) [30, 31], 0.5225 g formylpyrrole **21** (2.5 mmol) [23], 1.21 g KOH (21.6 mmol), and 9 cm^3 CH_3OH. The reaction mixture was stirred at room temperature overnight under an atmosphere of N_2. The solvent was then removed on a rotary evaporator, and the residue was redissolved in 10 cm^3 water and 1 cm^3 CH_3OH and then heated to reflux for 1 h. A small amount of solid remained undissolved; so, the solution was filtered before it was cooled to 0 °C then acidified with glacial acetic acid. The precipitate was collected and triturated using CH_3OH to give the desired yellow dipyrrinone **22**. Yield: 0.347 g, 1.05 mmol (42 %); m.p.: 211–212 °C; ^{1}H NMR ($(CD_3)_2SO$): $\delta = 12.17$ (2H, bs, CO_2H), 10.93 (1H, s, NH), 10.56 (1H, s, NH), 5.89 (1H, s, C(5)H), 2.48 (4H, m $CH_2CH_2CO_2CH_3$), 2.38 (2H, q, $J = 7.1$ Hz, CH_2CH_3), 2.17 (3H, s, CH_3), 2.05 (3H, s, CH_3), 0.98 (3H, t, $J = 7.1$ Hz, CH_2CH_3) ppm; ^{13}C NMR ($(CD_3)_2SO$): $\delta = 173.3$, 171.9, 162.0 (PyrC=O), 142.4, 133.7, 129.4, 128.7, 126.4, 125.0, 121.7 (Ar), 96.0 (C(5)H), 32.2 ($CH_2CH_2CO_2CH_3$), 18.9 ($CH_2CH_2CO_2CH_3$), 16.6 (CH_2CH_3), 15.4 (CH_2CH_3), 9.8 (CH_3), 9.1 (CH_3) ppm.

1,10-Dihydro-7-ethyl-3,8-dimethyl-2-(methoxycarbonylethyl)-1-oxodipyrrin (**23**, $C_{17}H_{22}N_2O_3$)

In a 5 cm^3 round-bottom flask were placed a mixture of 0.147 g dipyrrylmethane diacid **22** (0.443 mmol), 0.658 g potassium acetate, and 0.614 g sodium acetate. The mixture was placed in an oil bath heated to 120 °C; then the bath temperature was increased further until bubbles were observed in the reaction flask at 130 °C. The reaction was allowed to proceed for 20 min more until the temperature reached 180 °C. After allowing the mixture to cool to room temperature, 10 cm^3 water was added to the yellow residue. The aqueous mixture was acidified with concentrated hydrochloric acid to pH 2. The greenish-yellow precipitate of the propionic acid of **23** was collected by filtration, washed with large amounts of cold water, and used in the next step without further purification. ^{1}H NMR ($(CD_3)_2SO$): $\delta = 12.19$ (1H, bs, CO_2H), 10.50 (1H, bs, NH), 9.79 (1H, bs, NH), 6.75 (1H, s, Ar–H), 5.98 (1H, s, C(5)H), 2.49 (4H, m, $CH_2CH_2CH_3$), 2.42 (2H, q, $J = 7.1$ Hz, CH_2CH_3), 2.09 (3H, s, C(3)CH_3), 1.97 (3H, s, C(8)CH_3), 1.03 (3H, t, $J = 7.1$ Hz, CH_2CH_3) ppm; ^{13}C NMR ($(CD_3)_2SO$): $\delta = 173.6$, 171.3 (C=O), 142.1, 129.6, 128.7, 126.7, 122.9, 120.2, 117.4 (Ar), 98.1 (C(5)H), 32.5 ($CH_2CH_2CO_2CH_3$), 19.0 ($CH_2CH_2CO_2CH_3$), 16.9 (CH_2CH_3), 15.9 (CH_2CH_3), 9.7, 9.3 (CH_3) ppm.

In a 500 cm^3 round bottom-flask were placed 800 mg of the propionic acid of dipyrrinone **23** (2.78 mmol), 250 cm^3 CH_3OH, and 7 cm^3 2 N sulfuric acid. The mixture was heated to reflux temperature under an atmosphere of nitrogen. After 30 min CH_2Cl_2 was added and the acid was removed by washing the organic solution with saturated aq. $NaHCO_3$. The solvent was removed using a rotary evaporator, and the residue was purified by flash chromatography using 5–8 % CH_3OH in CH_2Cl_2 by vol as an eluent. Recrystallization from CH_3OH–H_2O gave methyl ester **23**. Yield: 435 mg, 1.44 mmol (52 %); m.p.: 188–189 °C; IR (KBr): $\bar{\nu} = 3{,}387$, 3,000, 1,730, 1,672, 1,176 cm^{-1}; UV–Vis: λ^{max} (ε) = 392 (32,400) nm (mol^{-1} dm^3 cm^{-1}); ^{1}H NMR: $\delta = 11.2$, 10.38 (1H, bs, NH), 6.84 (1H, d, $J = 2.4$ Hz, Ar–H), 6.18 (1H, s, C(5)H), 3.69 (3H, s, CO_2CH_3), 2.66 (4H, m, $CH_2CH_2CH_3$), 2.57 (2H, q, $J = 7.6$ Hz, CH_2CH_3), 2.18, 2.06 (3H, s, CH_3), 1.13 (3H, t, $J = 7.6$ Hz, CH_2CH_3) ppm; ^{13}C NMR: $\delta = 173.5$, 173.4 (C=O), 143.4, 131.6, 129.1, 125.8, 123.4, 122.1, 118.8 (Ar), 102.2 (C(5)H), 51.6 (CO_2CH_3), 32.7 ($CH_2CH_2CO_2CH_3$), 19.5 ($CH_2CH_2CO_2CH_3$), 17.8 (CH_2CH_3), 16.1 (CH_2CH_3), 10.0, 9.8 (CH_3) ppm; FAB-MS: *m/z* calcd for $C_{17}H_{22}N_2O_3$ 302.4, found 302.4.

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