Biosynthesis of Cochlioquinones [1]

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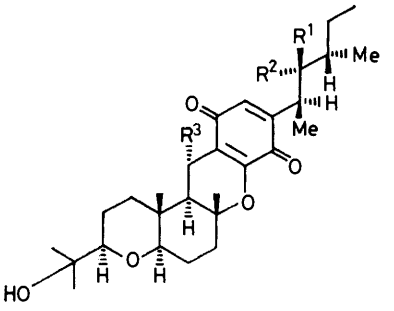
The biosynthesis of cochlioquinones (1) and (2) was studied by isotopic tracer experiments with ^{13}C- and ^{14}C-labelled compounds. The sequence of intermediates involved in the biosynthesis of the acetogenin moiety of (1) and (2) was assessed by incorporation of ^{14}C-labelled precursors. The use of doubly-labelled ^{13}C-acetate and ^{13}C n.m.r. spectroscopy made it possible to determine the rest of the pathway as follows: prenylation of the acetogenin-derived aromatic nucleus, decarboxylation and hydroxylation in the same position, and finally cyclization of the terpenoid moiety. Details of the cyclization process were obtained by the use of $^{18}O_2$ and mass spectrometry.

A PARASITIC mould of rice, *Cochliobolus miyabeanus*, has been found to produce ophiobolins [2] and, as minor metabolites, two yellow pigments, cochlioquinones A (1) and B (2). Their structures have been determined by chemical, spectroscopic, and crystallographic evidence.[3]



(1) $R^1 = H; R^2 = OAc; R^3 = OH$

(2) $R^1, R^2 = O; R^3 = H$

Previous studies have elucidated the biosynthetic origin of the carbon skeleton of cochlioquinones: their mixed biosynthesis occurs through the introduction of a farnesyl unit onto an aromatic precursor whose secondary methyl groups derive from methionine.[4] Our previous experiments indicated that the two oxygen atoms of the 2-(2-hydroxypropyl)tetrahydropyran system are derived from two different oxygen molecules in the course of two separate oxidative stages on the biosynthetic pathway.[5] Here we describe details of the biosynthesis steps and the most probable sequence of reactions and intermediates involved in the pathway to cochlioquinones.

RESULTS AND DISCUSSION

Incorporation of [1,2-$^{13}C_2$]acetate into cochlioquinones confirmed the biosynthetic origin of the carbon skeleton. Enriched (1) was isolated from cultures of *Cochliobolus miyabeanus* containing 90% [1,2-$^{13}C_2$]acetate. In the proton-noise-decoupled Fourier-transform ^{13}C n.m.r. spectrum of labelled (1), 24 signals with ^{13}C–^{13}C coupling were detected, indicating that 12 acetate units were incorporated intact. The labelled sites appeared as triplets with characteristic ^{13}C–^{13}C coupling constants for the carbon satellites (Table 1).

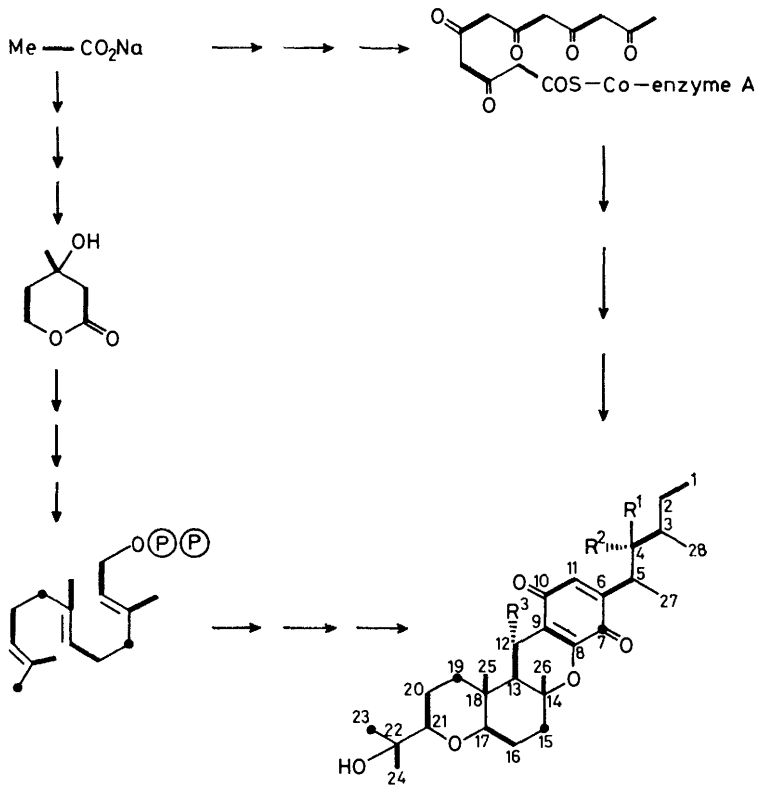
Detection of only 5 ^{13}C–^{13}C couplings in the acetogenin-derived part of (1) confirmed the cleavage of a ^{13}C–^{13}C bond in the biosynthesis (Scheme 1). Furthermore the enriched singlet due to C-7 shows that the decarboxylation and the hydroxylation of the aromatic nucleus involve the same carbon atom (Scheme 2). As in many other examples found in the literature,[6] a flavin-requiring hydroxylase may play a definite role in this process. It is worthy of note that all the substrates for these flavin-requiring hydroxylases have a phenolic OH group adjacent to the site where they are attacked.[6] The presence of a free adjacent phenolic group is always required for the direct replacement of a carboxy-group by a hydroxy-group.[7] Thus the cyclization process leading to the formation of the three rings of the terpenoid moiety

TABLE 1

^{13}C N.m.r. data for cochlioquinones A (1) and B (2)

Carbon atom	Chemical shifts Compound (1)	Compound (2)	^{13}C–^{13}C Coupling in (1) labelled with [1,2$^{13}C_2$]-acetate/Hz	Average enrichment (%) *
1	11.4	11.8	35.7	4.1
2	26.4	29.7	35.0	4.1
3	36.2	47.3	40.3	4.1
4	63.0	212.0	40.2	4.1
5	34.4	46.4	46.0	4.1
6	148.3	145.3	44.3	4.1
7	181.4	181.2		4.1
8	151.5	151.9	68.7	4.1
9	118.9	118.3	69.0	4.1
10	188.6	186.4	52.6	4.1
11	133.6	133.3	52.9	4.1
12	78.3	43.0	38.8	3.0
13	51.7	47.3	39.5	3.0
14	82.9	80.5	37.1	3.0
15	37.5	35.5		3.0
16	25.2	25.2	39.3	3.0
17	83.7	83.9	37.9	3.0
18	36.6	36.8	37.1	3.0
19	38.5	37.1		3.0
20	21.5	21.3	36.7	3.0
21	85.0	84.8	36.7	3.0
22	71.8	71.7	39.8	3.0
23	23.8	23.8		3.0
24	26.0	26.0	40.4	3.0
25	12.5	12.2	36.9	3.0
26	21.0	20.8	38.0	3.0
27	17.3	16.6		0
28	13.2	14.8		0
29	170.4		60.0	7.1
30	20.7		60.0	7.1

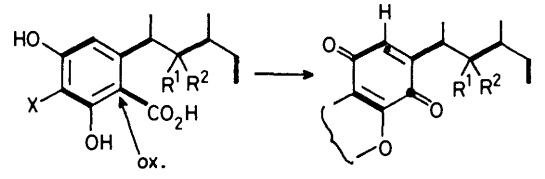
* A. G. McInnes, D. G. Smith, J. A. Walter, L. C. Vining, and J. L. C. Writh, *J.C.S. Chem. Comm.*, 1974, 282



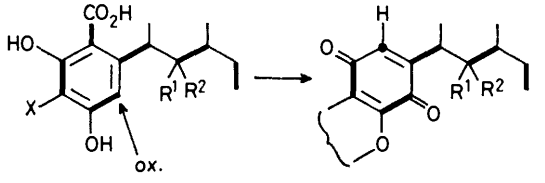
SCHEME 1

only occurs after the decarboxylation–hydroxylation step (Scheme 3). On the basis of many known examples,[8] the presence of a neighbouring carbonyl group is always required for the prenylation of an acetogenin-derived aromatic nucleus. Consequently it is possible to describe the biosynthetic sequence to cochlioquinones in this way: prenylation, decarboxylation–hydroxylation, and then cyclization of the farnesyl chain (Scheme 3).

(*a*) Decarboxylation and hydroxylation in the same position



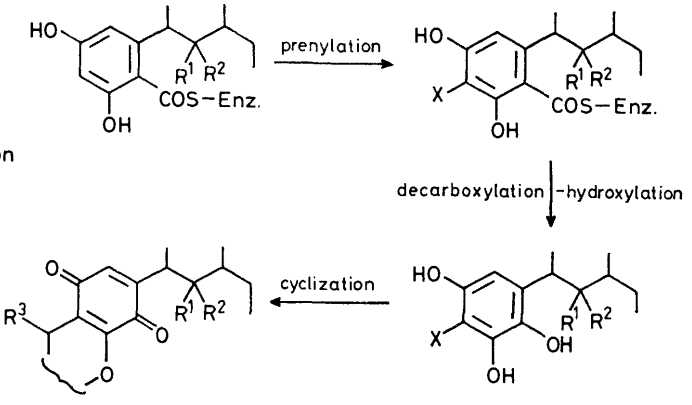
(*b*) Decarboxylation and hydroxylation in the other position



X = farnesyl

SCHEME 2

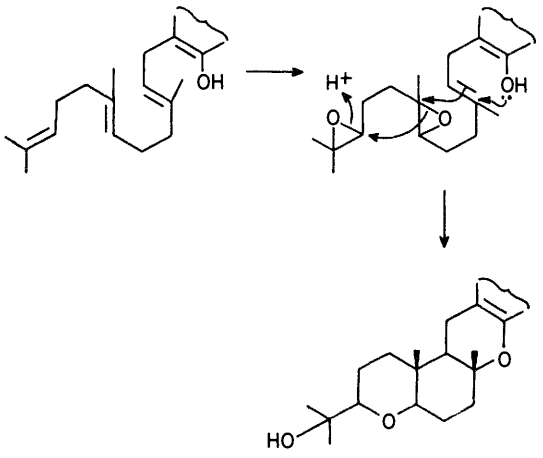
Our previous experiments, performed by using $^{18}O_2$ as isotopic tracer and mass spectrometry,[5] showed that the two oxygen atoms of the 2-(2-hydroxypropyl)-



X = farnesyl

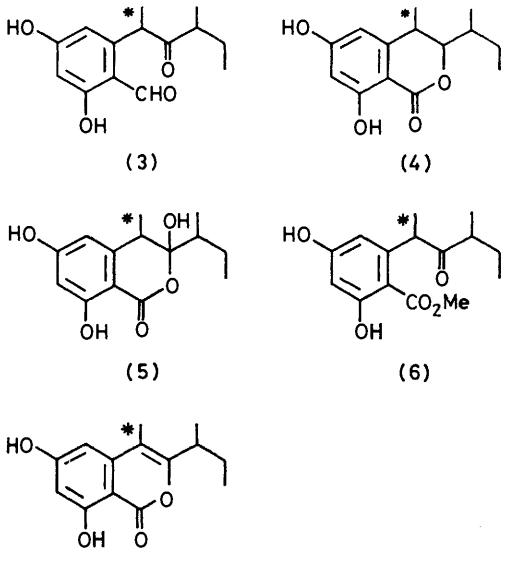
SCHEME 3

tetrahydropyran system are derived from two different oxygen molecules at separate steps on the biosynthetic pathway. Thus the three rings of the terpenoid moiety appear to be biosynthesised by the intervention of two epoxides, their opening being promoted by the cyclization process that starts from the attack of the phenolic hydroxy-group (Scheme 4). The identification of the acetogenin on which the prenylation occurs was carried



SCHEME 4

out through incorporation of the potential advanced precursors (3)—(7). These labelled compounds were obtained by alkylation with ^{14}MeI of the appropriate ketone, under phase-transfer conditions.[9] The specifi-



(3) (4) (5) (6) (7)

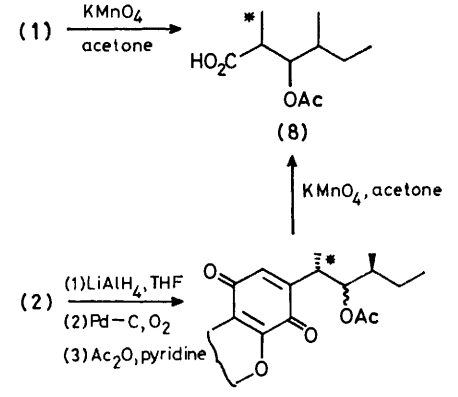
* denotes ^{14}C

city of incorporation into (1) and (2) was tested by oxidative demolition in order to give the acid (8),[3] isolated and counted as *p*-bromophenacyl ester (Scheme 5).

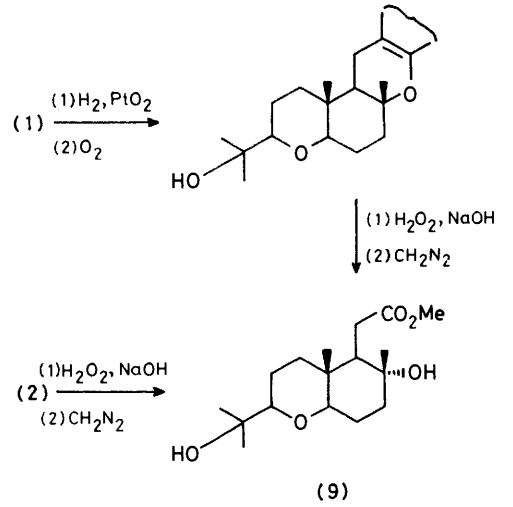
The incorporation into the terpenoid moiety was tested by alkaline hydrogen peroxide oxidation in order to afford compound (9) [3] (Scheme 6). The incorporation data of the precursors and the observed incorporation specificity are reported in Table 2.

The rate of formation of cochlioquinones seems to depend more on the biosynthesis of the acetogenin moiety than on that of farnesol: by feeding labelled acetic acid to the culture, the percentage recovery of label in the terpenoid moiety is higher (83—84%) under *a*-type conditions than under *b*-type conditions (54—55%) (Table 2). In the first days acetic acid is used mainly for terpene synthesis. Labelled cochlioquinone (2), obtained by feeding [2-^{14}C]mevalonolactone (Table 2, entry 1), gave a slightly labelled cochlioquinone (1). Furthermore this low radioactivity is derived from a degradation-resynthesis pathway: the percentage recovery of label in (8) and (9), obtained from cochlioquinone (1) derived by feeding of (2) or acetic acid, is similar (Table 2, entries 12 and 13). Therefore, as regards the sequence, the reduction of the side-chain carbonyl group is probably one of the first steps of the biosynthetic pathway to cochlioquinone (1).

The non-specific labelling of lactol (5)-derived cochlioquinones (1) and (2) indicates lactol degradation to acetate prior to incorporation (Table 2, entries 6 and 9; compare with entries 3 and 13). The specific incorporation of the unnatural methyl ester (6) shows that the micro-organism can hydrolyse the ester with no formation of lactol (5), and can apparently transform it into the enzyme-bound ester (10). The specificity of incorporation is higher under *b*-type conditions (Table 2, entries 7 and 10) which reduce the degradation pathway. The feeding of methyl ester (6) to the micro-organism represents the only way of obtaining information on this



SCHEME 5



(9)

SCHEME 6

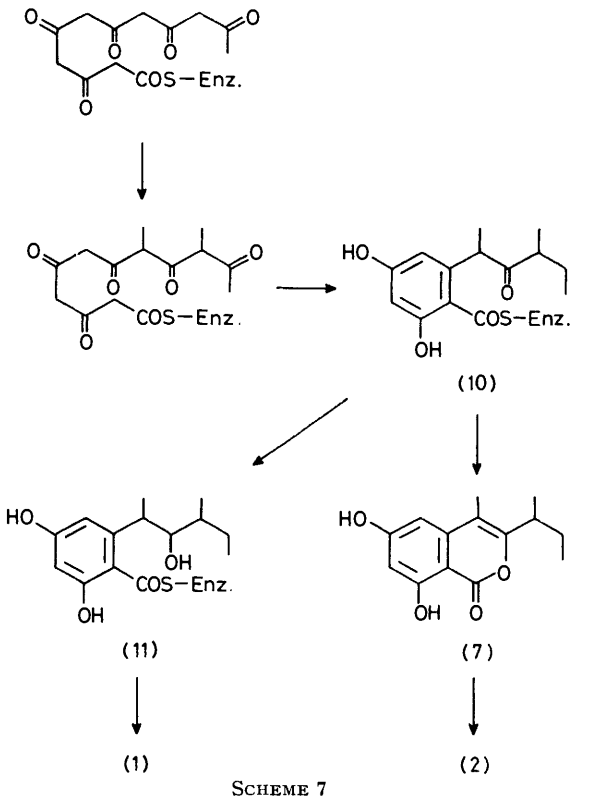
TABLE 2

Incorporation data for cochlioquinones A (1) and B (2)

Entry	Precursor	Percentage incorporation into (1)	Percentage recovery of label in (8)	Percentage recovery of label in (9)	Percentage incorporation into (2)	Percentage recovery of label in (8)	Percentage recovery of label in (9)
1 [a]	[2-^{14}C]Mevalonolactone	15		100	3		100
2 [a]	[Me-^{14}C]Methionine	1.59	96.5	2	0.86	96.5	2
3 [a]	[2-^{14}C]Acetic acid	50	7.0	84.1	9	8.7	83.2
4 [a]	(3)	0			0		
5 [a]	(4)	0			0		
6 [a]	(5)	7.1	7.5	85	1.4	8.1	84
7 [a]	(6)	0.25	80.2		0.15	81.5	
8 [a]	(7)	0			0.45	80.1	
9 [b]	(5)	7.0	25	50	1.3	20	59
10 [b]	(6)	0.20	94.2		0.10	95.0	
11 [b]	(7)	0			0.40	91.2	
12 [b]	Labelled (2) derived from [2-^{14}C]mevalonolactone	1.27	25	47			
13 [b]	[2-^{14}C]Acetic acid	39	26.5	54.5	7	21.3	54.9

[a] Labelled precursors were added to 4-day culture broths; 9 days after the addition, the fermentation was interrupted and (1) and (2) isolated. [b] Labelled precursors were added to 9-day culture broths; 4 days after the addition, the fermentation was interrupted and (1) and (2) isolated.

step of the biosynthesis, since the keto-acid exists only as the cyclised lactol structure (5).[9] Enol-lactone (7) is incorporated only into the cochlioquinone (2) (Table 2, entries 8 and 11): the carbonyl group of the side-chain is masked as the enol derivative and this is probably the way to protect it from the reduction. A double methylation of the hexaketide, followed by its aromatization to (10), is the common biosynthetic pathway for cochlioquinones (1) and (2) (Scheme 7). At this stage the biosynthetic pathways begin to differ: thus the carbonyl group reduction occurs in the case of (1), while enol-lactone formation takes place in the case of (2). The most probable sequence of reactions and intermediates involved in the biosynthesis of the acetogenin moiety is shown in Scheme 7.



SCHEME 7

EXPERIMENTAL

Incorporation of [1,2-$^{13}C_2$]*Acetate.*—Previously reported [10] cultural conditions were used. After five days fermentation, [1,2-$^{13}C_2$]acetate (90%) (0.1 g) was added to the cultures (two 750-ml Erlenmeyer flasks containing 150 ml of culture broth). After a further 24 h fermentation, another addition of doubly-labelled [$^{13}C_2$]acetate (0.1 g) was performed. Seven days after the addition, isotope-enriched cochlioquinones were isolated [40 mg (1); 8 mg (2)].

^{13}C N.m.r. spectra were recorded on a XL-100 Varian spectrometer operating at 25.2 MHz for *ca.* 0.1M solutions in $CDCl_3$, using a heteronuclear lock. All chemical shifts are quoted as p.p.m. downfield from internal $SiMe_4$.

Incorporation of ^{14}C-*Labelled Compounds and General Isolation Procedure.*—Labelled compounds were added to the cultures (two 750-ml Erlenmeyer flasks containing 150 ml of culture broth) after four days (*a*-type conditions, Table 2) or nine days fermentation (*b*-type conditions, Table 2). Nine days (*a*-type conditions) or four days (*b*-type conditions) after the addition, the mycelium in each flask was filtered off, crushed with Celite, and extracted with n-hexane until the extracts were colourless. The organic extracts were dried (Na_2SO_4) and evaporated to dryness *in vacuo*. Silica gel chromatography of this crude mixture does not efficiently separate cochlioquinones from ophiobolins. The residue was then hydrogenated in tetrahydrofuran (THF) over 10% palladium–charcoal catalyst for 8 h. After replacing hydrogen with air, some more 10% palladium–charcoal catalyst was added, and the solution was stirred overnight. Removal of catalyst and solvent gave a residue which was chromatographed on silica gel [dichloromethane–ethyl acetate (99 : 1—95 : 5)] to give cochlioquinone A (1) and B (2), efficiently separated from the degradation products derived from ophiobolins. Cochlioquinone A (1) and B (2) were crystallized (A from n-hexane; B from diethyl ether) to constant activity. Table 3 reports the amounts and the activities of labelled compounds incubated and of cochlioquinones obtained.

Specificity of Incorporation.—Oxidation of cochlioquinone (1) *with* $KMnO_4$. A solution of (1) (0.15 g) in acetone (20 ml) was treated with $KMnO_4$ (0.45 g). After 30 min at room temperature, the crude mixture was filtered through

TABLE 3

Compound	Precursor added/mg	Activity [c] of the precursor	Cochlioquinone (1) isolated/mg	Activity [c] of (1)	Activity [c] of (8) [d] derived from (1)	Activity [c] of (9) derived from (1)	Cochlioquinone (2) isolated/mg	Activity [c] of (2)	Activity [c] of (8) [d] derived from (2)	Activity [c] of (9) derived from (2)
[2-^{14}C]Mevalonolactone	12 [a]	18 516 667	580	57 466		97 320	155	43 000		64 402
[Me-^{14}C]Methionine	8 [a]	13 887 500	610	2 896	3 721	98	180	5 308	6 059	160
[2-^{14}C]Acetic acid	5 [a]	22 220 000	470	118 191	11 028	168 381	135	74 067	7 623	92 620
(5)	64 [a]	118 722	623	866	87	1 247	103	1 033	99	1 304
(6)	121 [a]	128 751	453	86	92		97	241	232	
(7)	41 [a]	92 515	706	0			108	158	150	
(5)	64 [b]	118 722	584	911	304	772	107	923	218	819
(6)	121 [b]	128 751	482	65	82		84	185	208	
(7)	41 [b]	92 515	658	0			103	147	159	
Labelled (2) derived from [2-^{14}C]-mevalonolactone	95 [b]	43 000	432	120	40	100				
[2-^{14}C]Acetic acid	5 [b]	22 220 000	512	84 627	29 894	78 130	128	60 758	15 310	50 134

[a] *a*-Type conditions: see Table 2. [b] *b*-Type conditions: see Table 2. [c] Units; disint. min^{-1} mg^{-1}. [d] Counted as *p*-bromophenacyl ester.

Celite. The Celite cake was washed with acetone until colourless, then treated with boiling water (50 ml) and refluxed for 20 min. The resulting mixture was filtered and the aqueous phase was extracted with ether. The aqueous layer was acidified with 3N HCl and then extracted with ether: the organic extracts were dried (Na_2SO_4) and evaporated. The residue (0.137 g) was dissolved in the minimum amount of ethanol and treated with 4% aqueous NaOH solution until the acid was just neutralised. The solution was warmed to 60 °C and treated with *p*-bromophenacyl bromide (0.185 g) in ethanol (6 ml). After 1 h reflux, the solution was allowed to cool and the solvent evaporated *in vacuo*. The residue (0.315 g) was chromatographed on silica gel [benzene–diethyl ether (99 : 1—93 : 7)] to give the *p*-bromophenacyl ester of (8) (0.03 g); *m/e* 301/299 (M^+, 7.5%), 185/183 (53), 157/155 (6.8%), 125 (29.3), 97 (13.7), 69 (12.1), and 43 (100).

p-*Bromophenacyl Ester of* (8) *from Cochlioquinone* (2).—Cochlioquinone (2) (0.14 g) dissolved in THF (14 ml) was added to a mixture of $LiAlH_4$ (0.035 g) in THF (15 ml), with stirring at 0 °C. After 1 h at room temperature, the usual work-up gave a product which was dissolved in THF, treated with 10% palladium–charcoal (0.015 g) under air, and stirred overnight. Removal of catalyst and solvent gave a residue which was acetylated under standard conditions. Oxidation of this product under the above-mentioned conditions ($KMnO_4$, acetone) gave the acid (8), isolated and counted as the *p*-bromophenacyl ester.

Oxidation of Cochlioquinone (2) *with* H_2O_2.—The previously reported conditions were used.[3]

Compound (9) *from Cochlioquinone* (1).—Cochlioquinone (1) was hydrogenated and then re-oxidised to quinone under the previously reported conditions.[3] Oxidation of this product under the above-mentioned conditions (H_2O_2–NaOH), and methylation gave (9).

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