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REACTIVITY OF PYRROLE PIGMENTS. Part 11[1]: ON THE STRUCTURE OF CHRYSINS AND OTHER RELATED BILE PIGMENTS

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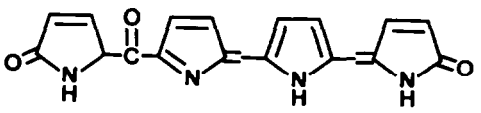
Abstract. The so-called chrysins described by Lemberg and Lockwood in 1939, which are obtained by treatment of biliverdin Zn^{++} complexes with alcoholic I_2, are demonstrated to be 5'-(pyrrol-2-ylmethyl)-5(1H)-pyrromethenones. Mesobilipurpurin XIIIα dimethyl ester (619nm) described by Siedel and Frõwis in 1940, which was obtained by treatment of mesobiliverdin XIIIα dimethyl ester with nitric acid containing nitrogen oxides, is demonstrated to be 5-nitromesobiliverdin XIIIα dimethyl ester. According to Siedel's results and the already known fragmentation pathway of such nitro derivatives, the structure of tripyrrinone-14-carbaldehyde is proposed for bilipurpurins of the keto type.

Some bile pigments with interesting structures have been described from the oxidative degradation of biliverdins (bilatrienes-abc) and bilirubins (biladienes-ac). In spite of having been reported at an early stage, these bile pigments are poorly studied and it has already been suggested that, taking advantage of the modern techniques of structural determination, more work should be performed in order to establish their correct structures[2]. The substances in question are the so-called bilipurpurins (keto or dialkoxy type), bilichrysins, and choletelins (diketo or tetraalkoxy type). Scheme 1 shows the basic skeleta described in the literature for them.

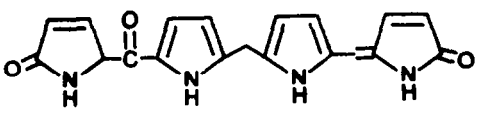
Bilichrysin and mesobilichrysin obtained from biliverdin IXα (1a) and mesobiliverdin IXα (1b), respectively, by treatment of their Zn^{++} complexes with alcoholic I_2, were described as structurally unidentified pyrrole pigments by Lemberg[3] in 1939. However, chrysins have also been isolated among the degradation products of the autoxidation in the dark of bilirubin IXα. To our knowledge chrysins have not been reinvestigated. Tentative structures for chrysins have been suggested in the literature[2,4]: type Ib (ref.[4] does not agree with the elemental analysis reported by Lemberg[3]; type Ia (ref.[2]) would be expected to give rise to a purpurin electronic spectrum, similar to biladienes-ab, i.e. a violinoid structure with three π conjugated rings as in the so-called violins and rhodins[2]. On the contrary the absorption spectra described for chrysins (gold colour as the name indicates) more closely resembles that of systems with two conjugated end rings (biladienes-ac), i.e. 5(1H)-pyrromethenones or bilirubins. In this work we present a structure for chrysins in agreement with the established knowledge in optical properties of bile pigments, and relate the chrysins to the bilipurpurins[3,5b].

The so-called purpurins (red - violet) and choletelins (yellow) had been studied by Siedel[5] in order to understand the transformations that bile pigments suffer in the Gmelin reaction. This work is an important, but early, study on bile pigment reactivity: because of its early date, criticism arises for some of the proposed structures. Purpurins and choletelins of the alkoxy type are certainly correct structures, because they have been reported several times in the



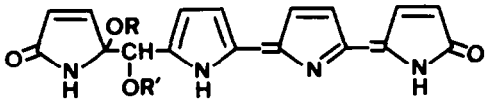
Ia

Bilipurpurin(keto type)*. Chrysin**



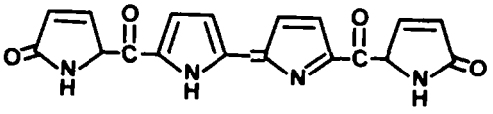
Ib

Chrysin***



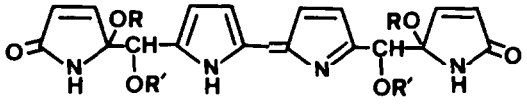
II

Bilipurpurin(alkoxy type)*.



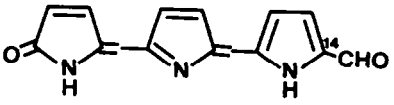
III

Choletelin (keto type)*.



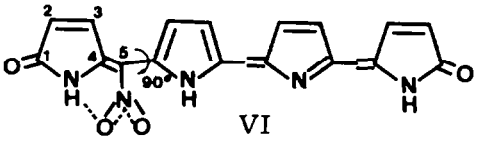
IV

Choletelin(alkoxy type)*



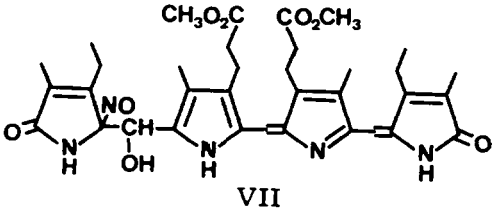
V

Tripyrrinone 14-carbaldehyde



VI

5-Nitrobiliverdin



VII

Mesobilipurpurin XIIIα dimethyl ester (619 nm)*

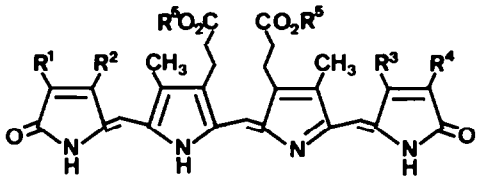
* ref.[5]. ** ref.[2]. *** ref.[4]

SCHEME 1

literature[6,7b] after Siedel: they have always been obtained by the reaction of a biliverdin with bromine in alcohol. We have already reported[8] that such compounds appear after regiospecific electrophilic addition (bromine at C5 and alkoxy group at C4) and subsequent nucleophilic substitution of the bromine by an alkoxy group. Siedel reported also a purpurin with a hydroxy group at the bridge carbon atom C5 (see scheme 1) and an alkoxy group at carbon atom C4; such a structure can also be explained through this reaction pathway. However, bromhydrin addition, i.e. a hydroxy group on C4, would lead to a fragmentation giving the corresponding maleimide. Bilipurpurins and choletelins of the keto type were obtained by reaction of mesobiliverdin XIIIα dimethylester (1c) with nitric acid containing nitrogen oxides: the isolation of the bilipurpurin XIIIα dimethyl ester (619 nm) - defined by its absorption maximum in presence of Zn^{++} - from this reaction has been described by Siedel, who propose for them structure VII (scheme 1). Such a structure is a very improbable one since tautomerism would lead to fragmentation of the terminal ring. Furthermore, it is known that in experimental conditions similar to those reported by Siedel, nitro derivatives with structure of type VI are obtained[7,8]. Bonnett has shown that the electronic spectrum of such nitro derivatives is of the violinoid type owing to the 90° twist of the terminal ring, which is due to the configuration at the exocyclic double bond and the internal hydrogen bond between the nitro group and the lactam hydrogen[7]. Consequently, a structure of type VI is expected for mesobilipurpurin XIIIα dimethyl ester (619 nm) a structure of type VI and this is demostrated in the present paper.

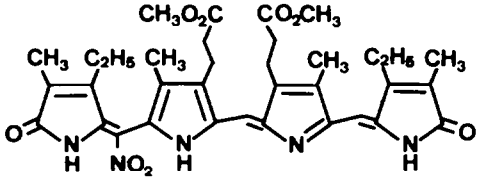
Mesobilipurpurin XIIIα Dimethyl Ester (619 nm: Zn^{++})

The Siedel procedure to obtain the title compound is based on the treatment of mesobiliverdin XIIIα dimethyl ester (1c) in a $CHCl_3$ solution with a sodium nitrite-nitric acid mixture. However, since this procedure was described for 1 g of starting substance, we have instead followed a

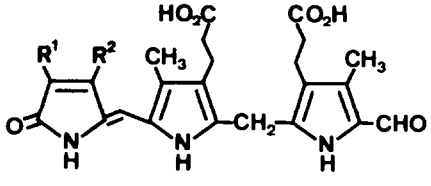


1

	R^1	R^2	R^3	R^4	R^5
a	CH_3	C_2H_3	CH_3	C_2H_3	H
b	CH_3	C_2H_5	CH_3	C_2H_5	H
c	CH_3	C_2H_5	C_2H_5	CH_3	CH_3
d	CH_3	C_2H_5	C_2H_5	CH_3	H



2



3

	R^1	R^2
a	CH_3	C_2H_3
a′	C_2H_3	CH_3
b	CH_3	C_2H_5
b′	C_2H_5	CH_3

FORMULA SCHEME

semimicro (30 mg) method[9] already adapted from ref.[7]. In this way we have obtained in small yield the corresponding nitro derivative 2, which was identified spectroscopically, on the basis of the known analogous derivatives[7,9]. The identity of 2 with the mesobilipurpurin described by Siedel[5b] is based on a comparison of the absorption band maxima in zinc acetate solution. The behaviour of the title compound in the presence of Zn^{++} agrees with our nitro derivative, i.e. 619 nm maximum, and appearance of a band at 670 -690 nm after long standing. Moreover, addition of iodine to the Zn^{++} solution produces a shift with time of the band at 619 nm towards 627 nm. (See experimental part for a detailed comparison).

Bonnett[7a] has already proposed that such nitro derivatives are Gmelin reaction intermediates, and we have demonstrated that such nitro derivatives fragment in the Gmelin reaction to give the cyclic imide and the corresponding tripyrrinone-14-carbaldehyde (structure V in scheme 1), following the Nef reaction[9]. In consequence reasonable doubts arise about the structure of the mesopurpurins of the keto type: Siedel obtained them[5a] after a similar treatment of the corresponding biliverdin with nitrating mixture and final column chromatography in Al_2O_3. In such conditions degradation of the nitro derivative in the terms described in ref.[9] is not to be excluded. Siedel himself considered a tripyrrolic structure for mesobilipurpurin XIIIα dimethyl ester (622nm), and in fact the corresponding tripyrrinone aldehyde ($C_{28}H_{33}N_3O_6$) agrees better than

the tetrapyrrolic keto structure with the elemental analysis, especially with the methoxy group analysis (Zeisel). Following the same reasoning, choletelins of keto type are probably pyrromethene dicarbaldehydes.

TABLE. MESOBILICHRYSIN AND BILICHRYSIN DATA FROM THE LITERATURE) (a) AND FROM THIS WORK (b)*

		MESOBILICHRYSIN (3b + 3b')	*BILICHRYSIN (3a +3a')*
M.p.	*a*	*dec. 240° C*	
	b	*dec. 235° C*	*dec. 225-235° C*
HPLC	*b*	*CH_3CN/H_2O, NH_4AcO 0.2 N (75:25; 1 ml/min)*	*CH_3CN/H_2ONH_4AcO 0.2 N (70:30; 1 ml/min)*
	(t_R)	*23.06 and 24.36 min (two peaks)*	*4.55 and 5.51 min (two peaks)*
Elemt. Analysis	*Calc.*	*$C_{26}H_{31}N_3O_6$ C, 64.85; H, 6.44; N, 8.73*	*$C_{26}H_{29}N_3O_6$ C, 65.12; H, 6.10;N, 8.76*
	Found		
	a	*C, 64.94; H, 6.51; N, 9.25*	
	*b**)*	*C, 64.79; H, 6.10; N, 8.62*	
MS (FAB) (m/e)	*b*	*(Xe; DTT); 482 (M^++1), 481(M^+)*	*(Xe; DTT); 480 (M^++1), 479 (M^+)*
UV-VIS	*a*	*(CH_3OH); 416*	
$\lambda_{max}^{nm}(\varepsilon)$	*b*	*($CHCl_3$); 417 (11700), 312 (6800)*	*($CHCl_3$); 440 (10500), 312 (6500)*
*^1H-NMR**) (δ,ppm)*	*b*	*($CDCl_3$); 1.06(t,3H; $\underline{CH_3}$-CH_2 exo), 1.14(t,3H,$\underline{CH_3}$-CH_2 endo),1.88 and 2.09(two s,two CH_3),2.16 and 2.29 (two s,two CH_3), 2.36-2.88(m,20H -CH_2-), 4.04(s,4H,bridge CH_2), 6.06 and 6.07(two s,2H,=CH-),9.28 (s,2H,-CHO), 9.13, 10.3 and 10.6 (broad s).*	*($CDCl_3/CD_3OD$); 1.99, 2.16, 2.17, 2.18(3s, each 3H,4 CH_3), 2.33(s,6H,two CH_3), 2.65--2.89(m,16H,-CH_2), 4.06(s,4H,bridge CH_2), 5.34-6.71 (m,6H,two ABX:exo H_A=5.37, H_B= 6.11, H_X=6.51, J_{AB}=2 Hz, J_{AX}=11.4 Hz, J_{BX}=17.8 Hz: endo H_A=5.61, H_B=5.62, H_X= 6.64, J_{AB}=1.6 Hz, J_{AX}=12 Hz, J_{BX}=17 Hz), 6.18(s,1H,=CH;exo C5),6.25(s,1H,=CH;endo C5), 9.34 (s, -CHO).*

**) ref.[3]. **) determined on pure 3b.*

Chrysins

We have repeated several times the chrysin preparations described in the literature, our results and yields being similar to those indicated by Lemberg[3]. The table shows the data obtained for bilichrysin and mesobilichrysin, and compares them with the data originally reported. Chrysins appear as by-products in the reaction of the corresponding biliverdin Zn^{++} complex with alcoholic iodine. The reaction product -or products- first obtained have violin type electronic spectra (i.e. three conjugated rings). These violins are partially transformed during the work-up into chrysins (yellow gold colour) and into other more stable violinoids, which are the principal products of the reaction. "Violin" formation during the reaction is clearly manifested in the electronic spectra, and in the characteristic electronic spectra and fluorescence of the Zn^{++} complexes. Lemberg's experimental procedure is analogous to an old chemical test for biliverdins[10], which accounts for such changes of the visible spectra: the electronic spectra of biliverdin Zn^{++} complexes change, after I_2 addition, to the typical spectrum with red fluorescence of the violin Zn^{++} complexes. In fact, the reaction is so fast and quantitative that such treatment can be considered as a biliverdin titration (see experimental part). In the absence of Zn^{++} free biliverdins react with alcoholic I_2 giving the same initial change on the electronic

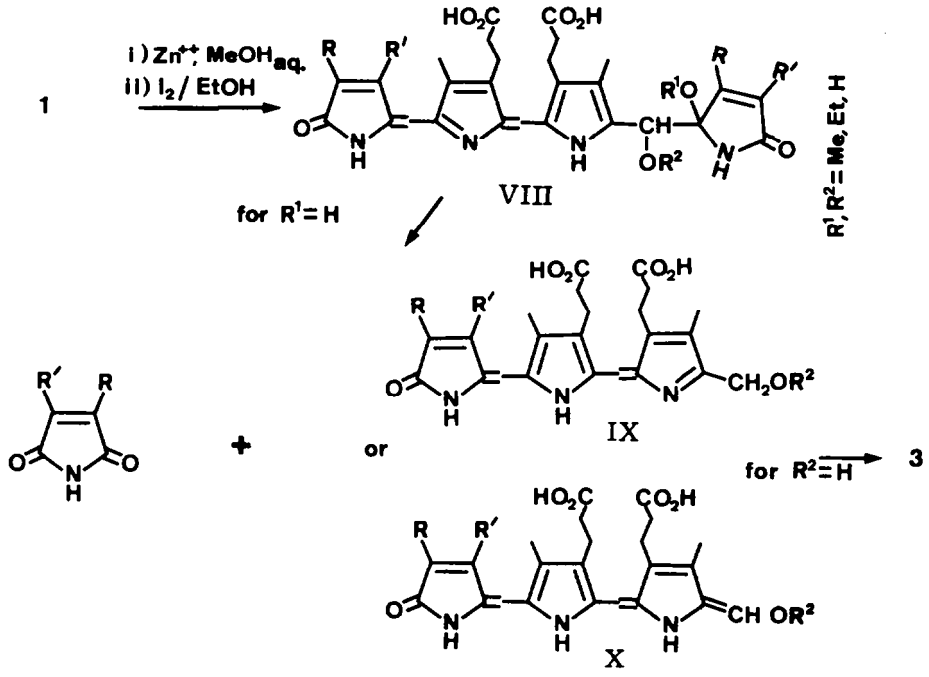
spectra, but slowly. As Siedel had already reported[5b] iodine, unlike bromine, does not react with biliverdins: our results agree with this statement. Zn^{++} enhances the reactivity towards electrophiles of the biliverdin system, but does not seem to modify qualitatively the basic reactivity of the biliverdin π skeleton.

The electronic spectra (see table) give the more striking characteristic of chrysins, i.e. typical spectra of a bile pigment with two conjugated rings, which cannot be explained through a 5(1H)-pyrromethenone or bilirubin type of structure, because the elemental analyses (see table) do not agree with any reasonable di- or tetrapyrrolic structure. The UV-Vis spectra of bilichrysin and mesobilichrysin show also in their absorption band the typical effect of the substitution of vinyl by ethyl groups . Furthermore, chrysins do not have basic nitrogen (in contrast to violins chrysins cannot be extracted from the organic phase with acid), which is also in agreement with a pyrromethenone or a rubin like type of structure. HPLC analysis of the chrysin obtained from 1a and 1b shows that they each consist of a mixture of two substances -not easily resolved chromatographycally - approximately in a 1:1 ratio. The ^{1}H-NMR spectra (200 MHz) of the mixture of these two substances (see table) allow the structural identification of the chrysins. They are the mixture of the two possible tripyrrolic 5'-[(5-formyl-1H-pyrrol-2-yl)methyl]-5(1H)-pyrromethenones of structure 3 (3a + 3a' for bilichrysin and 3b + 3b' for mesobilichrysin) obtained by degradation of one of the terminal rings of the constitutionally asymmetric tetrapyrrolic bile pigment (IXα substitution pattern). Such a structure agrees with Lemberg's results for the elemental analysis, and is confirmed by mass spectrometry (FAB). We have corroborated such results by obtaining pure mesobilichrysin 3b from mesobiliverdin XIIIα (1d), which can only give, owing to its symmetric constitution, a unique chrysin. The spectroscopic and physical data for this agree with those obtained for the original mixture of mesobilichrysins (see table and experimental part). As a complementary structural test, an analytical amount of chrysin 3b was oxidized with dichlorodicyanoquinone: the "violin" obtained showed the electronic spectrum expected[11,12] for the corresponding tripyrrinone carbaldehyde (V) (CH_2Cl_2: λ_{max}; 535, 504, 480 inf, and 320 nm; relative intensity 0.32, 0.34, 0.25, and 1.00 respectively).

The chrysin structure (3) accounts for the easy oxidation to "violin" type pigments and also for the absence of basic nitrogen (behaviour in front of acids[3]). Structures of type 3 implicate the existence of fragmentation processes, which have also been corroborated by us by the identification of the corresponding maleimides in the reaction mixtures. It is proposed (Scheme 2) that chrysins are formed by tautomerization of a tripyrrolic system (e.g. IX or X in scheme 2), which in turn is obtained from a tetrapyrrolic violin (VIII scheme 2; biladiene-ab; purpurin structure) by fragmentation. We have already described the formation of such purpurins through regiospecific electrophilic addition at the terminal ring exocyclic double bond followed by nucleophilic substitution[8]. In the case of "hydroxy" purpurins (VIII and R^1=H) with a hydroxy group at carbon atom C4, fragmentation can occur giving the corresponding maleimide and IX or X. Scheme 2 represents only a formal mechanism, because the structure of the Zn^{++} complexes is unknown. Similar oxidative fragmentations of bilin-1,19-diones to tripyrrinone 14-carbaldehyde and maleimide are already reported in the literature[11,12].

Tautomerization to chrysin (from IX or X) must be thermodynamically favoured, because of its similarity to a violin-rubin tautomerization process. However, it cannot be excluded that in the presence of Zn^{++} the thermodynamically favoured form could be violinoid IX or X, because chrysin absorption bands appear only in the work-up procedure after the elimination of zinc. Equilibrium between biladiene-ac and biladiene-ab seems to be always displaced towards the bilirubin system (biladiene-ac), but probably in special situations the relative stability can be reversed: e. g. by Zn^{++} complex formation. This interpretation must be taken into account to explain the old test for rubins consisting in the formation of a typical violin zinc complex spectrum by addition of traces of iodine to a bilirubin/Zn^{++} mixture[10]. A rubin to violin tautomerization, made possible through the stabilization by protonation of the violin form, has recently been described[13]. More work is in progress to study such a fragmentation pathway, and the identity of the intermediate violins.

The electronic spectra of chrysins show their absorption band at an intermediate position between that of single dipyrrolic 5(1H)-pyrromethenones and that of two pyrromethenone coupled spectra, i.e. bilirubins. The band at 312 nm, which is independent of the presence of vinyl or ethyl substituents (see table), agrees with the absorption band of alkyl susbstituted pyrrole-2-carbaldehyde[14].



SCHEME 2

As far as we know, bile pigment structures of type 3 have not been described in the literature. Furthermore, in tetrapyrrolic pigment reactions which implicate oxidative fragmentation of a terminal ring (e.g. singlet oxygen degradations), unidentified yellow coloured compounds (choletelin fraction[5a]) having an electronic absorption analogous to pyrromethenones or rubins are often detected. These compounds could be structurally more related to the here described pyrrol-2-ylmethyl 5' substituted pyrromethenones described here than to dipyrrolic systems.

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EXPERIMENTAL PART

Melting points were determined on a Kofler-Reichert microhot stage apparatus. HPLC was carried out on RadialPak silica or C18 columns with a Waters double pump using a variable wavelength detector 5FA 339. Preparative HPLC (PHPLC) at the semimicro scale was carried out through repetitive injection using the same system and conditions as for analytical HPLC. UV/Vis spectra were recorded on a Perkin Elmer Lambda 5 instrument. IR spectra were recorded on a Perkin Elmer 681 spectrometer. MS on a Hewlett-Packard 5988A instrument equipped for FAB analysis with a Capillaritron Frasor. ^{1}H-NMR spectra were determined on a Varian XL-200 (200 MHz) instrument.

Synthesis and properties of following compounds are described in the literature: Biliverdin IXα (1a)[15]; Mesobiliverdin IXα (1b) [16] was obtained from mesobilirubin IXα[17] following ref.[15]; Mesobiliverdin XIIIα dimethyl ester (1c)[18] from mesobiliverdin XIIIα (1d) following ref.[19], which was obtained from mesobilirubin XIIIα[19,20].

5-Nitromesobiliverdin XIIIα dimethyl ester (4): (Z,Z,Z)-8,12-Bis(2-methoxycarbonylethyl)-3,17-diethyl-5-nitro-2,7,13,18-tetramethyl-21,24-dihydro-bilin-1,19-dione

To a stirred solution of 33 mg of 3 in 9 ml tetrahydrofuran - acetic acid (3:1), at 0° and under Ar atmosphere, are slowly added 79 mg sodium nitrite in 4 ml water (Ar saturated). The reaction is followed by UV-Vis (disapparence of 343 and 610 nm absorption maxima, and appearence of 319 and 530 nm peaks). After 90 min, 20 ml water are added to the red solution, and it is extracted with 20 ml CH_2Cl_2, washed with 20 ml cold water, dried with sodium sulphate, filtered and evaporated to dryness under vacuum. Column chromatography (11.5 x 2.2 cm) in Polygosyl 60-4063 C19 (Machery Nagel) with methanol affords four coloured fractions (pink, orange, red and violet). The red fraction (11 mg), after purification by PHPLC ($CHCl_3$, 1.5% Ethanol; silica column) affords as the major product 5-nitromesobiliverdin XIIIα dimethyl ester as an red solid (4 mg, 11 %). Data are compared, when possible, with the data of ref.[5b] for the product described as mesopurpurin XIIIα dimethyl ester (619 nm).

M.p. 182 - 184° ; (ref [5b] 189°).

UV-Vis (λ_{max} nm): Ethanol; 319, 531 (ref.[5b] violet-red solution), standing of this solution a band appears at 670 -690 nm (ref.[5b]670nm): $Zn(AcO)_2$/Ethanol; 335, 573 sh, 619 (ref.[5b] 619nm): Addition of a small amount of iodine to the Zn^{++} solutions gives a slow shift of the 619 nm band to 627 nm, as described in ref.[5b];

MS (FAB): Xe, DTT; 660 (M^++ 1), 614 (M^++ 1 - NO_2).

Elemental Analysis ref.[5b]:

Cal. $C_{35}H_{43}O_8N_5$ (structure proposed in ref.[5b]).	C 63.50	H 6.55	N 10.59	(OCH_3 9.4)
Cal. $C_{35}H_{41}O_8N_5$ (5-nitro derivative)	C 63.72	H 6.26	N 10.62	(OCH_3 9.4)
Exp. ref.[5b]	C 63.58	H 6.04	N 10.91	(Zeisel OCH_3 10.3)

IR (KBr, cm^{-1}): 3420, 1740, 1710, 1605, 1520, 1320.

^{1}H-NMR (δ, ppm; $CDCl_3$ filtered through neutral alumina): 6.85 (s, H-C10), 5.84 (s, H-C15), 3.70 (s, one CH_3OCO), 3.66 (s, one CH_3OOC), 3.00 (t, J=7.2 Hz, one $\underline{CH_2}CH_2COO$), 2.92 (t, J=7.2 Hz, one $\underline{CH_2}CH_2COO$), 2.58 (m, two CH_2 of $CH_2\underline{CH_2}COO$-), 2.48 (q, J=7.8 Hz, $\underline{CH_2}CH_3$), 2.40 (q, J=7.8 Hz, $\underline{CH_2}CH_3$), 2.08 (s, CH_3-C13), 1.99 (s, CH_3-C18 or C7), 1.97 (s, CH_3-C7 or C18), 1.90 (s, CH_3-C2), 1.16 (t, $\underline{CH_3}$-CH_2-C17), 0.81 (t, $\underline{CH_3}$-CH_2-C3).

Chrysins

They are obtained[3] as by-products of the reaction of biliverdin Zn^{++} complexes with iodine (e.g. 30 mg 1b in 30 ml methanol containing 50 mg zinc acetate; a small amount of NH_3 is added to help in the solubilisation of the verdin, and 0.1 N alcoholic iodine is added). The reaction progression can be followed by UV-Vis. The reaction is fast and quantitative to such a degree that can be considered as a titration of the verdin. The Zn complex absorption of biliverdin IXα (703, 393 nm) is transformed into absorption at 635, 588 (small) and 349 (For mesobiliverdin IXα the initial absorptions at 679 and 369 are transformed into 622, 574 -small- and 336)i. e. typical violin Zn^{++} complexe absorption of biliverdins. In the same experimental conditions, but in the absence of Zn^{++}, only partial and not so fast reaction has been observed; however, the reaction products absorb as violinoids (ca. 540 nm).

Lemberg describes a different work-up for biliverdin IXα than for mesobiliverdin IXα, however we have interchanged both procedures and the results are the same. One of the procedures seems to be made in order to investigate the nature of the reaction products taking advantage of the small solubility in acid of chrysins (no basic nitrogen), the other work-up tries to increase the yield (always very low 5-10 %). In the first part, both procedures extract the Zn^{++} (either with acetic acid or with 1 % carbonate solution): after Zn^{++} elimination the absorption band of chrysin can already be observed. However, the rest of the reaction mixture seems to be exclusively composed by violinoids (635 and 349 nm or 622 and 336 nm; for vinyl or ethyl substituted biliverdin). Chrysins can only be obtained pure by final crystallization in methanol. The cyclic imides were detected from the reaction mixture by thin layer chromatography (TLC): methylvinylmaleimide was isolated by preparative TLC and identified by its physical and spectroscopical data[21]

Physical and chemical data of bilichrysin IXα and mesobilichrysin IXα are shown in the table.

5'-[(5-formyl-4-methyl-3(2-methoxycarbonylethyl)-1H-pyrrole-2-yl)methyl]-3,3'-dimethyl-4-ethyl-4'(2-methoxycarbonylethyl)-(Z)-5(1H)-pyrromethenone: 3b.

Since both derivatives above were mixtures of two substances, the preparation of one of the components of mesobilichrysin was undertaken by treatment of the symmetrical mesobiliverdin XIIIα.

To a solution of 77 mg (0.13 mmol) of 1c and 74 mg (0.34 mmol) of $Zn(OAc)_2$ and 0.1 ml conc. NH_3 in 15 ml methanol, one equivalent of alcoholic iodine (0.1 N solution) was added. 50 ml $CHCl_3$ were added and the solution was left standing at r.t. for 3 days. Extraction with 1 % sodium carbonate solution (4 x 20 ml) took all pigments into the water phase, which could then be extracted with ether. Acid extraction (8 x 20 ml 10 % HCl) of this organic phase removed the "violins", while the yellow pigments were retained in the organic phase. The latter, after drying, was vacuum evaporated. The yellow residue gwais purified by PHPLC [C18; CH_3CN/ 0.2 N NH_4AcO (25/75)]. The major fraction (8 mg), after filtration through microcrystalline cellulose (hexane followed by $CHCl_3$) and crystallization in methanol, affords 3b (3 mg).

M. p. 211 - 214° (dec.).

UV-Vis [λ_{max}(ε)]: $CHCl_3$; 420 (12 000), 312 (7 000).

IR (KBr, cm^{-1}): 3420, 3320, 1710, 1690, 1610.
HPLC : Under the same experimental conditions as on the table, its retention time coincides with the first peak of the two.
^{1}H-NMR: The spectrum agrees with the signals corresponding to 3b, given in the table. Bile pigments with free carboxylic acid groups require solvent mixtures to be solubilized for NMR spectroscopy. Their chemical shifts depending very much on the solvent mixture proportion and on the concentration. In some conditions they do not give well resolved spectra. This must be attributed to the formation of inversed micelles[22], and will be published elsewhere. The spectra reported on the table and those obtained with this pure substance were high resolution spectra, but, because of the different conditions of the recorded spectra (proportion of the solvent mixture) in some cases differences were observed between chemical shift values (ca. 0.1 ppm) .

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