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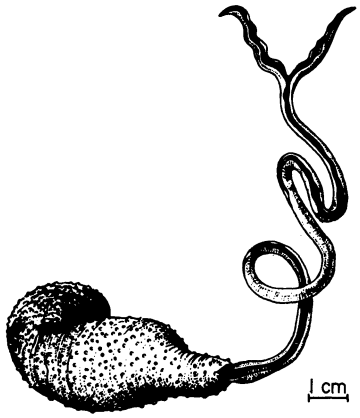
THE STRUCTURE AND PHYSIOLOGICAL ACTIVITY OF BONELLIN - A UNIQUE CHLORIN DERIVED FROM BONELLIA VIRIDIS

L. Agius[1], J.A. Ballantine[2], V. Ferrito[1], V. Jaccarini[1], P. Murray-Rust[3], A. Pelter[2*], A.F. Psaila[1,2] and P.J. Schembri[1].

The echurian worm Bonellia viridis has attracted the attention of both biologists and chemists over a long period of time. A drawing of the female organism is shown in Fig 1. The animal is a deep green all over its surface and is negatively phototactic. The trunk of the animal is generally housed in a burrow system and the proboscis is extended for feeding purposes.



Bonellia viridis Rol. (female)

Fig. 1

There are several points of interest regarding this organism. The first is the marked sexual dimorphism: the female is much larger than the male which is only 1 - 3 mm long. The males live parasitically inside the female after an initial period of settlement on the proboscis. There are usually several males in each female. The eggs hatch into sexually undifferentiated larvae. It has been repeatedly observed that if the indifferent larvae settle on a female they differentiate into males, whilst if they settle away from the female they develop into females. Thus it is clear that an exogenous agent is influencing the determination of the sex of the larvae, rather than this being wholly determined genetically.

The larvae seem to be specially attracted to the proboscis of the female on which they settle. When the larvae leave their site of attachment on the female, they leave behind a clear area from which all pigment has been removed,[4] something we have been unable to achieve by chemical extraction. This removal of pigment suggests that it is being actively taken up by the larvae and raises the question of whether or not the pigment has anything to do with their masculinisation. Such a question is also raised by the observation that the degree of masculinisation depends on the length of contact between the adult and larvae,

* Lecture delivered by Professor A. Pelter.

fairly short contact times resulting in intersexes.[5] Another observation of some interest is that irritation of the adult female produces a green secretion with potent masculinising properties.[4,6]

Naturally the proboscis is relatively vulnerable to attack, but in general is left unmolested. We have shown[4] that the flesh of B.viridis is highly unpalatable to a wide range of small predators. Thus B.viridis affords good material for the study of chemical products with marked biological activity.

A history of the study of the extracts from B.viridis is shown in Fig.2.

History of Bonellia viridis Extracts.

1874	E.R. LANKESTER	:	Obtained a green extract of the worms in sea water.
1875	H.C. SORBY	:	Obtained a visible spectrum of an alcoholic solution. Not chlorophyll.
1928	F. BALTZER	:	Showed that the extract had masculinising properties and was toxic to a variety of marine larvae.
1931	F. MICHEL	:	Suggested that extract was a chemical defence mechanism.
1939	E. LEDERER	:	Obtained crystalline pigment from extracts. $C_{31}H_{36\pm 2}N_4O_4$ Made methyl ester, Zn, Cu and Fe complexes. Visible spectrum almost identical to mesopyrrochlorin ($C_{31}H_{36}N_4O_2$). Suggested that pigment was a dioxymesopyrrochlorin formed by breakdown of chlorophyll.
1955	R. LALLIER	:	Showed that the extract immobilised sea urchin sperm and was lethal at higher concentrations. The toxic effects were enhanced by light. The green pigment was reponsible for the toxicity.
1962	R.F. NIGRELLI	:	The extracts were effective in inhibiting the growth of many cellular organisms including oral carcinoma at 5 p.p.m. in tissue culture.

Fig. 2.

Over 100 years ago, Sorby[7] studied the spectrum of an ethanol extract of B.viridis and showed that the green pigment was not chlorophyll. Incidentally in this paper, he first defined on bonellin, the use of the millimicron for the measurement of wavelength and for the plotting of spectra.[8]

The first real chemical work was carried out by E. Lederer [9] who obtained a crystalline green pigment from the extracts. The visible spectrum was almost identical to that of mesopyrrochlorin (see Fig.6), this being most significantly a chlorin lacking a C-15 substituent and with only simple alkyl or hydrogen substituents at all other positions of the chlorin system. Lederer was able to suggest an empirical formula for the pigment, as well as making various derivatives. As bonellin had two more oxygen atoms than mesopyrrochlorin, Lederer made the tentative suggestion that bonellin retained two oxygen atoms at C-13 and C-15, these being derived from the oxidation of the carbocyclic ring of chlorophyll.* Over a period of time this preliminary suggestion assumed the status of a proof, and the formula gained such acceptance that bonellin was treated as a fully characterised natural product.[10] However, a brief reading of the literature shows that the spectrum

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* IUPAC numbering for porphyrins is used throughout. cf. Formula 1.

observed and the suggested structure are incompatible with each other inasmuch as oxygen substituents at C-13 and, in particular at C-15, would be expected to profoundly modify the simple chlorin spectrum of mesopyrrochlorin.[11,12] Thus the accepted structure for bonellin was undoubtedly incorrect and in view of the high interest in the possible physiological properties of bonellin [13], a re-investigation was felt to be in order.

All our B. viridis were collected from Marsalokk bay on the Maltese coast. As the proboscides are easy to collect, and can regenerate, it was decided to concentrate initially on the pigment collected from this portion of the animal.[14] The proboscides were macerated in ethanol to give a solution with a spectrum virtually identical to that of pure bonellin. The extraction procedure for bonellin and dimethyl bonellinate is shown in Fig. 3. In the event, bonellin dimethyl ester (BDME) proved to be purer than the 'pure' bonellin and hence isolation was concentrated on the former. Bonellin was produced from the ester by simple hydrolysis, and it was this material that was used in our biological assays.

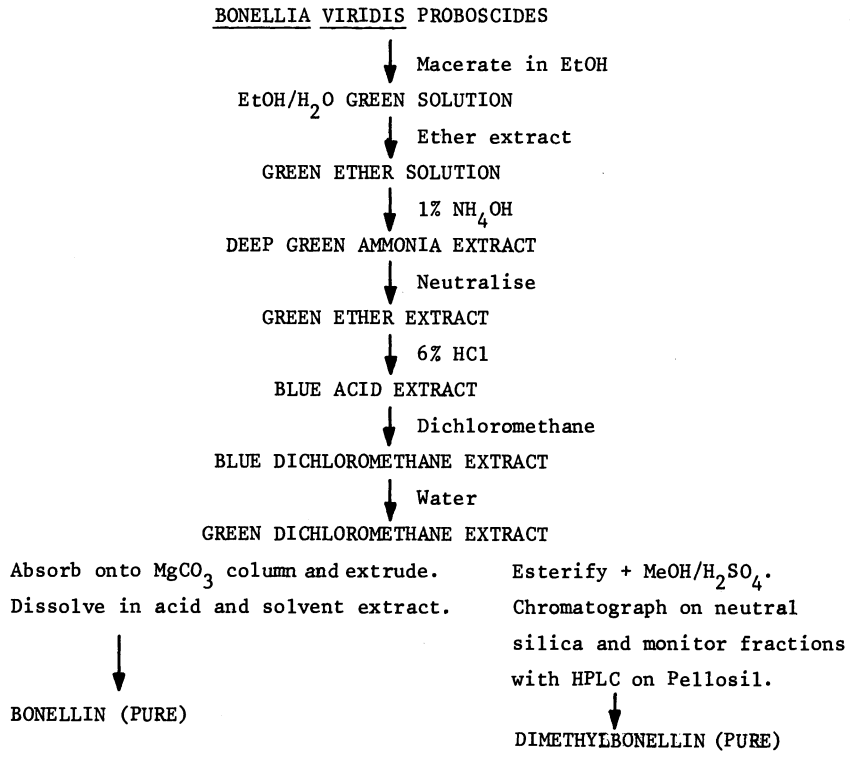

Fig. 3

BDME had an intense molecular ion at m/e 554.2928 ± 0.004 corresponding to $C_{33}H_{38}N_4O_4$, a formula in line with the elementary analysis (Fig. 4). Bonellin itself was the corresponding diacid of formula $C_{31}H_{34}N_4O_4$ (Fig. 4).[14] Various metal derivatives both of bonellin and BDME were made and had spectra differing strongly both from bonellin and the crude extracts from B.viridis. Thus, at this early stage a most unusual feature of bonellin emerged. It is a naturally occurring chlorin uncomplexed to any metal.[15] As far as we can tell from examination of the spectrum of the crude extract, this is the way in which the material occurs in the animal. Ethanol treatment is certainly not sufficient to destroy any of the metal complexes that we have made. It must be stated that our BDME is always contaminated by ca 1% of the copper derivative and ca 0.01% of the iron derivative. These may be present naturally but are more probably produced by extraction from the solvents used, as

bonellin readily complexes with copper and iron. In addition to simple derivatives a compound, anhydrobonellin, with an extra carbocyclic ring, was prepared together with its derivatives (Fig. 5). The relationship of various bonellin derivatives to bonellin is shown in Fig. 5.

ELEMENTAL MICROANALYSES

	% Carbon		% Hydrogen		% Nitrogen		
	found	requires	found	requires	found	requires	
BONELLIN	70.5	70.7	6.5	6.5	10.6	10.6	$C_{31}H_{34}N_4O_4$
DIMETHYLBONELLIN	71.3	71.5	7.0	6.9	10.4	10.1	$C_{33}H_{38}N_4O_4$
DIETHYLBONELLIN	72.4	72.1	7.6	7.3	9.3	9.6	$C_{35}H_{42}N_4O_4$

Fig. 4.

BONELLIN DERIVATIVES

BONELLIN	$C_{31}H_{34}N_4O_4$	
BONELLIN DIMETHYL ESTER	$C_{33}H_{38}N_4O_4$	$B + 2CH_2$
BONELLIN DIMETHYL ESTER Zn COMPLEX	$C_{33}H_{36}N_4O_4Zn$	$B + 2CH_2 - 2H + Zn$
BONELLIN DIETHYL ESTER	$C_{35}H_{42}N_4O_4$	$B + 2C_2H_4$
ANHYDRO-BONELLIN	$C_{31}H_{32}N_4O_3$	$B - H_2O$
ANHYDRO-BONELLIN METHYL ESTER	$C_{32}H_{34}N_4O_3$	$B - H_2O + CH_2$

Fig. 5.

The absorption spectrum of bonellin is shown in Fig. 6 and compared with the spectra of known porphyrins and chlorins (for each column the formula drawn is the full structure of the compound immediately beneath bonellin). Clearly bonellin is a chlorin and the relationship to mesopyrrochlorin (bottom of right hand column) on the basis of the spectra is very clear. Any formula proposed for bonellin must take this into account.

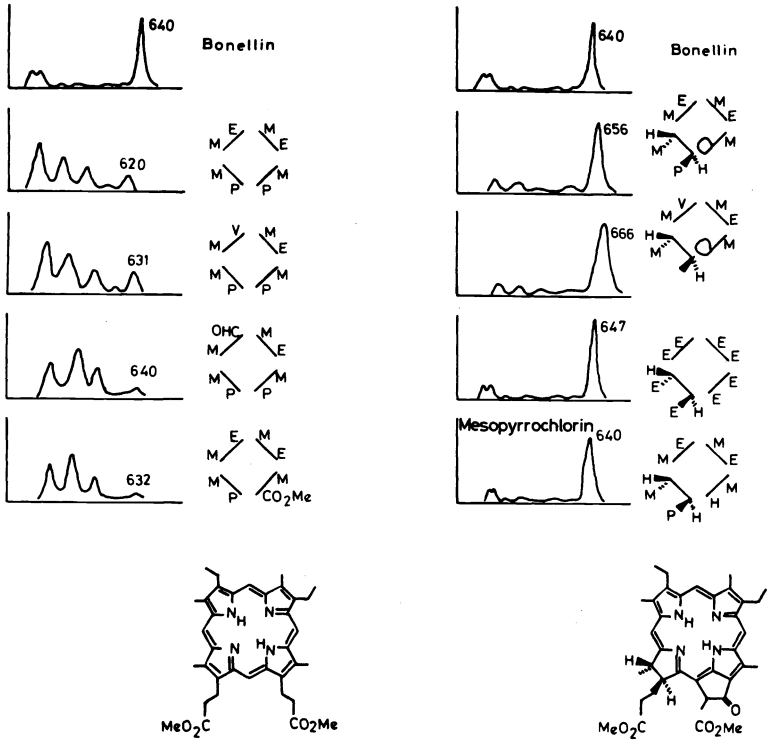

Fig. 6.

The C.D. curve for BDME shows that it is optically active* (Fig. 7).

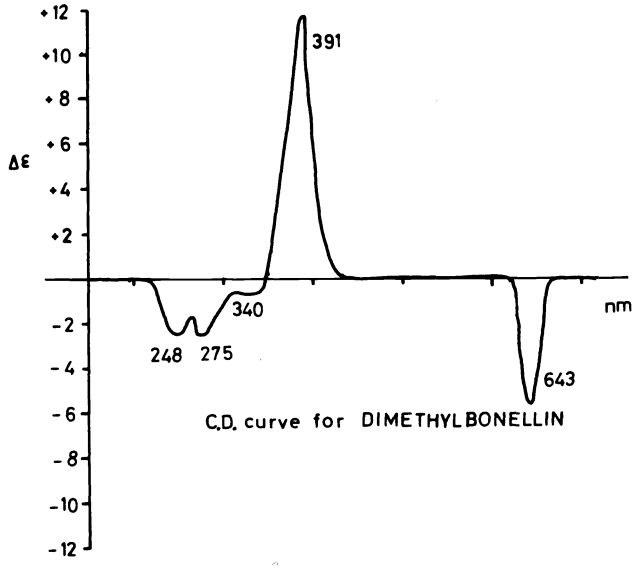

Fig. 7.

The ^{1}H n.m.r. of bonellin revealed some unusual features as compared with some model chlorins and with deuteroporphyrin IX dimethyl ester (Table I, Fig. 8). Instead of the usual doublet at τ8.28 indicative of the 18a methyl group, there are a pair of sharp singlets at τ7.88 and 8.20. A gem-dimethyl grouping in the reduced ring of the chlorin can be inferred and this was confirmed in that irradiation of the multiplet stretching from τ7.2-8.1 (Fig. 8,9) (17a CH_2, 17b CH_2) reduced the triplet at τ5.6 due to H-17 to a singlet. Hence H-17 cannot be adjacent to any ring carbon atoms bearing a proton and sub-unit (i) (Fig. 10) is defined. Such a unit is known for vitamin B_{12} but is unique in chlorin chemistry.

Clearly bonellin contains no vinyl substituents and has four meso protons in two groups of two at 0.34, 0.44 and 1.09 and 1.22. The positions are similar to those of known chlorins[16] but unlike all natural chlorins related to chlorophyll, bonellin lacks a substituent at C-15, this being another unique feature of the molecule. A noteworthy point is the appearance of two low field protons at 1.30 and 1.18 allylically coupled to two of the three 'aromatic' methyl groups at τ6.52 - 6.56. The position is strikingly like that of deuteroporphyrin IX dimethyl ester (DPME) (Fig. 9). Figure 9 also shows some decoupling experiments relating H-17, H-17a and H-17b and also similar experiments that delineate an 'aromatic' propionate group at C-13, as in most naturally occurring porphyrins. These signals together with the remaining 'aromatic' methyl singlet define the standard sub-unit (ii) together with the sub-units (iii) and (iv) (Fig. 10) which are unique in naturally occurring chlorins.

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* We thank Dr. M. Scopes of Westfield College, London for this spectrum.

TABLE I

^{1}H n.m.r. ($CDCl_3$,τ) of BDME and related compounds

SIDECHAINS	BDME	Deuteroporphyrin IX DME[1]	Chlorin e_6 TME[2]	Isochlorin e_4 DME[2]
2a	6.58	6.54	6.47	6.40
7a	6.61	6.60	6.66	6.53
12a	6.62	6.69	6.88	6.70
18a(cis)	7.94	6.76	-	-
18a(trans)	8.26		8.27	8.29
3a	-	-	2.17	1.89
3b	-	-	3.94	3.84
8a	-	-	6.27	6.27
8b	-	-	8.31	8.31
13a	5.86t(7)	5.90t	-	-
13b	6.91t(7)	6.93t	-	-
13d(CO_2Me)	6.42 or 6.52	6.47	5.77	-
17a	7.42 - 7.8m	5.90t	8.36	8.31
17b		6.93t	7.72	7.69
17d(CO_2Me)	6.42 or 6.52	6.47	6.41	6.41
'SATURATED' RING				
17	5.6 br.t.	-	5.60	5.49
18	-	-	5.60	5.49
'AROMATIC' RINGS				
3	1.24 br.s.	1.27 br.s.	-	-
8	1.48 br.s.	1.31 br.s.	-	-
13	-	-	-	1.17
MESO				
5	0.40	0.42	0.60	0.28
10	0.50	0.39	0.40	0.28
15	1.15	0.39	-	-
20	1.12	0.34	1.29	1.13
NH				
21/23	12.50 br.	14.44 br.	12.50 br.	12.70 br.

1) Y. Chang, P.S. Clezey and D.B. Morell, Austr.J.Chem., 1967, 20, 959. 2) H.H. Inhoffen, G. Klotmann and G. Jeckal, Ann.Chem., 1966, 695, 113. c.f. W.S. Caughey and W.S. Koski, Biochemistry, 1962, 1, 923.

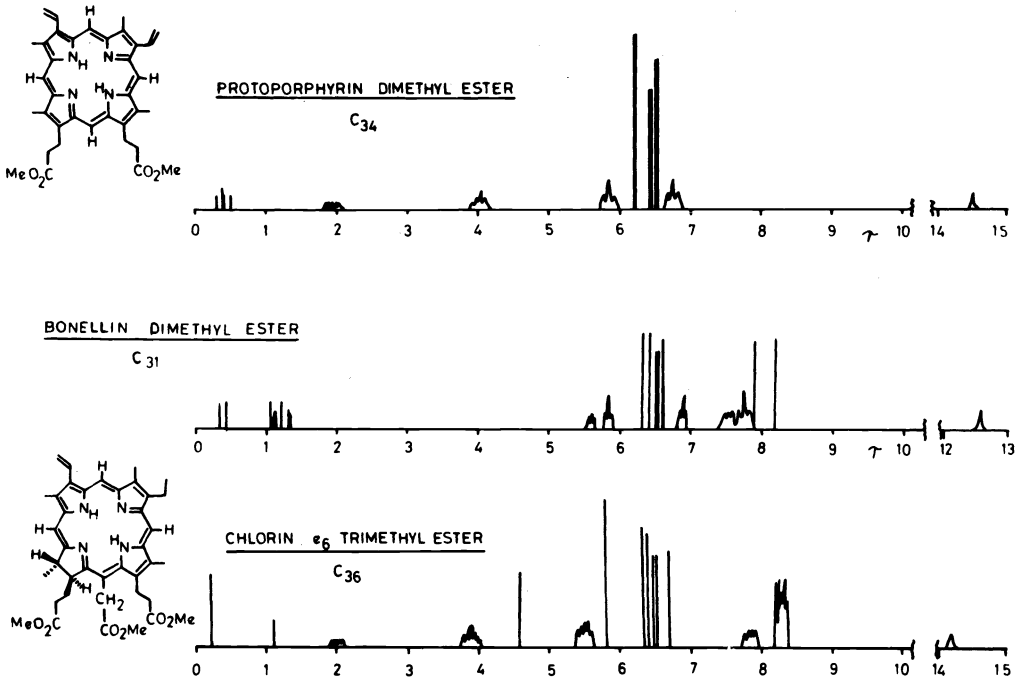


Fig. 8.

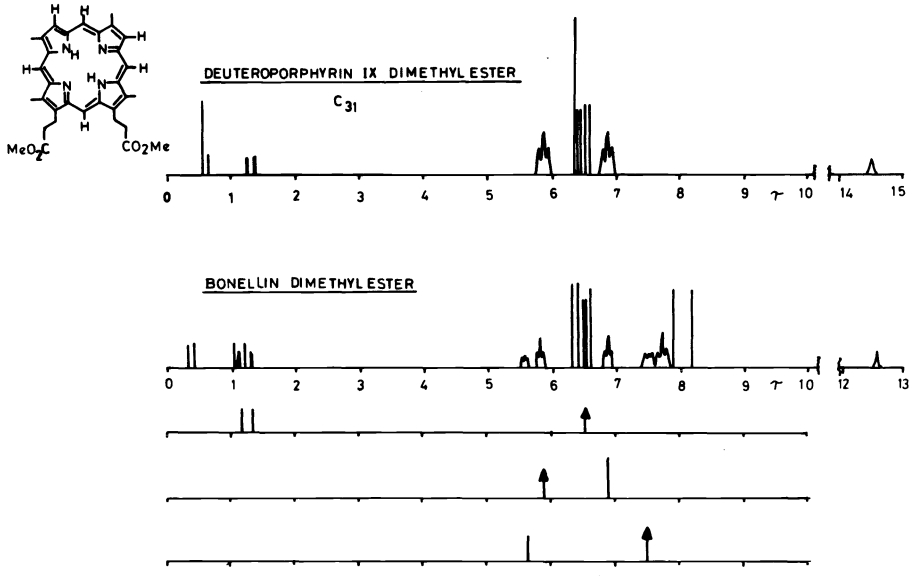


Fig. 9

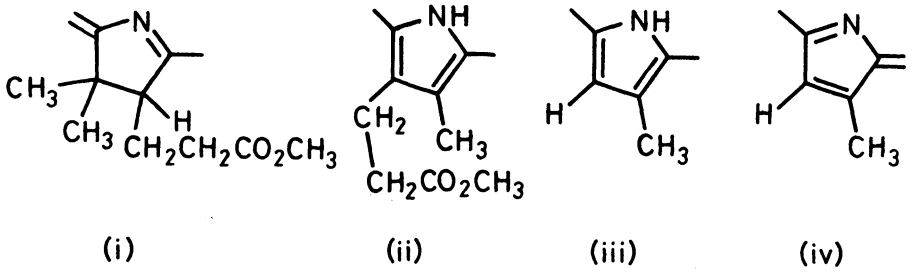
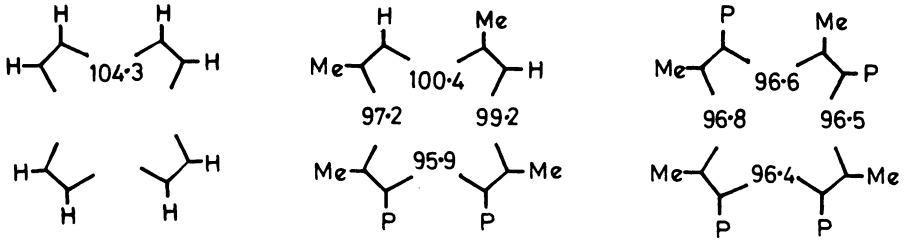


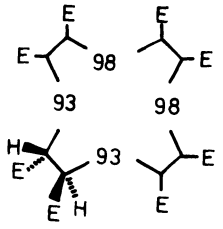
Fig. 10.

The sub-units (i) - (iv) can be assembled via the four meso-CH atoms, in a large variety of ways, none of which shows any very close relationship to chlorophyll, the parent of other known chlorins. In order to preserve a biogenetic relationship between bonellin and known naturally occurring porphyrins and chlorins, we confined our structural formulations to type III porphyrins. On this basis, BDME can be formulated as either (1b) or (2b), formally corresponding to the addition of methane across the peripheral positions of either ring D or C of deuteroporphyrin IX dimethyl ester. Bonellin itself would, of course, correspond to either (1a) or (2a).



H	H	103 – 104·5
Me	H	99 – 100·5
Me	Me	96 – 97·5

Fig. 11.



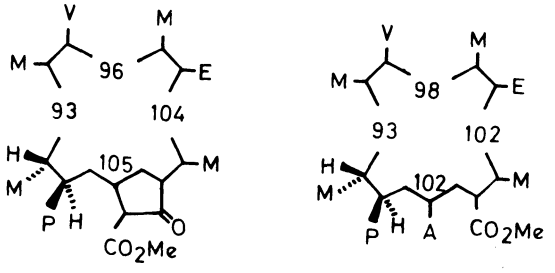
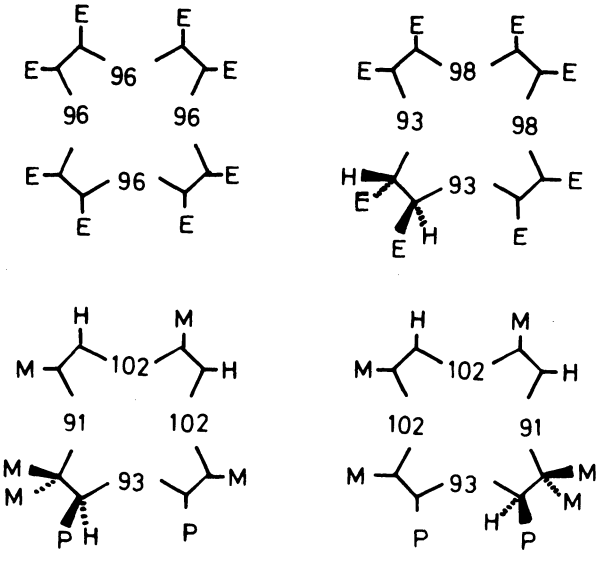


Fig. 12.



Position	Shift
5	+2
10	+2
15	−3
20	−3

Fig. 13.

TABLE II

^{13}C n.m.r. of BDME and related compounds.[a]

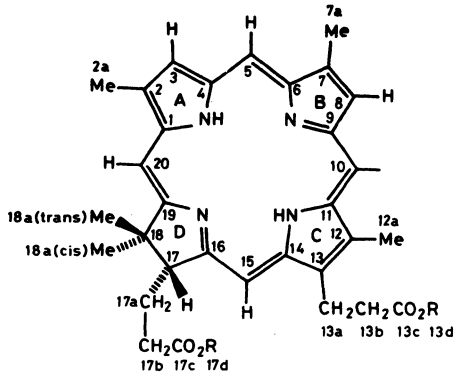
SIDECHAINS	BDME	Deuteroporphyrin IX DME	Chlorin esters[b,1,2]
2a	13.4	13.4	10.8 - 12.0
7a	13.4	13.4	10.7 - 11.3
12a	11.2	11.3	11.7 - 12.3
18a(cis)	32.0	11.3	-
18a(trans)	23.4		22.7 - 23.4
3a	-	-	128.3 - 128.8
3b	-	-	121.0 - 122.6
8a	-	-	18.1 - 19.4
8b	-	-	17.1 - 19.2
13a	21.5	21.5	-
13b	36.6	36.7	-
13c	173.8 or 174.2	172.8	-
13d	51.5 or 51.7	51.4	-
17a	31.8	21.5	30.8 - 31.2
17b	27.6	36.7	29.5 - 31.3
17c	173.8 or 174.2	172.8	172.4 - 172.8
17d	51.5 or 51.7	51.4	51.2 - 51.5
'SATURATED' RING			
16	165.7 or 172.1	Aromatic 128-144.2	159.0 - 173.4
17	57.8		50.8 - 52.0
18	49.7		49.2 - 50.0
19	165.7 or 172.1		159.0 - 173.4
AROMATIC RINGS			
3	126.3	130.2	122.0 - 154.5
8	130.3	136.0	
MESO			
5	102.1	98.7	95.4 - 102.1
10	102.5	99.5	101.7 - 105.9
15	91.2	95.1	92.5[c]
20	93.0	96.2	91.8 - 93.3

a) All spectra run in $CDCl_3$, chemical shifts in p.p.m. downfield from $SiMe_4$.
b) Wide range of chlorins[1,2].
c) trans-Octaethylchlorin as comparison to eliminate effects due to carbocyclic ring.

1) R.J. Abraham, G.E. Hawkes and K.M. Smith, J.C.S. Perkin II, 1974, 628. 2) K.M. Smith and J.F. Unsworth, Tetrahedron, 1975, 31, 367.

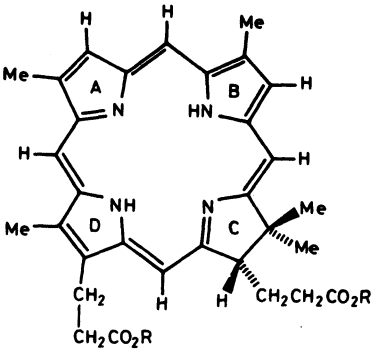
We attempted to distinguish between (1) and (2) by examining the chemical shifts of the C-5 and C-10 carbon atoms of BDME, which do not correspond well with those of octaethylchlorin (Table II). For porphyrins, it has recently been shown that meso-carbon atoms flanked by two protons give rise to signals in the ^{13}C n.m.r. at ca 104 p.p.m. compared with ca 96 p.p.m. when flanked by two simple alkyl groups[18]. Furthermore, in deuteroporphyrin IX, the signals due to C-5 and C-10 are ca 3 - 3.5 p.p.m. downfield of C-15 and C-20 (Fig 11) and the same trend would be anticipated in the electronically similar chlorins (Fig 11).

Comparison of the positions of the signals from the meso-carbon atoms of octaethylporphyrin and octaethylchlorin gives an index of the corrections to be applied to the meso-positions of a porphyrin on saturating one of the four heterocyclic rings (Fig. 13). We took octaethylchlorin rather than any chlorophyll derivative as our model compound as it lacked a C-15 substituent and also a carbonyl group attached to C-13 which we have shown in model experiments to strongly affect the signal due to C-10, the chemical shift of which is crucial to our argument (Fig 12).



(1)

(a) R = H; Bonellin
(b) R = Me; BDME.



(2)

The ^{13}C n.m.r. of BDME is assigned as in Table II, assignments being supported by the off-resonance spectrum. Signals for all the carbon atoms were observed and most could be specified by comparison with compounds of known structure. The 'aromatic' methyl groups at 12a, 2a and 7a appear at positions comparable with those of deuteroporphyrin IX as expected. Furthermore the unsubstituted ring atoms C-3 and C-8 of BDME appear at the corresponding positions with the correct multiplicity, thus confirming completely the structure of rings A and B. The saturated carbon atoms C-17 and C-18 (structure 1) appear at the expected postitions. Most importantly the signal assigned to C-18 remains a singlet in the off-resonance spectrum, thus proving the presence of a gem-dimethyl grouping whilst that of C-17 becomes a doublet. The signals assigned to the propionate groups attached to the reduced and non-reduced ring compare well with those for deuteroporphyrin IX and a wide range of chlorins.

The gem-dimethyl carbon atoms gave resonances at 23.4 (18a, cis) and 31.8 p.p.m. (18a, trans). The shift in the position of the trans- methyl group was confirmed by single frequency decoupling experiments using the well separated frequencies of the 'aliphatic' methyl groups and is in line with the stereoassignments made to dicyanocobalinamide[17]. Thus the ^{13}C n.m.r. of BDME is in accord with structures (1) and (2).

On this basis Fig. 13 shows the amendments needed for the values of the meso-carbon atoms in the conversion of a simple porphyrin to the corresponding chlorin. Also shown are the two possible sets of values for bonellin (structures (1) and (2)). In the case of structure (1) three of the four values are in line with those expected by applying the increments for the appropriate positions (Fig. 11) to deuteroporphyrin IX DME. Indeed if the effect of a gem-dimethyl group upon an adjacent meso-carbon atom, which is unknown, is similar to that of a CH_3-CH, then all four values fit. However in the case of structure (2), there is a meso-carbon atom at 102 p.p.m. between two flanking methyl groups. This should be found at 98 - 99.5 p.p.m. and the gross discrepancy led us to favour (1) for bonellin[14] despite the fact that it is ring C that is modified in vitamin B_{12} as in (2).

Naturally, as the spectroscopic investigations proceeded, we were attempting to obtain bonellin or one of its derivatives in a form suitable for X-ray analysis. Despite numerous attempts on many derivatives, none of the crystals obtained could be analysed. However, we had noticed that in the mass spectrum of bonellin there was a peak corresponding to loss of water. It was thought that this could be due to a dehydrative cyclisation of a propionate unit onto an adjacent meso-carbon atom i.e. C-15.[19] In an effort to emulate this reaction chemically, we dissolved bonellin in cold concentrated sulphuric acid, on which an irreversible change in the spectrum occurred. The product, anhydrobonellin, was obtained in high yield and purified as the monomethyl ester (ABME). On the basis of its ^1H and ^{13}C n.m.r. spectra,[20] (Fig. 14) as compared with BDME, it was clear that anhydrobonellin was derived specifically by cyclisation of the propionate group attached to C-17 rather than the similar grouping at C-13.

A summary of some of the spectral changes on passing from bonellin to anhydrobonellin is shown in Table III. Clearly it is the C-17 propionate group that is involved in the cyclisation.

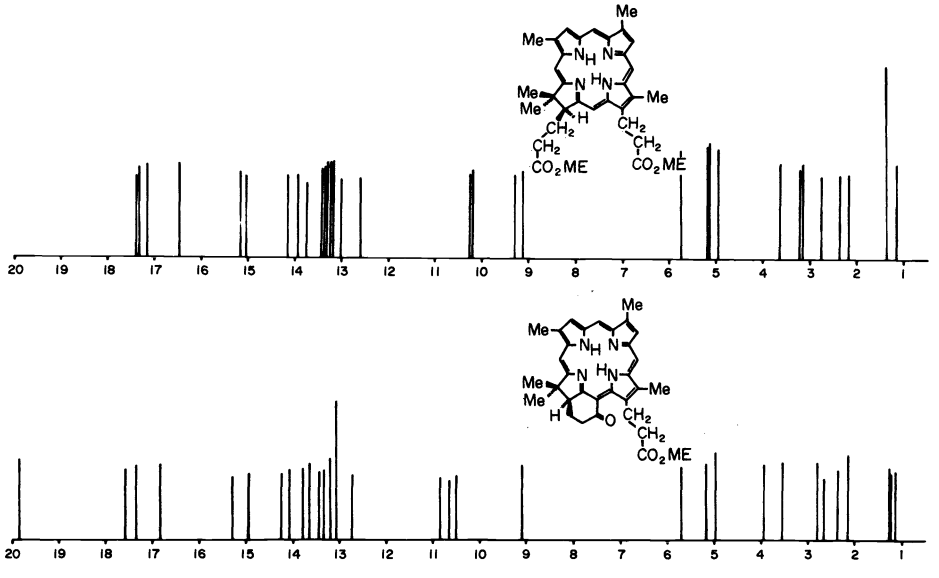


Fig. 14.

TABLE III

OBSERVED CHANGES OF NMR SPECTRA IN ANHYDROBONELLIN.

ATOMS	^{1}H (B-AB) * p.p.m.	^{13}C (B-AB)* p.p.m.
13a	+ 0.25	- 1.9
13b	- 0.11	+ 1.0
17	+ 0.28	+ 0.9
17a	- 0.20	- 7.2
17b	- 0.30	+ 6.0
17c	-	- 26.6
17d	no signal	no signal
18a cis	- 0.11	- 2.9
18a trans	+ 0.34	+ 3.9
15	no signal	- 11.1
12a	+ 0.1	- 0.3

* tentative assignments

\+ means shift to HIGH field in anhydrobonellin

The absorption spectrum of anhydrobonellin compares well with that of anhydromesopyrrochlorin (Fig. 15) which we found had been made by Fischer many years before[21] and anhydrobonellin and its monomethyl ester were assigned structures (3a) and (3b) on the basis of (1) for bonellin.

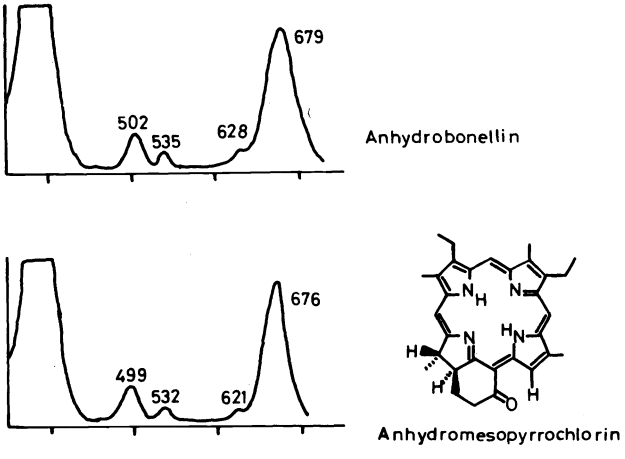
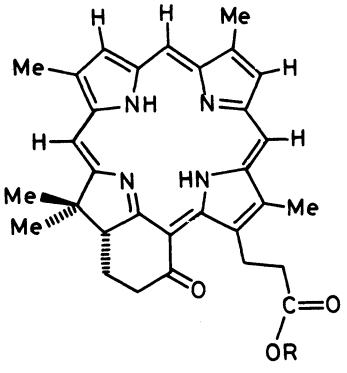


Fig. 15.



(3)

(a) R = H, Anhydrobonellin

(b) R = Me, ABME

A kinetic study of the dehydration carried out by Dr. P. Camilleri,* not only gave us its thermodynamic parameters, but assured us that the reaction took place in one step without any intermediate stages. An X-ray study on a single crystal (unfortunately racemic) of ABME gave the result shown in Fig. 16, and confirmed in every detail the structure (3b) for ABME and hence (1) for bonellin.[20] Another view of ABME is shown in Fig. 17. This shows that one methyl group at C-18 is almost at right-angles to the planar chlorin nucleus.

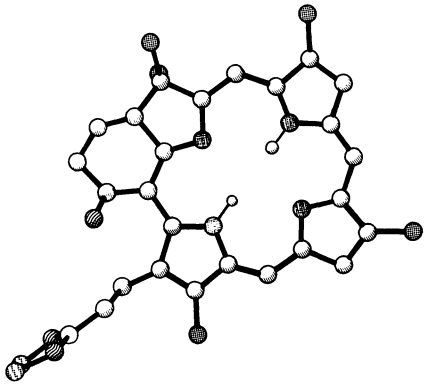


Fig. 16

* We thank Dr. P. Camilleri of the University of Malta for kindly supplying these results.

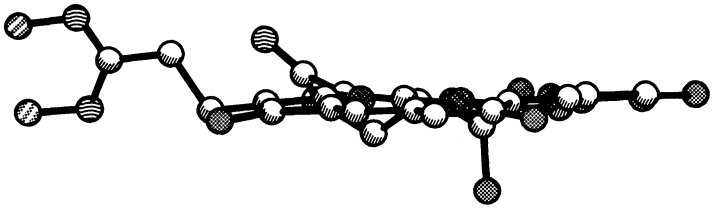


Fig. 17.

We had observed that there was present in the proboscis extract, a quantity (2%) of a pigment different from bonellin in chromatographic behaviour but with the same chromophore. Hence we examined the pigments of the body wall and viscera with the result shown in Table IV. (Peak 2 corresponds to the methyl ester(s) of the new pigment(s).) It can be seen that, in the body wall in particular there is a high proportion of this new pigment.

TABLE IV.

Distribution of Pigments in B.viridis.

	No. of animals	Wt. of purified material (mg)	BDME %	Peak 2 %
Body wall	20	57	50	50
Probosces	20	77	98	2
Viscera	20	0.5	71	29

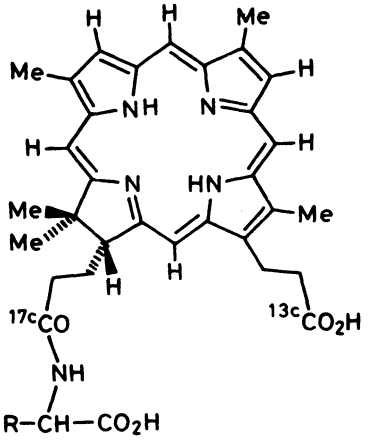
Examination of the mass spectra of the purified material showed that it was a mixture of amides produced by conjugation of single α-aminoacids to the C-17 propionic acid unit and that the parent compounds could be assigned structures (4).[22] We were unable to separate the mixture, but hydrolysis gave bonellin and a mixture of α-amino-acids with the composition shown in Table V. This composition was confirmed and the configuration of the amino-acids assigned, by g.l.c. of the N-pentafluoropropionylamino-acid-(-)-3-methyl-2-butyl esters.[23]

TABLE V.

Amino-acid Composition of Bonellin Conjugates.

Amino-acid	% of mixture	Configuration
Valine	62.7	L-
Isoleucine	23.0	L-
Leucine	5.9	L-
Alloisoleucine	4.0	D-

The biological significance of these amide conjugates of bonellin remains to be determined. We have been informed by Dr. G. Prota (University of Naples) that he has independently obtained the C-17d isoleucine conjugate of bonellin as the main component of the pigment of the body walls of B.viridis collected from the sea near Naples.[24]



Structure 4.

Of course, as the chemical work was progressing a parallel biological programme was being carried out.[25] As mentioned previously the larvae of B.viridis tend to settle on the proboscis of the female, and in the closed conditions of our tanks 98% became male. After settlement on the female, male characteristics appeared within 2h, after 3 days they were typical planariform males and after 6 days male differentiation was complete. Larvae isolated from the females, themselves became female at a much slower rate e.g. 33% in 2 days, 61% in 24 days and 78% in 31 days.

The results of tests with bonellin (98.5% pure) are shown in Table VI.[5] Clearly bonellin strongly increases the percentage of males, as compared with the pure sea-water control, though it is in no way as efficient as the adult female. This may be an effect of local concentration of active chemical or it may be that, in addition to bonellin, the female produces other active material. There would seem to be no question however that bonellin or its derivatives are involved in producing the masculinising effect, though the results are somewhat batch sensitive.

Table VI.

Results of masculinisation experiments giving the % distribution of the various sexual differentiations of the indifferent larvae.

Concentration of bonellin	♀	♂	(♂)	♀♂	Indifferent
1 ppm	64.6	19.1	12.1	3.0	1.0
0.5 ppm	61.1	32.6	3.2	2.1	1.0
0.2 ppm	77.3	6.2	8.2	7.2	1.1
0.01 ppm	44.6	20.7	23.8	8.7	2.2
With adult female	1.0	1.0	98.0	0	0
Seawater control	89.5	3.2	5.3	1.0	1.0

An effect we have studied in some depth is erythrocyte haemolysis. Fig. 18 shows that erythrocytes that have been exposed to bonellin in the dark or to pre-illuminated bonellin solutions in the dark do not lyse (even after periods much longer than shown in the Figure.) However erythrocytes which are exposed simultaneously to bonellin and light first swell, then rapidly lyse. The action spectrum of bonellin coincides with the absorption spectrum and whilst anhydrobonellin has some activity, copper bonellin has none.

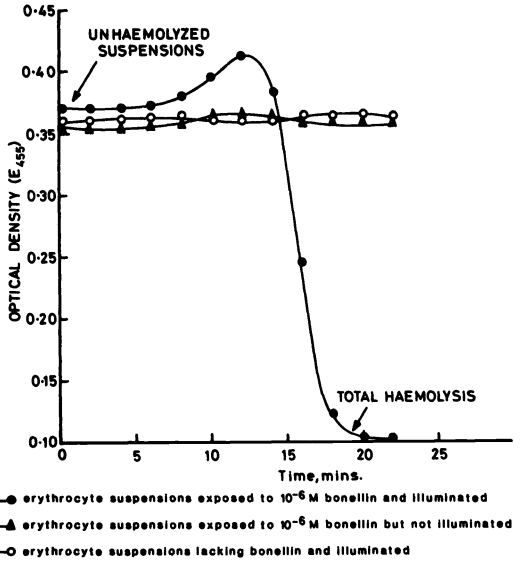


Fig. 18.

Absence of oxygen in the atmosphere halves the rate of haemolysis and the effect of $^1O_2$ quenchers shows that singlet oxygen is active in the reaction.

The results lead to the conclusion that neither bonellin nor its photo-oxidation product(s) are the active agents for lysis of erythrocytes. Possibly an unstable intermediate in the photo-oxidation (a hydroperoxide?) is the active agent and this problem is currently under investigation.

Suspensions of echinoid spermatazoa exposed to 10^{-6}M solutions of bonellin and illuminated for 15 min., sediment on centrifugation. Examination showed they were immobile and highly swollen and they eventually died. Control suspensions illuminated in the absence of bonellin or treated with bonellin in the dark or with pre-illuminated bonellin in the dark retained their normal shape, could be resuspended, and were highly active. (Table VII). Eggs also were strongly affected by a combination of bonellin and light, as was the development of fertilised eggs (Table VIII).

Table VII.

Effects of bonellin on fertilization of P.lividus eggs. In A the gametes were suspended in a 1.5 x 10^{-6}M solution of bonellin in seawater and kept in total darkness for 10 min before insemination. In B the gametes in bonellin solution were illuminated for 10 min before insemination. In A and B the final concentration of bonellin in the mixed egg/sperm suspension was 10^{-6}M.

Treatment of sperm or eggs in bonellin solution before insemination	Conditions of fertilization	% eggs with fertilization membrane 1 hr after insemination	% eggs forming normal blastulae after 6 hr
A. (i) Non-illuminated sperm	In the dark	60 - 80*	60 - 80*
	In the light	10 - 30	0
(ii) Non-illuminated eggs	In the dark	70 - 90	70 -90
	In the light	5 - 30	0
B. (i) Illuminated sperm	In the dark	0 - 5	0 - 5
	In the light	0 - 5	0
(ii) Illuminated eggs	In the dark	5 - 20	5 - 20
	In the light	0 - 5	0

* Controls in which (a) the gamete suspensions in seawater lacking bonellin were illuminated from 10 min before insemination till 6 hr afterwards; and (b) the gametes were kept in the dark for the same period; gave 70 - 90% formation of the fertilization membrane after 1 hr and 70 - 90% normal blastulae after 6 hr in both cases.

Table VIII.

Effects of adding bonellin to suspensions of fertilized P. lividus eggs at various times after insemination. Final concentration of bonellin in seawater, 10^{-6}M. Temperature 20°C.

Illumination after addition of bonellin	Time of addition of bonellin after insemination	Stage reached by at least 70% of eggs* 3 hr after insemination	48 hr after insemination
A. Absent	0 min	32-cell or later	plutei
	15 min	32-cell or later	plutei
	45 min	32-cell or later	plutei
	75 min	32-cell or later	plutei
	120 min	32-cell or later	plutei
B. Present	0 min	1-cell	1-cell
	15 min	1-cell; abnormal 2-cell	1-cell; abnormal 2-cell
	45 min	Abnormal 2-cell	Abnormal 2-cell
	75 min	Abnormal 4- and 8-cell	Abnormal 4- and 8-cell
	120 min	Abnormal 4- and 8-cell	Abnormal 4- and 8-cell

*The developmental stages reached by fertilized P. lividus in normal seawater at 20°C were monitored at intervals after insemination and gave the following results:- 0, 15 and 45 min, uncleaved; 75 min, 2-cell, 120 min, 4-cell; 3 hr, 32-cell or more advanced; 48 hr pluteus.

Clearly the biochemistry of bonellin remains to be explored. In addition the photochemistry of this unique molecule must be investigated in depth. What is clear however is that a characteristic of organisms that is normally self-determined i.e. its sex, can be strongly influenced by an external and naturally produced chemical or chemicals. The mode of sex-determination in Bonellia is thus of general interest for the understanding of sexual differentiation in animals.

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