The Structure of Bonellin and its Derivatives. Unique Physiologically Active Chlorins from the Marine Echurian *Bonellia viridis*

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The structure of bonellin, the physiologically active pigment of the marine echurian worm *Bonellia viridis*, has been established as (10a), a unique type of alkylated chlorin, by a combination of physical methods. The structure was confirmed by an *X*-ray crystallographic study on anhydrobonellin methyl ester (12b).

Bonellin is accompanied in the organism by a series of mono-amino-acid conjugate derivatives of general structure (14a) in which valine, leucine, isoleucine, and alloisoleucine are the major amino-acid constituents.

THE marine echurian worm, *Bonellia viridis*, has long been of particular interest to marine biologists, geneticists, and chemists [1] in that it produces indifferent larvae, the eventual sex of which is determined after hatching by environmental factors.[2,3] Furthermore, the otherwise highly vulnerable adult female has an integument (*a*) which is highly unpalatable to representatives of both invertebrate and vertebrate animals, and (*b*) extracts of which are highly toxic to a wide range of organisms from protozoa to amphibian tadpoles.[4-7]

The animal exhibits marked sexual dimorphism, as the female has a trunk *ca.* 5 cm long, with a forked proboscis that may be 1—1.5 m long when extended for feeding, whilst the male is but 1—3 mm in length. The trunk of the female is normally held in a burrow away from light, as the aminal, which is deep green all over, is negatively phototactic. For each female there are several males which live parasitically within the female after an initial period of settlement on the proboscis.[4]

Eggs are fertilised inside the female and hatch after laying into sexually undifferentiated larvae which have a marked tendency to settle on the proboscis of the female. If this occurs they rapidly differentiate male characteristics and cease to grow.[1,8-10] After leaving the proboscis a colourless spot is left at the site of settlement,[1] suggesting that pigment is actively ingested and may be associated with masculinisation. The suggestion is strengthened in that if larvae are removed from the proboscis after only a short stay, then intersexes result, with characteristics dependent on the contact time of the larvae with the proboscis.[11-13] Aqueous and other extracts of the proboscis of the *B. viridis* can produce masculinisation of the indifferent larvae,[14] whilst irritation of an adult female causes the release of a green secretion which is highly effective as a masculinising agent (L. Agius, unpublished results). If the larvae settle some way away from an adult female *B. viridis* then they slowly develop into females.

Whether the masculinisation of the larvae is associated with the inhibition of cell division is not clear, but it is interesting to note that crude extracts of *B. viridis* show anti-tumour activity.[7] Clearly the chemistry of *B. viridis* merits examination in order to understand the remarkable chemico-physiological mechanisms that enable the organism to survive.

Early investigations indicated that the green pigment of *B. viridis*, named bonellin, was not identical with chlorophyll [15,16] though it was suggested that dietary chlorophyll was the precursor for bonellin.[17] From the extracts, Lederer [18] obtained bonellin as a crystalline green pigment, $C_{31}H_{36\pm2}N_4O_4$, from which he made a crystalline dimethyl derivative and zinc, copper, and iron complexes. He noted that the absorption spectrum (Table 1) was almost identical with that of mesopyrrochlorin (1a) and tentatively suggested that bonellin had structure (1b), which is mesopyrrochlorin modified by

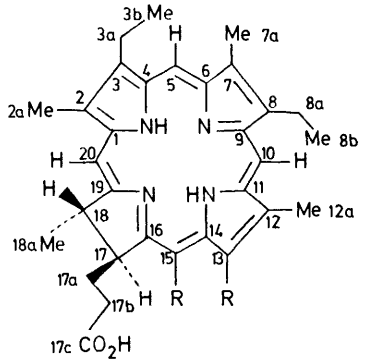
TABLE 1

Visible spectra of bonellin derivatives and related compounds (in $CHCl_3$) [a,b]

Bonellin (10a)	394 (149)	488 (sh) (8.0)	494 (8.3)	523 (1.9)	542 (1.3)	590 (2.5)	620 (sh) (2.7)	641 (15.9)
Mesopyrrochlorin (1a) (ref. 18) [c]		484	492	518	541	588	612	640
BDME (10b)	394 (197)	488 (14.6)		521 (2.4)	539 (1.4)	590 (3.3)	620 (sh) (5.7)	641 (38.6)
trans-0 octaethylchlorin (ref. 21)	391 (188)	487 (12.5)	496 (13.4)	520 (4.1)	544 (1.6)	593 (4.0)	617 (4.5)	647 (73.2)
Methyl phaeophorbide a (2a) (ref. 21)			506 (11.0)		535 (9.3)	560 (2.8)	610 (7.8)	666 (52.7)
Anhydrobonellin methyl ester (12b)	404 (218)	470 (sh) (3.4)	502 (11.8)	536 (7.6)	572 (sh) (1.7)	627 (5.9)	676 (373)	
Anhydromesopyrrochlorin (13) [c] (ref. 32)			499		532		621	676

[a] As bonellin and its derivatives do not obey Beers law, measurements were made in the concentrations range 5×10^{-6}—1.5×10^{-5} M. [b] $\lambda_{max.}$ in nm, 10^{-3} ε values in parentheses. [c] No ε values quoted in literature.

substitution of two hydroxy-groups at C-13 * and C-15.[18] Degradation of the carbocyclic ring of chlorophyll was suggested as the cause for oxygenation at C-13 and C-15. It is now quite clear that oxygenation of either C-13 or



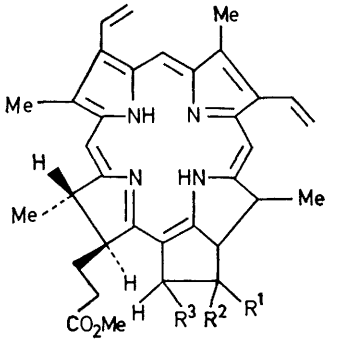
(1) a; R = H
b; R = OH

C-15 would profoundly modify the spectrum of mesopyrrochlorin [19-21] and the suggested structure for bonellin, though widely accepted in the literature,[22] could not be correct. In view of the possible involvement of bonellin with the defence mechanism [5] and masculinisation process [1] of *B. viridis* a structural reinvestigation was called for.

RESULTS AND DISCUSSION

Our material was initially derived from the proboscides of *B. viridis* collected in Marsaxlokk Bay on the Maltese coast. The proboscides were macerated with ethanol to give a green solution with a visible spectrum virtually identical with that of pure bonellin. The pigment was purified first by extraction into ammonia, then into dilute hydrochloric acid followed by esterification and column chromatography. From this process bonellin dimethyl ester (BDME) was obtained as a dark crystalline solid which could be hydrolysed to yield bonellin.

Bonellin dimethyl ester had an intense molecular ion



(2) a; $R^1, R^2 = O, R^3 = CO_2Me$
b; $R^1, R^2 = O, R^3 = H$
c; $R^1, R^3 = H, R^2 = OH$

at *m/e* 554.292 8 ± 0.004 corresponding to $C_{33}H_{38}N_4O_4$, a formula supported by elementary analysis. The diethyl ester, $C_{35}H_{42}N_4O_4$, was also produced. Bonellin itself is the corresponding di-acid, $C_{31}H_{34}N_4O_4$. Various metal derivatives (Cu, Zn, and Fe) of bonellin were made and had spectra differing strongly from both bonellin and the crude ethanol extracts of *B. viridis*. Hence *bonellin is a naturally occurring chlorin lacking a complexed metal atom*. Our pure samples of bonellin dimethyl ester were always contaminated with *ca.* 1% of the copper derivative and 0.01% of the iron derivative. We confirm the great similarity of the spectra of bonellin and mesopyrrochlorin, which, most significantly is a chlorin lacking a C-15 substituent and with only simple alkyl or hydrogen substitution at all positions of the chlorin nucleus.

The absorption spectra of bonellin and some of its derivatives are shown in Table 1 together with the spectra of some other chlorins.[21]

The presence of a carbocyclic ring as in methyl pheophorbide (2a) raises the long-wave absorption maximum to 660 nm, whilst even *trans*-octaethylchlorin, in which all the position (3, 8, 13, and 18) have methyl substituents has a bond at 647 nm. Only mesopyrrochlorin, (1a) lacking three β-substituents (at C-3, C-8, and C-13) has a spectrum similar to bonellin, suggesting that bonellin too lacks all or some β-substituents. Certainly the similarity must be accounted for in any proposal for the structure of bonellin.

Bonellin and its simple derivatives are optically active, and some c.d. measurements are shown in Table 2.

TABLE 2

Circular dichroism of bonellin and its derivatives *

Bonellin		BDME		Bonellin·2 HCl		ABME	
λ	Δε	λ	Δε	λ	Δε	λ	Δε
643	−6.00	643	−5.45	643	−0.19	685	+0.79
				618	−4.59	679	+1.27
391	+8.60	391	+12.00	392	+13.89	540	−0.85
343	−0.89	340	−1.20			405	−14.8
290	−0.76					354	+1.80
276	−2.16	275	−2.73	273	−0.37	326	+1.64
249	−2.41	248	+2.73	245	−1.49	291	+2.75
				223	+0.62	272	+1.27
						252	−0.32
						220	+1.91

* We thank Dr. P. M. Scopes, Westfield College, London, for taking these spectra.

The ^1H n.m.r. of bonellin dimethyl ester was compared with chlorin e_6 trimethyl esters (3),[21] isochlorin e_4 dimethyl ester (4),[23] and deuteroporphyrin IX dimethyl ester (5) [21,24] (Table 3).

Clearly bonellin dimethyl ester contains no vinyl or ethyl groups. There are however two groups of methyl singlets, five between τ 6.4 and 6.7 and two at τ 7.94 and 8.26. Chlorins exhibit a doublet at τ 8.29 for the C-18 methyl group, and hence the peaks at τ 7.94 and 8.26 may be assigned to a *gem*-dimethyl group on a reduced five-membered ring. This inference was confirmed in that irradiation of the multiplet stretching from τ 7.2 to 8.1 (17a- and 17b-CH_2) reduced the triplet at τ 5.6 (H-17) to a singlet, showing that this proton cannot be flanked by other ring protons. This evidence defines sub-unit (6), unique in chlorin chemistry but appearing as ring C in vitamin B_{12}.

The spectrum contains six one-proton absorptions between τ 0.40 and 1.48, of which four are sharp singlets (0.40, 0.50, 1.12, and 1.15) in the correct region for the

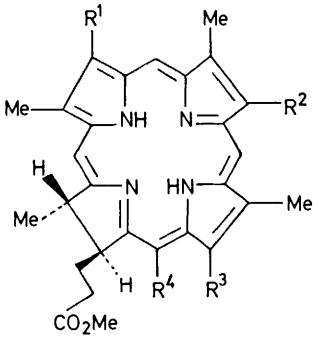
* See formula (1) for the numbering scheme used throughout.

TABLE 3

^{1}H N.m.r. data for bonellin derivatives and related compounds

Atom no. [see formula (1)]	BDME (10b)	ABME (12b)	DPDME (5)	Chlorin TME (3) [6]	Isochlorin e_4 DME (4)	Bonellin amino-acids conjugate esters (14b)
(*a*) Side-chains						
2a	6.58	6.85	6.67	6.47	6.40	6.57
7a	6.61	6.92	6.69	6.66	6.53	6.62
12a	6.62		6.60	6.88	6.70	6.62
cis-18a	7.94	7.95	6.54			7.89
trans-18a	8.26	8.70		8.27 (d)	8.39 (d)	8.29
3a				2.17	1.89	
3b				3.94	3.84	
8a				6.27	6.27	
8b				8.31	8.31	
13a	5.68 (t)	6.16 (m)	5.90 (t)			5.86 (t)
13b	6.91 (t)	6.64 (m)	6.93 (t)			6.91 (t)
13d (CO_2Me)	6.42 or 6.52	6.49	6.47	5.77		6.43 or 6.51
17a	7.42—7.8 (m)	6.97—7.50 (m)	5.90 (t)	8.36 (m)	8.31	7.3—7.8 (br m)
17b			6.93 (t)	7.72 (m)	7.69	
17d (CO_2Me)	6.42—6.52		6.47	6.41	6.41	6.43—6.51
(*b*) 'Saturated' ring						
17	5.6 (br t)	6.10 (br m)		5.60	5.49	5.7 (br)
18				5.60	5.49	
(*c*) 'Aromatic' rings						
3	1.24 (br)	1.66 (br)	1.27 (br)			1.20 (br)
8	1.48 (br)	1.86 (br)	1.31 (br)			1.41 (br)
13					1.23 (br)	
(*d*) meso						
5	0.40	0.87	0.42	0.60	0.28	0.40
10	0.50	0.16	0.39	0.40	0.28	0.49
15	1.15		0.39			1.05
20	1.12	1.86	0.34	1.29	1.13	1.26
(*e*) NHs						
21/23	12.50br	br	14.44 (br)	12.50	12.70	12.38 (br)
(*f*) Amino-acid						
N–H						3.7 (br)
–CH<						5.7 (br)
$-CH_2-$						8.8 (br)
$-CH_3$						9.2

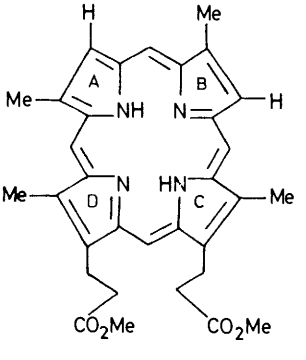
meso-protons (H-5, H-10, H-15, and H-20) of known chlorins.[21] There must therefore be a *meso*-hydrogen at C-15 in bonellin dimethyl ester, a further unique



(3) $R^1 = CH{=}CH_2$, $R^2 = Et$, $R^3 = CO_2Me$, $R^4 = CH_2CO_2Me$

(4) $R^1 = CH{=}CH_2$, $R^2 = Et$ $R^3 = H$, $R^4 = CH_2CO_2Me$

feature in a naturally occurring chlorin, as chlorophyll and its naturally occurring derivatives are substituted at the C-15. The remaining two low-field proton signals are two broad singlets which are sharpened considerably by irradiation of the 'aromatic' methyl region at τ 6.4—6.7. The situation is strikingly like that in (5) and suggests that bonellin dimethyl ester contains two allylically coupled protons at C-3 and C-8, and hence that units (7) and (8) are present, both rings being hitherto unknown in natural chlorins.

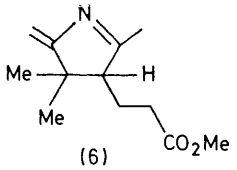


(5)

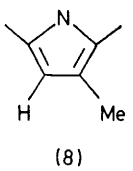
The structure of the remaining sub-units (9) could be deduced from the presence of a methyl group (τ 6.62) and a propionic acid side-chain attached to an 'aromatic'

ring as in compound (5). This sub-unit is standard in both porphyrins and chlorins.

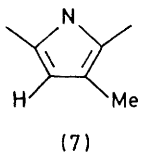
Units (6)—(9) can be put together *via* the four defined *meso*-CH groups to form a variety of chlorins, none of



(6)



(8)



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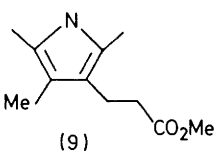
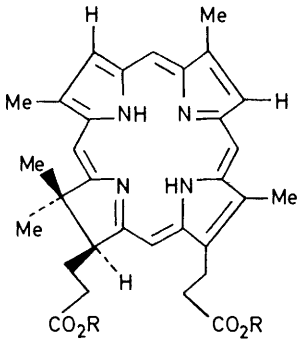
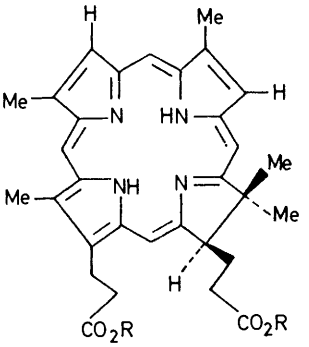


(9)

which shows a close relationship to chlorophyll, the parent compound hitherto for most known characterised chlorins. In order to preserve a relationship with known compounds and defined biogenetic pathways, we decided initially to confine our structural formulations to type III porphyrins.[21] Thus bonellin dimethyl ester can be formulated as either (10b) or (11b), which formally correspond to the addition of methane across the peripheral



(10) a; R = H
b; R = Me



(11) a; R = H
b; R = Me

positions of either rings C or D of (5). As bonellin dimethyl ester can be readily hydrolysed to yield bonellin, the latter must correspond to either (10a) or (11a).

TABLE 4

^{13}C N.m.r. data for bonellin derivatives and related compounds ($CDCl_3$, p.p.m.)

(*a*) Side-chains	BDME (10b)	ABME (12b)	DPDME (5) (ref. 25)	Range of various chlorin esters (ref. 26)	Bonellin amino-acid conjugate ester mixture (14b)
2a	13.4	12.9	13.4	10.8—12.0	13.4
7a	13.4	13.0	13.4	10.7—11.3	13.4
12a	11.2	11.4	11.3	11.7—12.3	11.3
cis-18a	32.0	27.9	11.3		31.8
trans-18a	23.4	26.3		22.7—23.4	23.2
3a				vinyl 128.3—128.8	
3b				vinyl 121.0—122.6	
8a				ethyl 18.1—19.4	
8b				ethyl 17.1—19.2	
13a	21.5	23.4	21.5		21.5
13b	36.6	35.5	36.7		36.3
13c	173.8 or 174.2	175.9	172.8		172.1—173.9
13d	51.5 or 51.7	51.5	51.4		51.7—51.9
17a	31.8	39.2	21.5	30.8—31.2	31.0
17b	27.6	21.6	36.7	29.5—31,3	27.9
17c	173.8 or 174.2	198.5	172.8	172.4—172.8	172.1—173.9
17d	51.5 or 51.7		51.4	51.2—51.5	51.7—51.9
(*b*) ' Saturated ' ring					
16	165.7 or 172.1	168.2 or 173.8	128.0—144.2 (aromatic)	159.0—173.4	165.3 or 172 1
17	57.8	56.8		50.8—52.0	57.9
18	49.7	49.1		49.2—50.0	49.9
19	165.7 or 172.1	168.2 or 173.8		159.0—173.4	165.3 or 172.1
(*c*) ' Aromatic ' rings					
3	126.3	127.4	130.2	122.0—154.4	126.0
8	130.3	130.7	136.0		130.1
Other 10 atoms	131.6—151.5	130.8—153.1	128.0—144.2		131.5—151.6
(*d*) *Meso*					
5	102.5	108.5	98.7	95.4—102.1	102.4
10	102.1	105.4	99.5	101.7—105.9	102.0
15	91.2	106.4	95.1	101.6—105.4	91.2
20	93.0	91.0	96.2	91.8—93.3	93.6
(*e*) Amino-acid					
CO–NH					173.9
CO_2R					172.5
CH_2					25.2/33.5/56.4
CH_3					18.9/17.8
CH					37.7

The ^{13}C n.m.r. spectrum of bonellin dimethyl ester is compared with that of (5) [25] and various chlorin esters [26] in Table 4.

The signal assigned to C-18 is in line with that of other chlorins, but remains a singlet in the off-resonance spectrum, confirming the presence of a *gem*-dimethyl group at this position. The *gem*-dimethyl resonances were assigned as 18a-*cis* at δ 23.4 and 18a-*trans* at δ 32.0 by means of single-frequency specific proton-decoupling experiments, using the widely separated proton frequencies of the 'aliphatic' methyl resonances for irradiation. The assignments are in line with those made to dicyanocobinamide.[27] Sub-unit (6) is thus confirmed.

The 'aromatic' methyl groups at C-2a, C-7a, and C-12a appeared at comparable positions to those of rings A and B of compound (5), as do the doublets due to C-3 and C-8, confirming the sub-units (7) and (8).

The signals assigned to the propionate units attached to both reduced and non-reduced rings compare well with those for a range of chlorins and for compound (5) (Table 4), confirming the final sub-unit (9).

The ^{13}C n.m.r. spectrum of bonellin dimethyl ester substantiates all the sub-units delineated by the ^{1}H n.m.r. spectrum and is in accord in either (10b) or (11b). In an attempt to distinguish between (10b) and (11b) we extended observations that the chemical shifts of the *meso*-carbon atoms of porphyrins depend markedly upon the substitution of the two flanking β-substituents.[28] When flanked by two alkyl groups, the *meso*-carbon atom gives a signal between δ 96 and 97.5, when by one alkyl group and a proton the signal shifts to *ca.* δ 99—100.5, and when flanked by two protons a further shift occurs to bring the signals to *ca.* δ 103—104.5.

Table 5 gives the signals for the *meso*-carbon atoms of

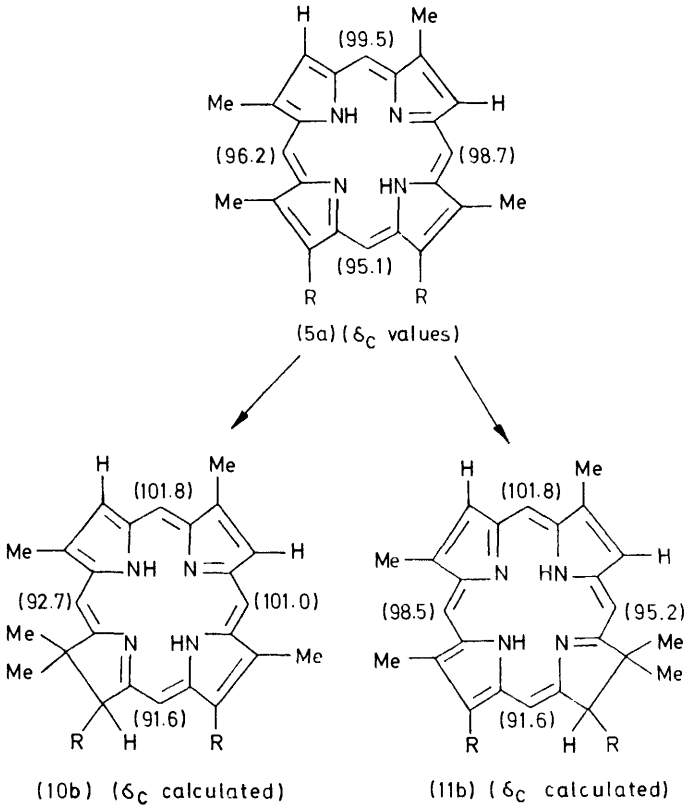
TABLE 5

^{13}C N.m.r. chemical shifts for *meso*-carbon atoms of porphyrins and chlorins

	C–5	C–10	C–15	C–20
Porphin (ref. 28)	104.4	104.4	104.4	104.4
DPDME (5) (ref. 25)	99.5	98.7	95.1	96.2
trans-Octaethylchlorin (ref. 26)	98.3	98.3	92.5	92.5
Chlorin e_6 trimethyl ester (3) (ref. 26)	98.2	101.7	101.7	93.2
Methyl pheophorbide a (2a) (ref. 26)	96.4	103.6	104.8	92.6
Methyl pyropheophorbide a (2b) (ref. 26)	96.4	103.2	105.4	92.4
Compound (2c)	98.1	99.2	109.1	93.0

various porphin and chlorin derivatives. Unfortunately most of the known chlorins had either substituents at C-15 or a carbonyl group at C-13a. The substituent at C-15 would clearly affect the chemical shift of this carbon atom, but rather less obviously the carbonyl group at C-13a influences the chemical shift of C-10, as we showed by reducing methyl pyropheophorbide (2b) to the corresponding mixture of hydroxy-compounds (2c). We therefore took octaethylchlorin as our base-line for calculating the changes to be expected in chemical shifts on reduction of one ring of a porphyrin. The signal for the *meso*-carbon atoms of octaethyl porphin is at δ 96.0, giving shifts on reduction of one ring of C-5, C-10 (remote from reduced ring) +2.3 p.p.m. and C-15, C-20 (adjacent to reduced ring) −3.5 p.p.m.

Addition of methane across ring D of compound (5) (Scheme 1) gives rise to (10b) with the expected chemical



SCHEME 1

shifts shown, whilst addition across ring C of (5) gives (11b) again with the expected chemical shifts displayed. These shifts assume that a *gem*-dimethyl grouping is similar to that of a saturated CHMe group as seems probable, but this is not necessary for the argument. Clearly the calculated shifts for the *meso*-carbon atoms of structure (10b) are much closer to those for bonellin dimethyl ester (102.5, 102.1, 91.2, and 93.0) than are the shifts calculated for (11b). In particular the *meso*-proton flanked by two methyl groups in (11b) is 3 p.p.m. downfield from the nearest low-field signal. This gross discrepancy led us to favour (10b) rather than (11b) for bonellin dimethyl ester,[29] although it is ring C in vitamin B_{12} that is similarly modified. The assignment made was later confirmed by *X*-ray analysis of anhydrobonellin methyl ester [30] (see later).

The mass spectrum of bonellin (Experimental section) contains a prominent $M - 18$ peak, ascribed to a dehydrative cyclisation of a propionic acid unit on to an adjacent *meso*-position.[31] To emulate this reaction

chemically, bonellin was dissolved in concentrated sulphuric acid, when an irreversible change in the spectrum occurred. One product only, anhydrobonellin, was obtained in high yield and purified as the monomethyl ester. The absorption spectrum of anhydrobonellin methyl ester (12b) compares well with that of the previously prepared [32] anhydromesopyrrochlorin (13) (Table 1). Analysis of the ^{1}H and ^{13}C n.m.r. spectra (Tables 3 and 4) of anhydrobonellin methyl ester shows that the major differences in chemical-shift positions compared with bonellin dimethyl ester are associated with the C-17 propionate side-chain, C-18 and, of course, C-15 (Table 6).

TABLE 6

Chemical-shift changes in the n.m.r. spectra of anhydrobonellin methyl ester relative to bonellin dimethyl ester *

Position	^{1}H n.m.r. (p.p.m.)	^{13}C n.m.r. (p.p.m.)
13a	−0.3	+1.9
13b	+0.27	−1.1
17	−0.5	−1.0
17a	+0.3	+7.4
17b	+0.45	−6.0
17c		+24.5
15		+13.4
cis-18a	−0.1	+4.1
trans-18a	−0.44	−2.9
12a	−0.2	−0.2

* A plus sign indicates a shift to low field in ABME.

Thus although there are two propionate units flanking the C-15 position, cyclisation has specifically involved the C-17-propionate group, leading to structure (12b) for anhydrobonellin methyl ester. This remarkable selectivity must be due to the extra flexibility in the transition state conferred by the sp^3 carbon at C-17 as compared with the sp^2 carbon at C-13. A kinetic study indicated that the transformation from bonellin to anhydrobonellin (12a) was direct, without any intermediates.*

analysis was racemic and hence the absolute configuration of anhydrobonellin methyl ester (and bonellin) remains to be established. Presumably some degree of racemisation can occur in acid media by a series of protonation–deprotonation reactions as in Scheme 2.

An *X*-ray study of a single crystal of anhydrobonellin methyl ester [30] led to the projection formulae shown in Figures 1 and 2 (*R*-factor of 14%). The ester group

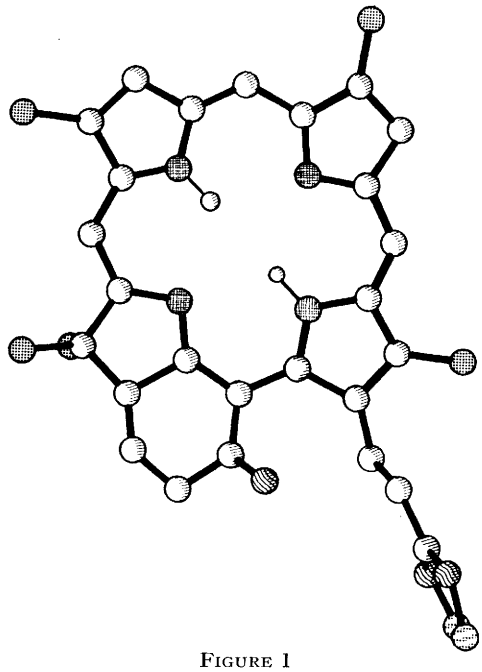
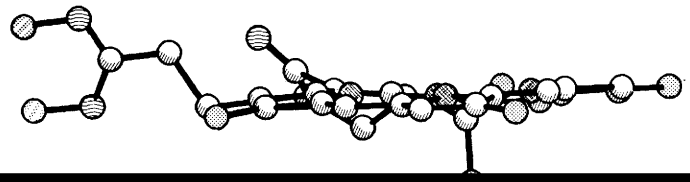
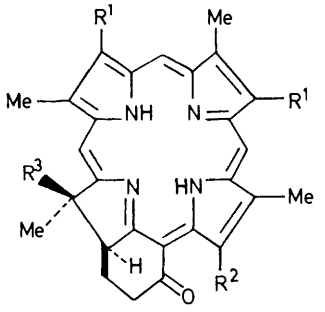


FIGURE 1

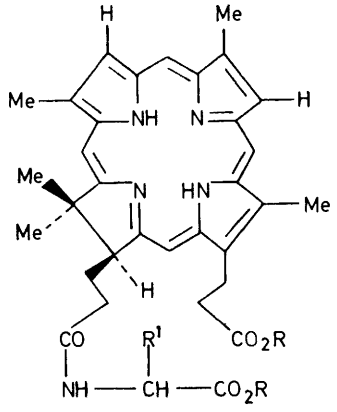


spectrometry indicated that although the new material ran as a single spot on t.l.c. and gave one band only on h.p.l.c. using a wide variety of systems, it was nevertheless a mixture. Major molecular ions at *m/e* 667.373 2 ± 0.003 3 and 653.357 4 ± 0.003 3 indicated that the new



(12) a; R^1 = H, R^2 = $CH_2CH_2CO_2H$, R^3 = Me
b; R^1 = H, R^2 = $CH_2CH_2CO_2Me$, R^3 = Me
(13) R^1 = Et, R^2 = H, R^3 = H

pigments had valine and leucine residues conjugated to bonellin, and also that no dipeptides were present in the mixture. We were unable to resolve the mixture into its components but acid hydrolysis gave bonellin together with an α-amino-acid mixture consisting of valine (62.7%), isoleucine (23.0%), leucine (5.9%), and alloisoleucine (4.0%), together with some minor components totalling 4.4%. The proportions of amino-acids present were confirmed and their configurations assigned by g.l.c. analysis of the *N*-pentafluoropropionyl amino-acid (−)-3-methylbut-2-yl esters * on a high efficiency glass capillary column. †,[33] The amino-acids present were L-valine, L-isoleucine, L-leucine, and D-alloisoleucine.



(14) a; R = H
b; R = Me

An intense peak at *m/e* 467.244 7 ± 0.002 3 ($C_{29}H_{31}N_4O_2$) resulting from loss of a propionyl amino-acid side-chain (CH_2CH_2·CONH·CHR·CO_2Me) was observed in the mass spectrum of the mixture. It has previously been noted that chlorins lose the complete propionate side chain from C-17, but that porphyrin esters undergo a benzylic cleavage with loss of CH_2CO_2Me.[31] In the mass spectrum of the amino-acid conjugate esters there was no significant benzylic loss of $CH_2CONHCHRCO_2Me$ to give an ion at *m/e* 481.

Hence the amino-acids are attached only to C-17c and the conjugates must be represented by (14a), the esters being (14b). This formulation is in accord with the ^1H n.m.r. of the mixture of amino-acid conjugate esters (Table 3) in which the signals due to the C-13 propionate side-chain are unaffected by conjugation, whereas those due to the protons on C-17a and C-17b are both broadened and modified in shape.

Whilst our work on the amino-acid conjugates was in progress,[34] and after our preliminary publication of the structure of bonellin itself [29,30] we were informed by Dr. G. Prota (University of Naples) ‡ that he had independently examined the body walls of *B. viridis* collected near Naples and characterised the 17c-isoleucine conjugate of bonellin as the overwhelming component of the mono-peptide conjugates present (*ca.* 93%).[35] Thus the composition of the amino-acids conjugated to bonellin can vary with the geographic provenance and presumably genetic constitution of the *B. viridis* studied.

Parallel investigations, showing that bonellin in the light is capable of lysing erythrocytes and immobilising echinoid spermatozoa, and presumably is involved in masculinisation of the larvae of *B. viridis*, are being reported separately.[1,36] The effect of light is of interest and it is possible that an intermediate in the oxidation of bonellin rather than bonellin itself is the active physiological chemical. This possibility is under active investigation.

The biochemistry of bonellin remains to be explored. Clearly, however, bonellin is a chemical that is certainly responsible for the defence of *B. viridis*, and is possibly involved in the mechanism of sex-determination. This external influence on a differentiation which in most organisms is preponderantly genetically determined is of wide general importance.

EXPERIMENTAL

All melting points were measured on a hot stage microscope. Visible spectra were recorded on a Pye-Unicam model SP 800 spectrophotometer. (Solutions of bonellin and its derivatives were found to deviate significantly from Beer's law and measurements were made for solutions in the range 5×10^{-6}—1.5×10^{-5}M). Circular dichroism graphs were obtained by Dr. M. Scopes at the S.R.C. c.d. unit at Westfield College, University of London. ^1H N.m.r. spectra were recorded on a Varian model HA-100D instrument and ^{13}C n.m.r. spectra on a Varian model XL-100 instrument fitted with a Gyrocode module. Mass spectra were measured on a modified AEI model MS 9 spectrometer with a Mass Spectrometry Services console and Instem Maxi

* We thank Professor W. A. König for his generous gift of a sample of (−)-3-methylbutan-2-ol.

† We thank Drs. R. J. Laub and K. Williams for technical assistance with the g.l.c. determination.

‡ We are grateful to Dr. Prota for supplying us with this information prior to publication.

data system. H.p.l.c. apparatus employed Altex pumps and flow-control systems and a Cecil model CE 272 variable-wavelength spectrophotometer as detector. Experimental *X*-ray crystallographic information for anhydrobonellin methyl ester has been reported,[29] and atomic co-ordinates are available on request from the Director of the Cambridge Data Centre, University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW.

Extraction of the Chlorin Pigments from Bonellia viridis.—Female specimens of the marine worm *Bonellia viridis* (20 animals) were collected in Marsaxlokk Bay on the Maltese coast at depths of 1—20 m and were dissected to obtain separate samples of the proboscides, viscera, and body wall which were extracted separately as follows. The wet tissue was macerated in ethanol (200 cm^3) and after removal of the cell debris, the filtrate was diluted with water (400 cm^3) and then extracted with ether (3 × 150 cm^3) until the last ether extract was colourless. Both the aqueous fraction A and the ether fraction E were retained in each case.

(a) *Treatment of the ether fraction E.* The green ether layer (450 cm^3) was extracted with hydrochloric acid (3 × 100 cm^3 of a 6% solution), the resultant blue aqueous layer was neutralised with ammonium hydroxide solution (3%) and the pigment extracted into ether (2 × 100 cm^3). This green ether extract was shaken with ammonium hydroxide (3 × 100 cm^3 of a 1% solution) and the aqueous layers neutralised with hydrochloric acid (6%) before the pigment was extracted into ether (2 × 100 cm^3). The ether layer was washed with water (100 cm^3) and the solvent removed under reduced pressure to leave a green residue of crude pigments which were esterified by treatment with a solution of 10% concentrated sulphuric acid in absolute methanol (50 cm^3) overnight at room temperature. The solution was shaken with a mixture of water (100 cm^3) and dichloromethane (100 cm^3). The solvent layer was washed with water (100 cm^3) and on evaporation yielded a mixture of crude fraction E esterified pigments as a green solid.

(b) *Treatment of the aqueous fraction A.* The green aqueous solution was run through a short column (2 × 5 cm) of neutral alumina (Merck), the pigments being strongly absorbed. After washing the column with water, the column was dried, extruded, and the pigment recovered from the alumina by treatment with a solution of 10% concentrated sulphuric acid in absolute methanol (50 cm^3). The esterification was allowed to proceed at room temperature overnight and the solution was shaken with a mixture of water (100 cm^3) and dichloromethane (100 cm^3). The solvent layer was washed with water (100 cm^3) and on evaporation yielded a mixture of crude fraction A esterified pigments.

(c) *H.p.l.c. analysis of the crude esters.* Each of the crude ester mixtures was analysed on a 10μ Partosil column (25 cm × 4 mm i.d.) using a solvent system of toluene–methanol (97.5/2.5) with a flow rate of 2.5 $cm^3\ min^{-1}$ at a pressure of 300 lb in^{-2} with u.v. monitoring at 400 nm. Each of the methylated fractions from *B. viridis* separated into two resolved bands which were quantitatively analysed (Table 7).

Bonellin Dimethyl Ester (BDME) (10b).—The combined methyl esters from the extraction of the proboscides (75 mg) were chromatographed on a column of neutral silica gel (500 g, Koch–Light 200—300 mesh) using dichloromethane as solvent. BDME was readily eluted leaving the other constituents as a green band at the top of the column. After evaporation of the eluant, the ester was further purified by chromatography on a column of Kieselgel G (10g, Merck for t.l.c. plates) in ether–pentane (3 : 1) at a nitrogen pressure of 10 lb in^{-2}. BDME was eluted in the first 20 cm^3 as a sharp green band and after evaporation of the solvent was deposited as a green solid (65 mg) which was recrystallised from ether–pentane to give *bonellin dimethyl ester* as lustrous dark prisms, m.p. 53—55 °C (Found: C, 71.3; H, 7.0; N, 10.4%; M^+, 554.292 8 ± 0.004 0. $C_{33}H_{38}N_4O_4$ requires C, 71.5; H, 6.9; N, 10.1%; *M*, 554.288 3); $\lambda_{max.}$ (6% HCl) 402 (10^{-3} ε 119), 523 (3.4), 587(sh) (4.6), and 631 nm (24.4); *m/z* 554 (100%), 539 (13), 523 (4), 481 (4), 467 (9), 465 (3), 393 (3), and 379 (6).

Zinc Complex of BDME.—A solution of zinc(II) acetate hydrate (0.5 g) in methanol (20 cm^3) was added to BDME (100 mg) and allowed to react at 20 °C for 5 h. The resultant blue solution was poured into a mixture of water (50 cm^3) and dichloromethane (50 cm^3). The solvent layer was evaporated to dryness and the residue recrystallised from ether to furnish the *zinc complex* as deep blue prisms (87 mg), m.p. 70—73 °C (Found: C, 64.3; H, 5.9; N, 9.2. $C_{33}H_{36}N_4O_4Zn$ requires C, 64.3; H, 5.8; N, 9.1%); $\lambda_{max.}$ ($CHCl_3$) 402 (10^{-3} ε 119), 504 (2.2), 539 (0.7), 566 (0.7), 583 (2.8), and 612 nm (24.6); C.D. λ (Δε) 612 (−6.9), 404 (+14.90), 380 (+2.43), 363 (−2.43), 310 (+2.40), 276 (−0.61), 263 (+0.78), and 245 nm (−1.56).

Bonellin (10a).—BDME (100 mg) was hydrolysed by treatment with 25% hydrochloric acid (150 cm^3) for 2 d at 20 °C. The resultant blue solution was neutralised with sodium hydrogen carbonate and the di-acid was extracted into dichloromethane (2 × 100 cm^3), which was washed with water and the solvent removed. The crude bonellin was dissolved in ether (200 cm^3) and extracted into ammonium hydroxide solution (3%). After neutralisation with hydrochloric acid (6%), the pigment was extracted into dichloromethane (2 × 100 cm^3). Removal of the solvent gave *bonellin* (90 mg) which was recrystallised from ether to yield dark micro-crystals which decomposed at *ca.* 300 °C (Found: C, 70.5; H, 6.5; N, 10.6%. $C_{31}H_{34}N_4O_4$ requires C, 70.7; H, 6.5; N, 10.6%); $\lambda_{max.}$ (6% HCl) 402 (10^{-3} ε 135), 526 (2.6), 540 (0.6), 548 (0.5), 586sh (2.7), and 636 nm (15); *m/z* 526 (33%), 508 (45), 480 (100), 465 (78), 453 (20), 405 (16), 391 (15), 379 (10), 281 (14), 280 (23), and 241 (21).

TABLE 7

Extracts from 20 animals		Weight of crude methyl esters/mg	H.p.l.c. areas Peak 1 %	H.p.l.c. areas Peak 2 %	Estimated weights Peak 1 mg	Estimated weights Peak 2 mg
Proboscides	Ether extract E	72	97	3	74.7	2.3
	Aqueous extract A	5	96	4		
Body wall	Ether extract E	37	66	33	28.5	28.5
	Aqueous extract A	20	18	82		
Viscera	Ether extract E	0.35	85	15	0.36	0.14
	Aqueous extract A	0.15	39	61		
Whole animal	Ether extract E	109.4				
	Aqueous extract A	25.2			103.6	30.9

Bonellin Hydrochloride.—Bonellin (10 mg) was dissolved in 10% hydrochloric acid (1 cm³) and allowed to stand at room temperature overnight. Dark blue prisms of *bonellin hydrochloride* (12 mg) were deposited, m.p. 236—240 °C.

Bonellin Diethyl Ester.—Bonellin (50 mg) was esterified with ethanol (50 cm³) containing concentrated sulphuric acid (4 cm³) at 20 °C and the diethyl ester isolated as described for the dimethyl ester. The product was recrystallised from ether–pentane to furnish *bonellin diethyl ester* as green prisms (45 mg), m.p. 103—104 °C (Found: C, 72.4; H, 7.6; N, 9.5. $C_{35}H_{42}N_4O_4$ requires C, 72.2; H, 7.2; N, 9.6%); *m/z* 582 (100%), 567 (10), 554 (4), 537 (8), 509 (7), 495 (8), 481 (12), 393 (11), and 379 (14).

Anhydrobonellin Methyl Ester (ABME) (12b).—Bonellin (85 mg) was dissolved in concentrated sulphuric acid (98%, 2.5 cm³) at 20 °C. The blue solution gradually turned green and after two hours absolute methanol (25 cm³) was added. The solution was partitioned between dichloromethane (50 cm³) and water (50 cm³) and the dichloromethane layer was washed with water and the solvent evaporated. The crude product was purified by column chromatography on polyamide (2 × 25 cm) using dichloromethane as eluting solvent. A brown band was gradually eluted (30 cm³) and the recovered solid was dissolved in ether (20 cm³) and transferred into 6% aqueous hydrochloric acid (2 × 50 cm³). The aqueous layer was extracted into dichloromethane, and the organic layer washed with water and evaporated to furnish a brownish solid. Recrystallisation from ether–pentane furnished *anhydrobonellin monomethyl ester* as dark prisms, m.p. 216—217 °C (55 mg) (Found: C, 73.7; H, 6.3; N, 11.0. $C_{32}H_{34}N_4O_3$ requires C, 73.5; H, 6.6; N, 10.7%); *m/z* 522 (100%), 504 (48), 502 (13), 501 (22), 500 (46), 485 (30), 449 (16), 447 (15), 446 (37), 442 (38), 428 (11), 427 (33), and 224 (16).

Methyl Esters of Bonellin Amino-acid Conjugates (14b).—The combined methyl ester fractions from the body wall extracts of *B. viridis* (57 mg) were applied in ether (1.5 cm³) solution to a column of Kieselgel G (10 g, Merck for t.l.c. plates) and eluted with ether–pentane (3 : 1) under a nitrogen pressure of 10 lb in^{-2}. Two sharp bands were separated on elution; the first (15 cm³) was shown to be bonellin dimethyl ester by H.p.l.c., and the second (20 cm³) gave a mixture of the methyl esters of the amino-acid conjugates of bonellin as a green solid on evaporation (28 mg). One further pass through the column yielded the mixture of esters, as green crystalline material. Further chromatography merely caused decomposition to give bonellin (Found: M^+, 667.373 2 ± 0.003 3 and 653.357 4 ± 0.003 3. $C_{39}H_{49}N_5O_5$ requires 667.373 2; $C_{38}H_{47}N_5O_5$ requires 653.357 4); *m/z* 667 (40%), 654 (31), 653 (100), 639 (9), 636 (7), 621 (9), 607 (2), 593 (4), 555 (2), 480 (20), 467 (12), 465 (9), 453 (5), 407 (2), 393 (4), and 379 (8); $\lambda_{max.}$ ($CHCl_3$) 394, 494, 523, 539, 590, 620(sh), and 641 nm.

Hydrolysis and Amino-acid Analysis of Bonellin Amino-acid Conjugate Esters.—The mixture of bonellin amino-acid conjugate methyl esters (44 mg) was treated with 6M hydrochloric acid (2 cm³) in a sealed tube at 120 °C for 15 h. After opening the tube, the solid residue was removed by filtration and extracted into dichloromethane, which was washed with water and the solvent removed. The crude product was dissolved in ether and extracted into ammonium hydroxide solution (3%). After neutralisation the pigment was extracted into dichloromethane. Removal of the solvent gave a dark green solid (24 mg) which was identical in all respects (u.v., mass spectra) with bonellin.

The aqueous hydrochloric acid filtrate from the sealed tube was evaporated to dryness at 0.1 mmHg, and the residue dissolved in water and analysed on a Beckman Model 120 automatic amino-acid analyser. The retention times of the amino-acid peaks were compared with those of a mixture of standard amino-acids and indicated the presence of four significant amino-acids; valine (62.7%), isoleucine (23.0%), leucine (5.9%), and alloisoleucine (4.0%), as well as a number of minor constituents which together totalled 4.4% of the mixture.

Configuration of the Amino-acids.—The dry mixture of the amino-acid hydrochlorides above (5 mg) was heated with a saturated solution of dry hydrogen chloride in (−)-3-methylbutan-2-ol (1.0 cm³, *ca.* 96% optical purity, obtained from Professor W. A. König [33]) for 90 min at 100 °C. After removal of the excess of reagent *in vacuo*, the sample was acylated with pentafluoropropionic anhydride (0.5 cm³) in dichloromethane (2 cm³) for 30 min at 20 °C. Removal of the excess of reagent *in vacuo* gave the diastereoisomer mixture as a residue which was dissolved in ethyl acetate (1 cm³) for g.l.c. analysis. A Pye model-104 gas chromatograph, modified to accept a 25-m glass capillary PLOT column coated with SE-30 (LKB type 2101) and fitted with an all-glass falling-needle injector [37] was used for the analysis. A flow rate of 2 cm³ min^{-1} of helium was used with a temperature-rise programme of 2° min^{-1} from 85 to 220 °C with N_2 make-up gas (18 cm³ min^{-1}) being fed into the detector stream. The system was standardised with mixtures of derivatised DL-amino-acids when sharp completely resolved peaks were obtained for all of the D- and L-amino-acid diastereoisomers. The efficiency was 45 000 theoretical plates for the L-valine derivative. The elution order for the standards, with relative retention times (r.r.t.), was: L-valine (r.r.t. 1.0, 12 min), D-valine (r.r.t. 1.05), L-leucine (1.20), L-alloisoleucine (1.24), D-leucine (1.25), L-isoleucine (1.26), D-alloisoleucine (1.28) and D-isoleucine (1.31). The derivatives of the bonellin amino-acids gave four major peaks corresponding to L-valine (r.r.t. 1.0, *ca.* 60%), L-leucine (1.20, *ca.* 5%), L-isoleucine (1.26, *ca.* 25%), and D-alloisoleucine (1.28, *ca.* 5%).

Methyl 21-hydroxypyrophaeophorbide a.[38]—Methyl pyrophaeophorbide a [39] (50 mg) in dichloromethane (15 cm³) and methanol (15 cm³) was treated with a solution of sodium borohydride (20 mg) in sodium hydroxide (8 cm³ of 0.005M) under reflux for 2 h. After neutralisation the product was purified by chromatography on neutral alumina and recrystallised from ether–pentane to yield the mixture of (20*R*,*S*) epimers [43] as green microcrystals, m.p. 146—148°C (35 mg).

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