Biosynthesis of violacein:[1] oxygenation at the 2-position of the indole ring and structures of proviolacein, prodeoxyviolacein and pseudoviolacein, the plausible biosynthetic intermediates of violacein and deoxyviolacein

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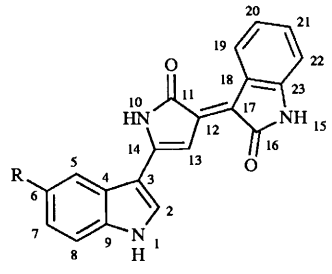
During a search for biosynthetic intermediates of violacein pigments **1** and **2**, employing enzyme inhibitors and blocked mutants, three novel tryptophan metabolites **3**, **4** and **6** were found. Compounds **3** and **4**, named prodeoxyviolacein and proviolacein, respectively, were red in neutral or basic medium, and blue in acidic solution. However, the red–orange colour of compound **6**, named pseudoviolacein, did not change with acidity. The structures were determined by chemical modifications and by the analysis of HRMS (EI) and 2D NMR spectra resulting from COSY (^1H–^1H and ^1H–^{13}C), HMBC and NOESY experiments. The structures of **3**, **4** and **6** were established as 3,5-di(indol-3-yl)pyrrol-2(2*H*)-one, 5-(5-hydroxyindol-3-yl)-3-(indol-3-yl)pyrrol-2(2*H*)-one and 5-(5-hydroxy-2-oxoindol-3-ylidene)-3-(indol-3-yl)pyrrol-2(1*H*)-one, respectively. The structure of **6** is similar to that of violacein **2**, differing only in that the other indolyl group is oxygenated. Compounds **3** and **4** do not have an oxoindole unit. The isolation of **3**, **4** and **6** together with **5** (named as pseudodeoxyviolacein, previously published)[1] lead to the proposition that the oxygenation at the 2-position of indole ring is a final step in the biosynthesis of violacein analogues.

Violacein **2** and its deoxy analogue, deoxyviolacein **1**, are blue–violet pigments, produced by the bacterial species *Chromobacterium violaceum*. They exhibit antibiotic activity against some microorganisms,[2] and strong cytotoxity against tumour cells such as L929, M5076 and HeLa.[3] Violacein consists of three structural units: 5-hydroxyindole, 2-oxoindole and pyrrol-2-one. In previous reports,[1,3–9] we have demonstrated that all the skeletal atoms were derived exclusively from L-tryptophan, and that all the oxygen atoms originated from aerial molecular oxygen.[5] Decarboxylation processes also occur during the biosynthesis[4,10] of which the most fascinating is the 1,2-shift of the indole ring occurring during the construction of the pyrrol-2-one nucleus between the side chains of two tryptophan molecules.[4] To elucidate the biosynthetic pathway and the mechanism of the indole shift, we have searched for intermediates and isolated various tryptophan metabolites with the aid of enzyme inhibitors and blocked mutants.[7–9] *N*,*N*-Diethyldithiocarbamate is known to be a copper-enzyme inhibitor.[11,12] Recently, by using *N*,*N*-diethyldithiocarbamate as a copper chelator, we have isolated an interesting compound, 5-(2-oxoindol-3-ylidene)-3-(indol-3-yl)pyrrol-2(1*H*)-one **5**, named as pseudodeoxyviolacein,[1] whose structure is quite similar to that of deoxyviolacein, differing only in that the oxygenation, by which the oxoindole ring is formed, has taken place at the other indole ring. We also isolated three other metabolites **3**, **4** and **6** related to violacein and deoxyviolacein by using the copper chelator and/or a blocked mutant. This paper describes their chemical structures and discusses the biosynthetic sequence of the oxygenation at the 2-position of the indole ring. Compounds **3** and **4** lack oxygenation at the 2-position of either of the indole ring.



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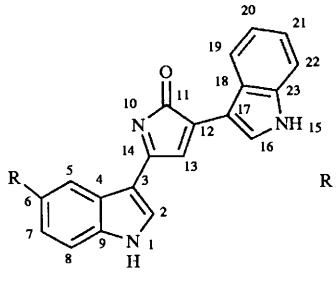

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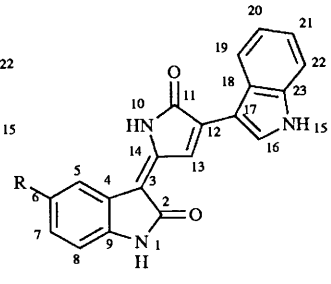


5 R = H
6 R = OH

Results and discussion

Administration of L-tryptophan into washed whole cells of *C. violaceum* in the presence of sodium *N*,*N*-diethyldithiocarbamate afforded a new red compound **3** along with pseudodeoxyviolacein **5**.[1] The metabolite **3** was purified as follows: the methanolic extract containing the pigments was adsorbed on Amberlite XAD 2, the resin was washed with 10% aq. methanol, and the pigments were eluted with abs. methanol, and then subjected to column chromatography over Sephadex LH 20 eluting with methanol. The following pigments were obtained in order of elution: compound **3**, compound **5**, deoxyviolacein and violacein. The colour of metabolite **3**, like a pH indicator, changed with the acidity. Fig. 1 shows the electronic spectrum of compound **3**: red (λ_{max} 493 nm) in neutral or basic medium, and blue (λ_{max} 609 nm) in acidic

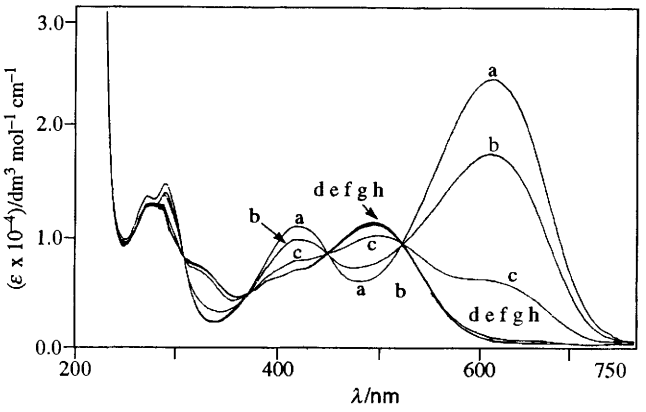


Fig. 1 Electronic spectra of compound **3** as a function of acidity. A stock solution of compound **3** in EtOH was diluted with an equal amount of aq. buffer solution, and then the electronic spectra were measured (final concentration of the pigment: 2.69 × 10^{-5} mol dm^{-3}). pH: a, 2.0; b, 4.0; c, 5.0; d, 6.0; e, 7.0; f, 8.0; g, 9.0; h, 10.0.

medium. The pK_a was determined to be *ca.* 4.6 from the titration curve, which was obtained from the plots of the absorbance at 609 nm against pH. Exact mass determination (EI) of compound **3** showed the molecular composition to be $C_{20}H_{13}N_3O$ (*m/z* 311.1062). The material was converted into its tetrahydro derivative (*m/z* 315) by catalytic hydrogenation over 5% Pd–C, suggesting the presence of two double bonds in molecule **3** other than the aromatic indole rings. The electronic spectrum of the hydrogenated product was as follows: λ_{max}(MeOH)/nm 220 (ε/dm^3 mol^{-1} cm^{-1} 62 430), 280 (11 100) and 289 (9300). This spectrum is typical for an indole ring, and did not change with pH. The molar absorptivity ε at 280 nm indicates that two indole rings are involved in compound **3**.[13] The NMR data of the hydrogenated material **7** (Fig. 2) in $CDCl_3$ were as follows: δ_H 2.54 (1 H, m), 3.12 (1 H, m), 4.18 (1 H, dd, *J* 11.6 and 8.6), 5.19 (1 H, dd, *J* 10.3 and 6.2), 6.02 (1 H, br s), 7.3–7.1 (6 H, m, ArH), 7.39 (2 H, dt, *J* 1.9 and 7.3), 7.69 (2 H, dt, *J* 1.9 and 7.3), 8.12 (1 H, br s) and 8.16 (1 H, br s); δ_C 38.8 (t), 40.4 (d), 49.6 (d), 111.3 (d), 111.5 (d), 113.2 (s), 116.4 (s), 118.9 (d), 119.1 (d), 119.6 (d), 119.9 (d), 121.3 (d), 122.1 (d), 122.3 (d), 122.6 (d), 125.4 (s), 126.7 (s), 136.5 (s), 136.7 (s) and 177.6 (s). Spin–spin decoupling experiments revealed that the two signals at δ_H 2.54 and 3.12 were non-equivalent CH_2 protons (*J* 13 Hz, geminal coupling). Three exchangeable protons (δ_H 6.02, 8.12 and 8.16) were found by addition of D_2O. The ^1H NMR spectrum of the tetrahydro product also proved that compound **3** has two indole rings (two indolic NHs at δ_H 8.12 and 8.16). The oxygen atom present in the molecule is part of an amide function (δ_C 177.6 and δ_H 6.02 for the tetrahydro derivative). ^1H–^1H COSY connectivities verified the presence of a 2-pyrrolidone moiety as shown in **7** (Fig. 2). To confirm the structure, the metabolite **3** was reduced with $NaBH_3CN$, a reagent for selective reduction of an imino group. The spectral data were as follows: *m/z* (EI) 313 (M^+); λ_{max}(MeOH)/nm 218 (ε/dm^3 mol^{-1} cm^{-1} 53 000), 276 (13 400), 288 (13 300) and 305–330 (br sh). The absorption at 305–330 nm is diagnostic of a vinylindole moiety.[13] A new high-field signal appeared in the NMR spectra of the dihydro product in [$^2H_6$]DMSO (Table 2): δ_H 5.59 (d, *J* 2.5 Hz) and δ_C 54.1 (d), suggesting that the sp^3 carbon is directly attached to the nitrogen atom. The ^1H–^1H COSY spectrum verified the presence of a =CH–CH–NH– moiety. The observed HMBC correlations ($^2J_{CH}$ and $^3J_{CH}$) from these protons (Fig. 2) supported the structure of **8** being the dihydro derivative of compound **3**. Thus, compound **3** was unambiguously assigned to be 3,5-di(indol-3-yl)pyrrol-2(2*H*)-one.

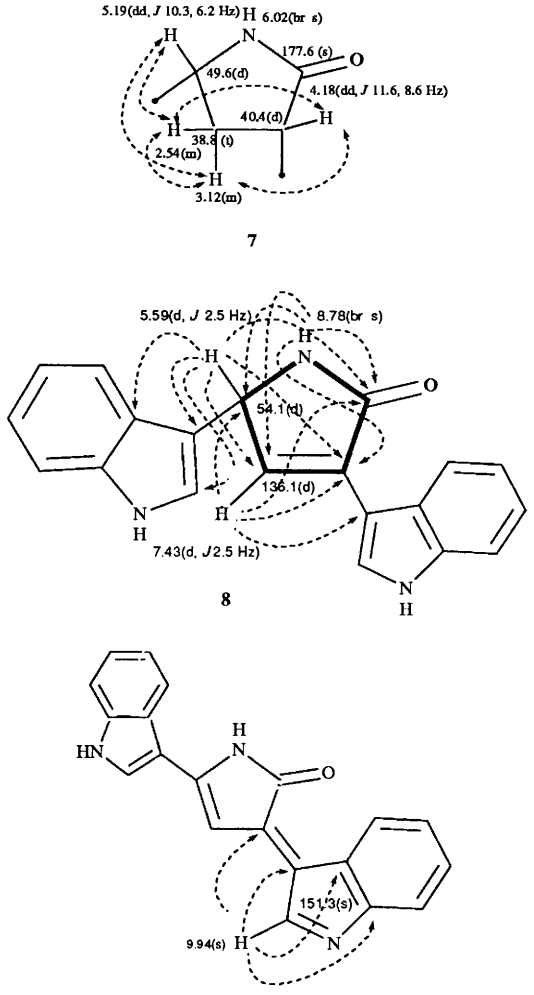


Fig. 2 2D NMR analyses for the derivatives of compound **3** and for the acidified species. Compounds **7** and **8** show the tetrahydro and dihydro derivatives, respectively. The two indole rings are not shown in **7**. The COSY correlations (←——→) observed between each proton of the pyrrolone moiety are shown in **7**. The observed HMBC correlations (- - - →) from three protons on the pyrrol-2-one ring are shown in **8**. The structure of compound **3** in acidic medium ([$^2H_6$]DMSO containing a trace of TFA) was determined to be **9** by HMBC analysis (- - - →) from the low-field resonance (δ_H 9.94).

The structural transformation caused by the acidity of the medium was investigated by NMR measurements, as solutions in [$^2H_6$]DMSO containing a trace of trifluoroacetic acid (TFA). Remarkable features of the ^1H NMR spectrum were as follows: one of the two indolic NHs was absent and a lower-field signal (δ_H 9.94, s) was present. The signal at δ_H 9.94 did not have any cross peak in the ^1H–^1H COSY spectrum, while it is correlated to that at δ_C 151.3 (d) in the ^1H–^{13}C COSY spectrum. It was inferred from HMBC correlations (Fig. 2) that the signal at δ_H 9.94 is assignable to the proton at the 2-position of the indole ring. Thus, the indolium **9** was assigned to the compound in an acidic medium. The indolium structure may contribute to the bathochromic shift to give the blue colour due to the extended conjugated system of π-electrons, the distribution of π-electrons of **9** being analogous to that of deoxyviolacein **1** [λ_{max}(50% aq. EtOH)/nm 570] (compare **1** with **9**). To measure the NMR spectrum in a basic medium, dry ammonia was bubbled into the [$^2H_6$]DMSO solution in an NMR tube. When this solution was set aside for *ca.* 1 day at room temperature the red colour disappeared to give an almost

Table 1 ^1H and ^{13}C NMR data of prodeoxyviolacein **3**, proviolacein **4** and pseudoviolacein **6** in [$^2H_6$]DMSO [δ_H(multiplicity, J/Hz)]

	3[a]		**4**[a]		**6**	
Position	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C
1	12.56 (br s)		12.38 (br s)		10.46 (br s)	
2	8.57 (d, 2.4)	133.5 (d)	8.56 (d, 2.9)	132.6 (d)		169.3 (s)
3		107.5 (s)		107.4 (s)		105.9 (s)
4		125.3 (s)		125.7 (s)[c]		122.6 (s)
5	8.28 (m)	121.0 (d)	7.94 (d, 2.0)	110.1 (d)	7.29 (d, 1.9)	109.3 (d)
6	7.31 (m)	122.2 (d)	9.92 (br s)	156.8 (s)	9.10 (br s)	152.4 (s)
7	7.31 (m)	123.9 (d)	7.00 (dd, 8.5, 2.0)	115.4 (d)	6.65 (dd, 8.3, 1.9)	114.9 (d)
8	7.53 (m)	113.1 (d)	7.57 (d, 8.5)	116.8 (d)	6.72 (d, 8.3)	110.5 (d)
9		137.1 (s)		134.8 (s)[c]		133.6 (s)
10	*d*		*d*		10.24 (br s)[f]	
11		173.2 (s)[b]		173.8 (s)		170.0 (s)
12		135.5 (s)[b]		134.8 (s)[c]		133.6 (s)
13	8.17 (s)	116.0 (d)[b]	8.15 (s)	117.4 (d)	7.65 (s)[f]	116.4 (d)
14		163.5 (s)[b]		160.3 (s)		143.6 (s)
15					11.96 (br s)[g]	
16	9.94 (s)	151.3 (s)	9.62 (s)	150.8 (d)	8.46 (s)[g]	130.0 (d)
17		110.9 (s)		112.4 (s)		106.8 (s)
18		123.4 (s)		125.8 (s)[c]		125.4 (s)
19	8.52 (m)	123.1 (d)	8.30 (m)	121.1 (d)	8.07 (m)	120.2 (d)
20	7.53 (m)	126.1 (d)	7.34 (m)	122.1 (d)	7.32 (m)	121.3 (d)
21	7.53 (m)	127.3 (d)	7.34 (m)	123.8 (d)	7.32 (m)	122.9 (d)
22	7.74 (m)	115.2 (d)	7.59 (m)	113.1 (d)	7.55 (m)	112.6 (d)
23		139.9 (s)		137.2 (s)		136.7 (s)

[a] Measured in [$^2H_6$]DMSO containing a trace of TFA. The ^1H NMR signals in neutral [$^2H_6$]DMSO solution were overlapping, therefore, NMR signals of the neutral species could not be assigned. [b] Satellite peaks were observed; $^1J_{C(12)\text{-}C(13)}$ = 65.2 Hz by the feeding experiment of [3-^{13}C]tryptophan and $^2J_{C(11)\text{-}C(14)}$ = 8.6 Hz by that of [2-^{13}C]tryptophans. The labelling patterns are illustrated in Scheme 1. The satellite signals of 13-H were observed as $^1J_{C(13)\text{-}H(13)}$ = 176 Hz in the ^1H NMR spectrum. In neutral medium ([$^2H_6$]DMSO), the signals at δ_C 172.9 (C-11), 131.8 (C-12), 119.6 (C-13) and 146.0 (C-14) were enriched ($^1J_{C(12)\text{-}C(13)}$ = 65.0 Hz, $^2J_{C(11)\text{-}C(14)}$ = 8.5 Hz). [c] These signals may be interchangeable. [d] The amide protons were not clearly observed. [e] Overlapping signals. [f] Long-range coupling arising from $^4J_{H(10)\text{-}H(13)}$ was observed in ^1H–^1H COSY spectrum. [g] Cross peak was observed in ^1H–^1H COSY spectrum.

Table 2 NMR data of the prodeoxyviolacein derivatives **8** and **11**, produced by $NaBH_3CN$ reduction and by saturation with dry ammonia in [$^2H_6$]DMSO [δ_H(multiplicity, J/Hz)]

	Dihydroprodeoxyviolacein **8**		Prodeoxyviolacein-NH_3 adduct **11**	
Position	^1H	^{13}C	^1H	^{13}C
1	11.0 (br s)		11.08 (br s)[c]	
2	7.27 (d, 1.7)	123.6 (d)	7.44 (s)[c]	123.3 (d)
3		111.2 (s)		116.3 (s)
4		125.5 (s)		125.1 (s)
5	7.41 (d, 8.0)	118.8 (d)	7.57 (d, 7.6)	119.92 (d)[a]
6	6.89 (t, 8.0)	118.5 (d)	6.91 (dd, 7.6, 7.9)	118.7 (d)
7	7.05 (t, 8.0)	121.3 (d)	7.05 (dd, 7.9, 8.0)	121.1 (d)
8	7.36 (d, 8.0)	111.6 (d)	7.37 (d, 8.0)	111.8 (d)
9		136.7 (s)		137.1 (s)
10	8.75 (br s)		8.76 (br s)[d]	
11		172.6 (s)		171.7 (s)[b]
12		129.8 (s)		127.6 (s)[b]
13	7.41 (d, 2.5)	136.1 (d)	7.19 (s)[d]	140.1 (d)[b]
14	5.59 (d, 2.5)	54.1 (d)		73.0 (s)[b]
15	11.35 (br s)		11.41 (br s)[c]	
16	8.33 (d, 2.7)	125.9 (d)	8.30 (s)[c]	126.5 (d)
17		106.6 (s)		106.4 (s)
18		118.8 (s)		125.1 (s)
19	7.84 (d, 8.0)	119.8 (d)	7.75 (d, 7.7)	119.97 (d)[a]
20	7.05 (t, 8.0)	119.8 (d)	7.05 (dd, 7.7, 8.0)	120.04 (d)[a]
21	7.14 (t, 8.0)	121.6 (d)	7.14 (dd, 7.3, 8.0)	121.8 (d)
22	7.44 (d, 8.0)	111.8 (d)	7.45 (d, 7.3)	112.1 (d)
23		136.1 (s)		136.4 (s)

[a] May be interchangeable. [b] $^1J_{C(12)\text{-}C(13)}$ = 69.0 Hz, $^2J_{C(11)\text{-}C(14)}$ = 8.1 Hz. These coupling constants were obtained by independent feeding experiment of [3-^{13}C]- and [2-^{13}C]-tryptophans as shown in Table 1. [c] Cross peaks were observed in ^1H–^1H COSY spectrum. [d] Long-range coupling due to ^4J.

colourless solution, which showed a new absorption at 305–330 nm (br sh). This suggests the presence of a vinylindole nucleus as described for the dihydro derivative **8**. The NMR data in basic medium are shown in Table 2. A striking feature is that a new signal ws present at δ_C 73.0 in addition to the signals for the sp^2 carbons from the original pigment. The chemical shift (73.0 ppm) is indicative of a geminal diamine structure **11** (Fig. 3). Thus, the amino group was directly

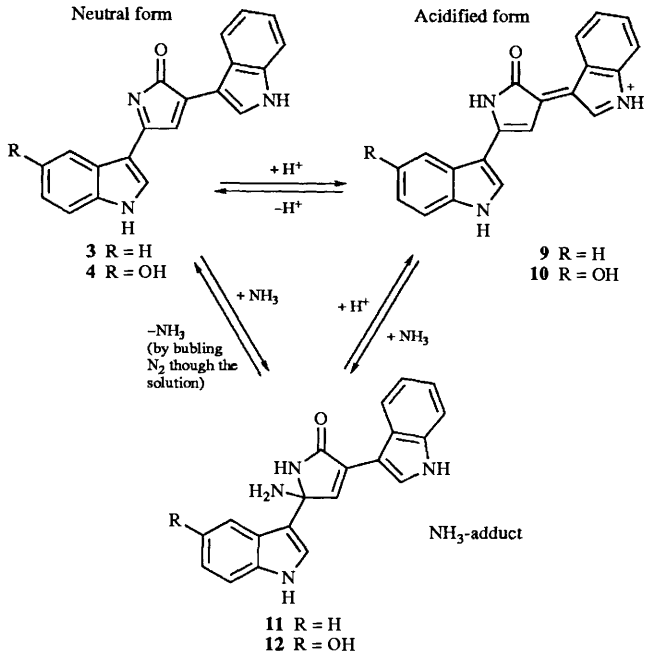


Fig. 3 Structural transformation of compound **3** induced by changing the acidity of the [$^2H_6$]DMSO solution

attached to C-14 by nucleophilic attack of ammonia. The diamine structure **11** was not isolated due to its instability; when the excess of ammonia was removed by bubbling nitrogen through the solution, the red colour gradually reappeared and the electronic absorption and the NMR spectra were superimposable on those of the original compound **3**. The structural transformation is summarized in Fig. 3.

During the purification with Sephadex LH 20, we found another material **4**, whose colour changed with pH like compound **3**; blue in acidic solution and red in neutral or basic medium. The R_f value of metabolite **4** on SiO_2 TLC was lower than that of compound **3**, indicating a higher polarity than compound **3**. The quantity of compound **4** produced by the treatment with *N*,*N*-diethyldithiocarbamate was very small. We searched for a blocked mutant, prepared with the mutagenic agent NTG, for the production of compound **4** in sufficient quantity for structural analysis; mutant No. 26 was selected. A tryptophan solution was metabolized at pH 8.5 with washed cells of mutant No. 26 to give a blue–green colour to the cells. Purification, which was essentially the same as for compound **3**, gave compound **4**: λ_{max}(50% aq. MeOH)/nm 602 (ε/dm^3 mol^{-1} cm^{-1} 23 000) in acidic solution; 493 (17 000) in neutral or basic medium. The pK_a was *ca.* 4.7. Exact mass measurements (EI) showed the molecular composition of **4** to be $C_{20}H_{13}N_3O_2$, suggesting that compound **4** has an extra hydroxy group compared with compound **3**. All the NMR data of **4**, including HMBC and NOESY, were essentially the same as those of compound **3** except for the presence of a hydroxy group at the 5-position of indole ring (Table 1). When compound **4** was set aside in a [$^2H_6$]DMSO solution saturated with ammonia it underwent, like compound **3**, nucleophilic addition of ammonia to give a colourless product **12**. Therefore, the structure of compound **4** was established as 5-(5-hydroxyindol-3-yl)-3-(indol-3-yl)pyrrol-2(2*H*)-one.

The above results for compounds **3** and **4** suggested the production of hydroxylated **5** (compound **6**, analogue of pseudodeoxyviolacein) by *C. violaceum*, possessing a 5-hydroxyindole ring. However, it was difficult to detect this metabolite after the incubation in the presence of *N*,*N*-diethyldithiocarbamate. By monitoring the same colour tone as compound **5** and the higher polarity on SiO_2 TLC, we could detect compound **6** amongst the various metabolites of mutant No. 26, although in very small quantity. Exact mass measurements (EI) showed the molecular formula to be $C_{20}H_{13}N_3O_3$ (*m*/*z* 343.0969), identical with that of violacein. The low resolution mass spectrum (EI) of the hydrogenated compound **6**, prepared by treatment with H_2–PtO_2, showed *m*/*z* 347, indicating the involvement of two double bonds in compound **6**. UV absorption of the tetrahydro derivative was as follows: λ_{max}(MeOH)/nm 250 (ε/dm^3 mol^{-1} cm^{-1} 7800), 275 (5400) and 300 (4700, sh). The maximum at 250 nm in the UV spectrum is characteristic of an oxoindole ring.[13] This absorption spectrum was quite similar to that of the tetrahydro derivative of violacein, indicating that compound **6** has the same chromophore as violacein, *i.e.* it contains the same three structural units. These structural units were further confirmed by the ^1H and ^{13}C NMR spectra of compound **6** (Table 1). The substitution pattern of the 5-hyroxyindole and oxoindole on the pyrrolone nucleus was determined by the HMBC spectrum (Fig. 4). The five cross peaks from the pyrrolidone proton in the HMBC spectrum played a crucial role in proposing the structure of compound **5** (pseudodeoxyviolacein) in comparison with four possible structures (see Fig. 1 shown in ref. 1), which are presumed on the basis of the substitution patterns of indole and oxoindole rings.[1] This has been discussed in detail in the previous paper.[1] The five cross peaks were also observed in compound **6** (Fig. 4). Thus, the structure of compound **6** was determined to be 5-(5-hydroxy-2-oxoindol-3-ylidene)-3-(indol-3-yl)pyrrol-2(1*H*)-one. On the basis of the clear observation of two cross peaks (5-H/13-H and 19-H/13-H) in the NOESY spectrum, it is proposed that the conformation of 5-hydroxyindole and the geometry of the oxoindole ring in the molecule **6** is that illustrated in Fig. 4 (the same as those of compound **5**).[1] Fig. 4 also shows that the spatial arrangements of the acidified forms of compounds **3** and **4**, **9** and **10** respectively, were identical with those of deoxyviolacein **1** and violacein **2**.

Scheme 1 (see top) illustrates the biosynthesis of violacein and deoxyviolacein from tryptophan, as has been elucidated with the aid of stable isotopically labelled tryptophans.[4,5] The feeding experiments with [3-^{13}C]- and [2-^{13}C]-tryptophans carried out independently showed that one of the two indole rings in tryptophan migrates to the 2-position of the side chain. This has been previously demonstrated by the observations of highly enriched ^{13}C signals at both 12- and 13-positions, which were accompanied by satellite peaks ($^1J_{CC}$ 54.8 Hz) as a result of the high incorporation rate of [3-^{13}C]tryptophan, while in case of feeding with [2-^{13}C]tryptophans, the carbons at both the 11- and 14-positions were labelled ($^2J_{CC}$ 7.1 Hz).[4] Similar feeding experiments were carried out in the presence of diethyldithiocarbamate to obtain labelled **3**, ^{13}C NMR spectra of which showed results identical with those of deoxyviolacein (Table 1): the same labelling pattern and the appearance of satellite signals due to $^1J_{C(12)\text{-}C(13)}$ 65.2 Hz and $^2J_{C(11)\text{-}C(14)}$ 8.6 Hz, respectively, from incorporation experiments with [3-^{13}C]- and [2-^{13}C]-tryptophans (Scheme 1, see bottom). This fact verified that compounds **3** and **4** were biosynthesized with a 1,2-shift of the indole ring in a way similar to that of deoxyviolacein and violacein. Comparison of the structures of **3** and **4** with those of deoxyviolacein **1** and violacein **2** strongly suggests that compounds **3** and **4** might be the biosynthetic intermediates of deoxyviolacein and violacein, and that oxygenation at the 2-position of the indole nucleus may be a final step. If this is so the oxygenation occurs *via* route a and deoxyviolacein **1** and violacein **2** will be formed as shown in Scheme 1. On the other hand, if the oxygenation occurs *via* route b at the alternative indole ring, compounds **5** and **6** will be biosynthesized. We propose, therefore, to name compound **3** as prodeoxyviolacein,

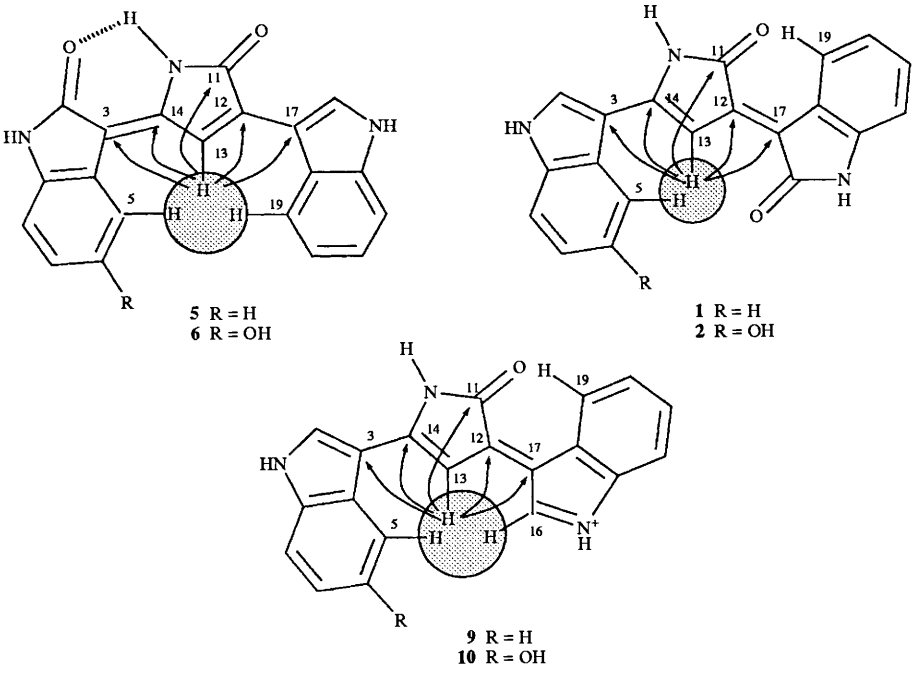
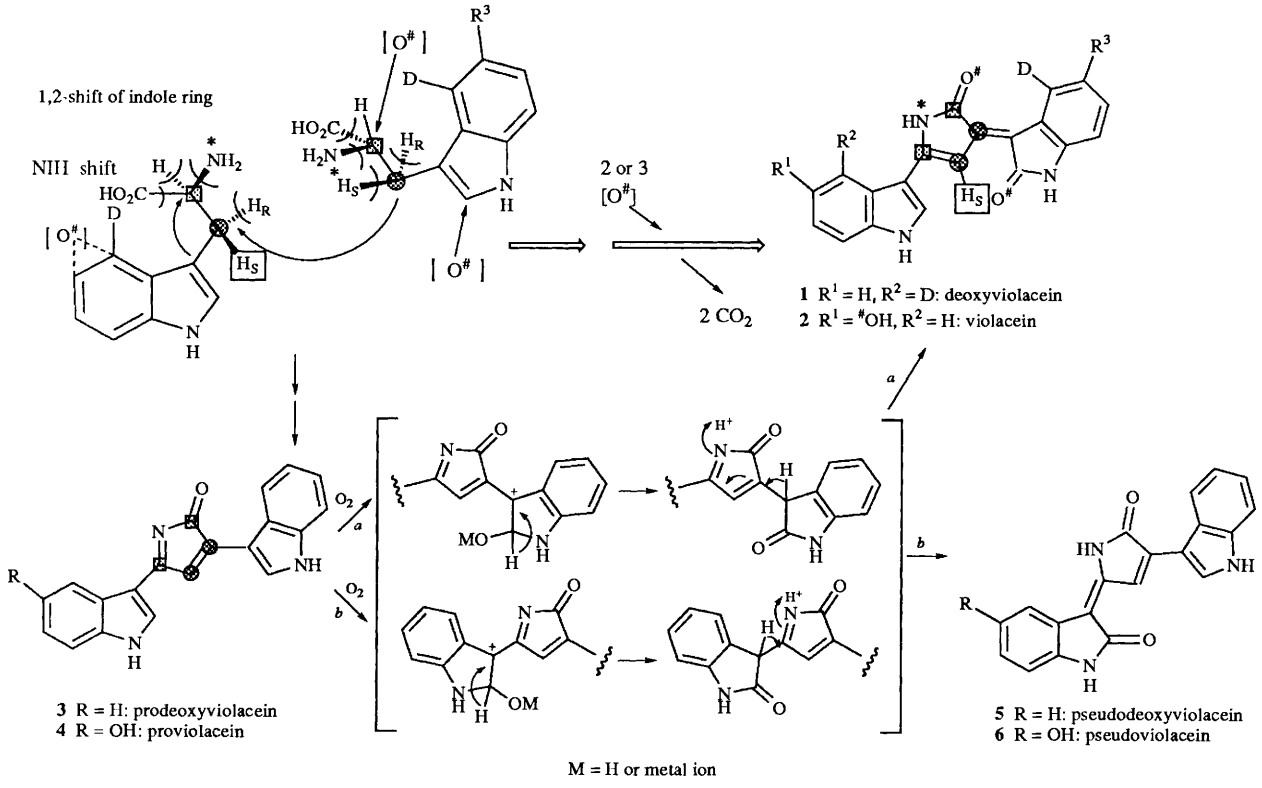


Fig. 4 Conformation analyses of the violacein-related compounds (**1**, **2**, **5**, **6**, **9** and **10**) in DMSO solution. The symbols of → and ● shows the observed HMBC and NOEs, respectively, from the proton of the pyrrolone moiety. The conformation of the acidified forms of compound **3** and **4** (**9** and **10**) in [$^2H_6$]DMSO was identical to those of deoxyviolacein **1** and violacein **2**.



Scheme 1 Proposed pathway of violacein pigments from tryptophan and the ^{13}C-labelling patterns. The symbols ■ and ● represent [2-^{13}C]- and [3-^{13}C]tryptophans, respectively. The ^{13}C–^{13}C coupling constants are shown in Tables 1 and 2. The pigments violacein and deoxyviolacein would be biosynthesized *via* route a.

compound **4** as proviolacein and compound **6** as pseudoviolacein. The amounts of **5** and **6** produced were negligibly small, compared to those of deoxyviolacein and violacein. Since the formation of **5** and **6** would arise as a result of incorrect specificity between the substrate (**3** or **4**) and the oxygenase, the amounts produced would have been very small. The copper chelator *N*,*N*-diethyldithiocarbamate may have more or less obstructed the correct molecular recognition, leading to increased amounts of **3** and **4** produced. The oxygenation reaction, therefore, may be catalysed by a copper-containing enzyme. Our hypothesis is that the enzyme mechanism might be a hydroxylation (*via* an epoxide intermediate) at the 16-position of either the neutral indole structure (**3** and **4**) or the indolium structure (**9** and **10**, acidified form), probably catalysed by a

monooxygenase. It has been reported that phenylalanine hydroxylase (a monooxygenase, epoxide intermediate) from *C. violaceum*,[14] which also acts as tryptophan hydroxylase to form 5-hydroxytryptophan,[15] is a copper-containing enzyme.[16] We have previously proved the biosynthetic intermediacy of 5-hydroxytryptophan for violacein.[6] The quantity of compound **4** produced in the presence of diethyldithiocarbamate was smaller, compared with that of compound **3**. This could be ascribed to the inhibition of the hydroxylation at the 5-position of the indole nucleus by the copper-chelating agent. This idea would be further supported by the finding that the reduction in yield of violacein **2** produced in the presence of the copper-chelator (*ca.* 0.25–0.2) was larger than that of deoxyviolacein **1** (*ca.* 0.5), when compared to their quantities produced without the additive. In any event, diethyldithiocarbamate would have inhibited, though not completely, two types of copper-containing oxygenases; one responsible for the oxygenation at the 2-position of the indole ring, and the other for the catalysis of the oxygenation at the 5-position. For mutant No. 26, the oxygenation at the 2-position would have been blocked during a series of tryptophan metabolisms to result in an accumulation of the metabolites **3** and **4**. To validate some of the assumptions discussed above, further investigations at enzymic level are necessary. Deuterium-labelled derivatives of **3** and **4** were prepared by feeding experiments of L-[2,4,5,6,7-$^2H_5$]tryptophan[17] in the presence of diethyldithiocarbamate into the parent strain and/or into mutant No. 26. Compounds **3** and **4** were hardly soluble in aqueous media, but could be dissolved with Triton X-100, after which re-incorporation experiments were carried out with the growing cells or the washed whole ones of the parent strain. However, the incorporation of deuterium atoms into violacein analogues failed. This unexpected result may be ascribed to problems of membrane permeability.

Our preliminary experiments have shown that a cell-free extract prepared just by ultrasonication could not biosynthesize violacein pigments from the tryptophan precursor and also failed to convert **3** and **4** into violacein pigments. Different conditions and/or some additives are necessary to establish the cell-free system for an efficient conversion of the tryptophan precursor into violacein pigments. The cell-free system is now under exploration in order to settle the problem of cell-membrane permeability frequently encountered in demonstrating the intermediacy.

Experimental

UV spectra were measured on a JASCO Ubest-30 spectrophotometer, IR spectra with a JASCO IR-700 spectrophotometer, ^1H and ^{13}C NMR spectra on a Varian Gemini 200, a Unity plus 400 and a Bruker AM 500 spectrometers, and mass spectra of (EI) on a JEOL DX 300 and a JEOL XS 100 mass spectrometer.

Isolation of compound 3

The bacterial cells (*Chromobacterium violaceum* JCM 1249) were harvested by centrifugation (9000 rpm) after incubation for 12 h in a 2 dm^3 Erlenmeyer flask containing the culture medium (nutrient broth; 1 dm^3).[4] The harvested cells were washed with a saline solution and then suspended in an aqueous solution (100 cm^3; pH 7.2) containing L-tryptophan (50 mg), K_2HOP_4 (100 mg), $MgSO_4 \cdot 7\ H_2O$ (20 mg) and sodium *N*,*N*-diethyldithiocarbamate (276 mg). The amount of diethyldithiocarbamate, under which compound **3** was efficiently produced, was measured by monitoring **3** by TLC (SiO_2; $CHCl_3$–MeOH 95:5). This solution was incubated at 25 °C for 24 h on a rotary shaker (200 rpm). D-Tryptophan was inert to the production of compounds **3**–**6** as well as violacein pigments. Compound **3** was an intracellular metabolite. The green–blue pigments were extracted with hot methanol, concentrated to a small volume, and adsorbed on Amberlite XAD 2. The resin was washed with 10% aq. methanol and then the pigments were eluted with abs. methanol. After concentration the residue was transferred onto a Sephadex LH 20 column. Eluting with methanol two red pigments and the blue pigments deoxyviolacein and violacein were separated. In order of elution compound **3**, compound **5**, deoxyviolacein and violacein were isolated. The red fractions containing compound **3** were concentrated under reduced pressure to give compound **3** as dark red crystals (41 mg; from 4 g of tryptophan), mp >275 °C (from MeOH); ν_{max}(KBr)/cm^{-1} (neutral) 3400, 3250, 3150, 3060, 2925, 2850, 1705, 1638, 1590, 1490, 1445, 1360, 1118, 999 and 740; (TFA salt) 3400, 3110, 2925, 2860, 1740, 1678, 1578, 1483, 1440, 1360, 1332, 1210, 1168, 1130, 1105, 1000, 765 and 745; λ_{max}(50% aq. EtOH)/nm see Fig. 1; δ_H(400 MHz; [$^2H_6$]DMSO) and δ_C(100 MHz) see Table 1 (Found: M^+, 311.1062. $C_{20}H_{13}N_3O$ requires *M*, 311.1059).

Derivatives of compound 3

Tetrahydro compound 3. A suspension of the neutral form of compound **3** (4.6 mg) and 5% Pd–C (20 mg) in EtOH (7 cm^3) was saturated with hydrogen and stirred for 20 h. The reaction product was purified by preparative TLC (SiO_2) to give the tetrahydro derivative of **3** (1.1 mg), λ_{max}(MeOH)/nm see text; δ_H(200 MHz; $CDCl_3$) and δ_C(50 MHz) see text (Found: M^+, 315.1393. $C_{20}H_{17}N_3O$ requires *M*, 315.1414); *m*/*z* (EI) 315 (M^+), 272, 245, 198 and 154.

Dihydro derivative. To a suspension of compound **3** (13.2 mg) in dry MeOH (10 cm^3) was added $NaBH_3CN$ and the mixture was stirred at room temperature. The reagent was added intermittently until no starting material remained. The product was purified on a Sephadex LH 20 column eluting with MeOH to give the dihydro derivative of **3** (9.3 mg); λ_{max}(MeOH)/nm see text; *m*/*z* (EI) 313 (M^+); δ_H(400 MHz; [$^2H_6$]DMSO) and δ_C(100 MHz) see Table 2.

Ammonia adduct of compound 3 in [$^2H_6$]DMSO. Dry ammonia was bubbled through a [$^2H_6$]DMSO solution of compound **3** and over a period of *ca.* 4 h the colour of **3** gradually changed from red to almost colourless. After initial NMR measurements, dry nitrogen was bubbled through the [$^2H_6$]DMSO solution in the NMR tube to remove the excess of NH_3, resulting in a red colour and NMR signals identical with those of the original compound **3**. λ_{max}(MeOH saturated with NH_3)/nm 279, 288, 313 and 475. When measured in DMSO, maximum absorption in the visible region was at 454 nm, which decreased gradually with time, whilst the absorption around 300–330 nm increased. When the ammonia was removed, as described above, the spectrum recorded was identical with the original electron spectrum of compound **3**; δ_H(400 MHz; [$^2H_6$]DMSO) and δ_C(100 MHz) see Table 2.

Isolation of compounds 4 and 6

The blocked mutant No. 26, unable to biosynthesize violacein, which was prepared with the treatment of the mutagenic reagent NTG,[7–9] was grown for 12 h in a nutrient medium. To the harvested cells, obtained after centrifugation, was added a tryptophan solution in NH_4OH–NH_4Cl buffer (0.1 mol dm^{-1}; pH 8.5) and the whole was then incubated for 24 h. The amount of **4** produced was higher at pH 8.5 than at pH 7.2. The cells, which gradually turned green during the incubation, were extracted with abs. MeOH and the green–red pigments were adsorbed on an Amberlite XAD 2 column. The resin was washed with 20% aq. MeOH, then the adsorbed pigments were eluted with hot MeOH, and concentrated to a small volume. This was left aside overnight at room temperature, during which time the green pigments precipitated out. The red supernatant

was subjected to column chromatography on Sephadex LH 20 eluting with methanol to give four products ranging from orange to red.

Compound 4: mp >240 °C (from MeOH); λ_{max}(H_2O–EtOH, 50:50)/nm (pH 2) 600 (ε/dm^3 mol^{-1} cm^{-1} 22 800), 415 (9400), 303 (14 200) and 272 (11 300); (pH 7) 492 (16 700), 412 (8500), 385 (8050), 290 (13 000) and 271 (12 070); (pH 10) 492 (16 700), 412 (8600), 385 (7900), 292 (12 900) and 271 (11 850); ν_{max}(KBr)/cm^{-1} (neutral) 3400, 3280, 1710, 1630, 1575, 1490, 1440, 1350, 1105, 995 and 938; (TFA salt) 3410, 1710, 1680, 1635, 1580, 1490, 1442, 1425, 1350, 1295, 1240, 1200, 1105, 998 and 940; δ_H(500 MHz; [$^2H_6$]DMSO) and δ_C(125 MHz) see Table 1 (Found: M$^+$, 327.1020. $C_{20}H_{13}N_3O_2$ requires *M*, 327.1008.

Tetrahydro derivative of compound 4: λ_{max}(MeOH)/nm 280 (ε/dm^3 mol^{-1} cm^{-1} 9700), 289 (8800) and ~310 (sh); *m*/*z* 331 (M$^+$).

Compound 6: mp >240 °C (from MeOH); λ_{max}(MeOH)/nm 472 (ε/dm^3 mol^{-1} cm^{-1} 11 100), 413 (8900), 394 (8500), 290 (9500) and 275 (9100) (Found: M$^+$, 343.0969. $C_{20}H_{13}N_3O_3$ requires *M*, 343.0957); ν_{max}(KBr)/cm^{-1} 3425, 1670, 1630, 1585, 1470, 1430, 1355, 1300, 1240, 1198 and 1105; δ_H(500 MHz; [$^2H_6$]DMSO) and δ_C(125 MHz) see Table 1.

Tetrahydro derivative of 6: λ_{max}(MeOH)/nm see text.

Preparation of the ^{13}C-labelled compound 3

DL-[2-^{13}C]Tryptophan (90% ^{13}C) was purchased from MSD, Canada. A mixture of labelled and unlabelled DL-tryptophan (18.8 mg and 6.4 mg, respectively) was dissolved in the buffer solution (50 cm^3; pH 7.2) described above. The solution was incubated with agitation for 24 h at 25 °C on a rotary shaker at 180 rpm in the presence of sodium diethyldithiocarbamate (138 mg) by using the washed whole cells, which had been harvested from a culture (0.5 dm^3) grown in a nutrient broth medium for 12 h. Labelled **3** was purified according to the method described above. The yield was 0.35 mg. The ^{13}C content at both C-12 and C-13 was determined to be *ca.* 60% from the ratio between the satellite peaks and the central peak. The optically pure L-[3-^{13}C]tryptophan was prepared from H^{13}CHO (97.8%, ^{13}C, MSD).[4] The incubation conditions and the ratio of labelled L-tryptophan to the unlabelled one was the same as that of DL-[2-^{13}C]tryptophan. The ^{13}C content of labelled **3** thus obtained was also the same as that of the [2-^{13}C]precursor.

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