Studies on the biosynthesis of violacein. Part 9.[1] Green pigments possessing tetraindole and dipyrromethene moieties, chromoviridans and deoxychromoviridans, produced by a cell-free extract of *Chromobacterium violaceum* and their biosynthetic origins†

A. Z. M. Ruhul Momen,[a] Takaaki Mizuoka[a] and Tsutomu Hoshino*[ab]

[a] *Graduate School of Science and Technology and*[b] *Department of Applied Biological Chemistry, Faculty of Agriculture, Niigata University, Ikarashi, Niigata 950-2181, Japan*

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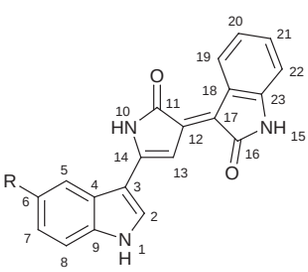
Novel green-coloured pigments, **5** and **6**, are reported, which are concomitantly produced under a cell-free system together with the pigments of violaceins **1**, **2** and proviolaceins **3**, **4**. The metabolites **5** and **6** have been named deoxychromoviridans and chromoviridans, respectively. The structures of **5** and **6** were established by analyses of the FABMS and various types of 2D-NMR spectra as 3-[2-{(*Z*)-[3,5-di(1*H*-indol-3-yl)-1*H*-pyrrol-2-yl]methylidene}-5-(1*H*-indol-3-yl)-2*H*-pyrrol-3-yl]-1*H*-indole and 3-[2-{(*Z*)-[3,5-di(1*H*-indol-3-yl)-1*H*-pyrrol-2-yl]methylidene}-3-(1*H*-indol-3-yl)-2*H*-pyrrol-5-yl]-1*H*-indol-5-ol, respectively, and are closely related to those of violaceins **1**, **2** and proviolaceins **3**, **4**, although they have an extra C_1 unit at the dipyrromethene moiety and were not oxygenated at any position. The C_1 unit of the dipyrromethene moiety was demonstrated to come from serine by feeding labelled [3-^{13}C]serine, indicating that serine hydroxymethyltransferase and methylenetetrahydrofolate are responsible for the biosyntheses. The green pigments also have metal chelation capability with iron, copper, zinc and cobalt ions.

Introduction

Violacein **2** and deoxyviolacein **1** are tryptophan metabolites produced by *Chromobacterium violaceum*. In the preceding paper in this series,[1] we reported a cell-free synthesis of violacein pigments; when tryptophan was incubated under the cell-free system, green pigments were always produced together with blue pigments **1**, **2** and red pigments of proviolacein **4** and prodeoxyviolacein **3**.[2] To understand the cell-free system in more detail, the structures of the green metabolites need to be elucidated. This paper describes the characterizations of the structures of the green compounds **5** and **6**. The core structure is composed of two structural units with the whole structure containing four indole rings and one dipyrromethene skeleton. This characteristic structure has never been found before in nature. The biosynthetic origins of this interesting dipyrromethene structure are also reported.



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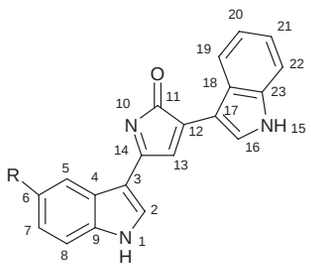
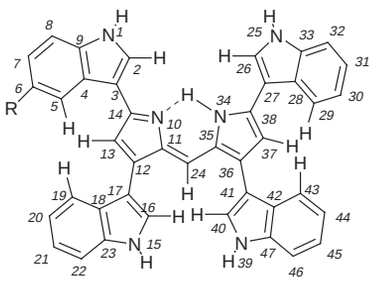
2 R = OH



3 R = H
4 R = OH



5 R = H
6 R = OH

Results and discussion

The amounts of green pigments **5** and **6** produced by the cultivation of *Chromobacterium violaceum* were negligible, however, significant quantities were produced in a cell-free system. To isolate the green pigments in a large quantity, a cell-free system was employed without the use of an NADPH cofactor, because addition of NADPH stimulated violacein production, resulting in a decreased production of green compounds.[1] Cell-free extracts were prepared by an ultrasonication protocol as described in the Experimental section. Incubation of tryptophan in the enzyme solution and purification gave pure **5** in a yield of 2.5 mg from 200 mg of tryptophan. To obtain green compound **6**, we used the blocked mutant No. 26, prepared with the mutagenic agent of NTG,[2,3] because the cell-free system mentioned above did not produce **6** in a sufficient quantity for its structure to be determined. The green compounds were allowed to accumulate within the cells and then extracted with methanol to give a solution containing various pigments including prodeoxyviolacein **3**, proviolacein **4** *etc*. The methanolic solution was concentrated into a small volume and left to stand for a few days at room temperature, leading to precipitation of the green metabolites **5** and **6**. Compound **6** was purified in a way similar to compound **5**, yielding 4 mg of pure compound **6** from 3 g of tryptophan.

* Correspondence should be addressed to the Faculty of Agriculture.

† *Abbreviations*: FH_2, dihydrofolate; FH_4, tetrahydrofolate; NADPH, nicotinamide-adenine dinucleotide phosphate, reduced form; NTG, *N*-methyl-*N*′-nitrosoguanidine.

The FABMS spectrum of **5** showed 605 as the highest ion, whilst its acetate, prepared using Ac_2O–Et_3N, exhibited *m*/*z* 773 (tetraacetate), demonstrating the molecular weight to be 604. Exact mass determination (positive HRFABMS) showed the molecular composition of **5** to be $C_{41}H_{29}N_6$ (*m*/*z* 605.2517, $M^+ + H$) and that of its acetate to be $C_{49}H_{37}N_6O_4$ (*m*/*z* 773.2878, $M^+ + H$). The ^{13}C NMR spectrum of **5** in [$^2H_6$]-DMSO containing a trace of trifluoroacetic acid (TFA) showed only 21 signals (Table 1). DEPT experiments indicated that compound **5** was composed of nine quaternary and twelve methine carbons, one of the latter being correlated to 1 × H, while the others to 2 × H, judging from the integration of the ^1H NMR and HMQC spectrum. These findings suggested that compound **5** is a symmetrical molecule. Assignments of the ^1H and ^{13}C NMR signals of the indole rings were established from the ^1H–^1H COSY, HMQC and HMBC spectra,[2,4] and showed the presence of two indole rings, however compound **5** in fact has four indole rings because it is a symmetrical molecule. The assignments of protons and carbons of indole nuclei are shown in Table 1. The resonances for the unidentified protons and carbons other than those of the indole rings were as follows: δ_H 7.41 (2H, s), 7.50 (1H, s), 12.67 (1H, br s, exchangeable with D_2O); δ_C 111.6 (d), 119.5 (d), 128.4 (s), 142.2 (s), 147.7 (s). The HMQC spectrum showed the following correlations: δ_H 7.41/δ_C 111.6 and δ_H 7.50/δ_C 119.5; the ^1H NMR spectrum revealed that the former could be assigned to 2 × H and 2 × C, and the latter to 1 × H and 1 × C. The HMBC cross peaks clarified the presence of the pyrrole ring and the assembly pattern of the four indole nuclei with the pyrrole moiety. Some of the HMBC correlations are depicted in Fig. 1. The fact that an HMBC cross peak was clearly found between the signal at δ_H 7.50 (24-position) and the carbon signal of the pyrrole ring (11/11′-positions) was a determining factor in the structure determination of compound **5**. From the above HMBC correlation results and the fact that it is a symmetrical molecule, the structure of **5** shown in Fig. 1 can be proposed. This structure was further supported by the NOESY spectrum, as illustrated in Fig. 1; correlations were observed between the protons of 5-H, 13-H and 19-H, between 16- and 16′-H and 24-H, and between 2- and 2′-H and 10-H. The dipyrromethene unit was also demonstrated by the ^1H–^{15}N HMBC spectrum (field gradient); clear cross peaks due to three-bond ^1H–^{15}N couplings were observed for both H24/N10(10′) and H13(13′)/N10(10′) (Fig. 1). Full analysis of the tetraacetate of **5** by various 2D NMR spectra proved that acetylation occurred at the indolic NH and supported the structure of **5** (Table 1). Thus, the structure of **5** was established as 3-[2-{(*Z*)-[3,5-di(1*H*-indol-3-yl)-1*H*-pyrrol-2-yl]methylidene}-5-(1*H*-indol-3-yl)-2*H*-pyrrol-3-yl]-1*H*-indole.

The molecular composition of **6** was determined to be $C_{41}H_{29}N_6O$ (*m*/*z* 621.2440, $M^+ + H$) from HRFABMS, *i.e.* an increment of 16 mass units compared to **5**, suggesting that **6** is a monohydroxy-derivative of **5**. Compound **6** showed individual signals for all 41 carbons in its ^{13}C NMR spectrum (Table 2), unlike symmetrical molecule **5**. It was shown that the hydroxy group was attached to the 5-position of one of the indoles, by the fact that the hydroxy proton at δ_H 9.22 (exchangeable with D_2O) in [$^2H_6$]DMSO was correlated to three carbons at the 4-, 5- and 6-positions of one indole nucleus in the HMBC spectrum. Assignments for the ^1H and ^{13}C signals of the four indole rings in **6** were determined in the same way as for **5**. Unidentified signals of three methine and six quaternary carbons other than those in the indole rings, clarified by DEPT and HMQC experiments, were as follows: δ_H 7.28 (1H, br)/δ_C 111.2(d), 7.38 (1H, s)/111.4(d), 7.45 (1H, s)/119.4(d) for methine groups, and 128.39(s), 128.35(s), 141.7(s), 142.4(s), 146.9(s), 148.3(s) for quaternary carbons. The HMBC correlations from the two protons at δ_H 12.66 (NH, br, 10- or 34-H) and δ_H 7.45 (1H, s, 24-H) were the same as those of compound **5** (Fig. 1), indicating that the dipyrromethene moiety is also present and is bonded to three indole rings and one 5-hydroxyindole ring to form structure **6**. The position of attachment of the 5-hydroxyindole to the pyrrole moiety was further clarified by the correlations between H-5, H-13 and H-19 in the ROESY spectrum (Fig. 1). Thus, the structure of **6** was established as 3-[2-{(*Z*)-[3,5-di(1*H*-indol-3-yl)-1*H*-pyrrol-2-yl]methylidene}-3-(1*H*-indol-3-yl)-2*H*-pyrrol-5-yl]-1*H*-indol-5-ol.

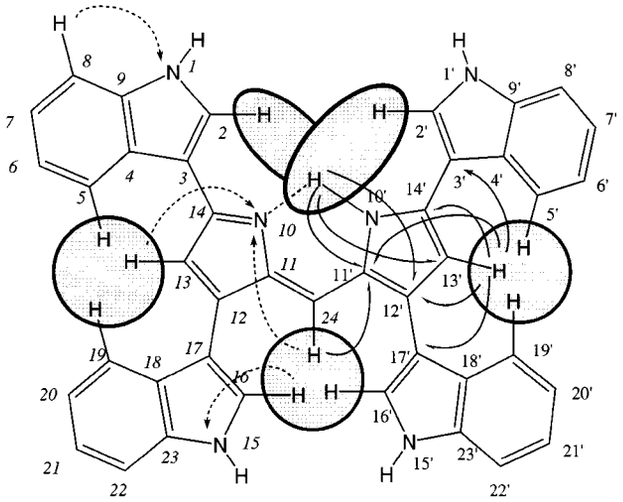


Fig. 1 2D NMR analyses for compound **5**. Arrows ⟶ and ---→ show the observed ^1H–^{13}C and ^1H–^{15}N HMBC, respectively. The observed NOESY cross peaks are illustrated by the shaded areas. The same cross peaks as compound **5** were observed for compound **6** in the ^1H–^{13}C HMBC and ROESY spectra.

Fig. 2 shows that the colour of the pigments **5** and **6** changes on altering the acidity; they are green in acidic and neutral media, but red in a basic medium. Compound **5** had two pK_a (*ca*. 5.3 and 8.5) values in 50% aq. dioxane. In a strongly acidic medium of pH 2, λ_{max} in the visible region was at 640 nm and its molar absorptivity (ε/dm^3 mol^{-1} cm^{-1}) had the remarkably large value of 53800. When the [$^2H_6$]DMSO solution was acidified by adding TFA, the signals of the pyrrolic NH at the 10- and 10′-positions of compound **5** were intensified to exactly 2 × H as judged by the integration and shifted to a lower field (δ_H 12.67) (Table 1), compared to that (δ_H 12.32) measured in the neutral solution, while the chemical shifts for the other signals were a little ($\Delta\delta_H$ ±0.03) shifted; the lower pK_a 5.3 may suggest an equilibrium between protonation and deprotonation of the nitrogen atom at the 10-position. The ^1H signal at the 10- or 10′-position of **5** disappeared when measured in [$^2H_6$]DMSO solution containing a trace of ammonia gas, indicating that the pyrrolic N*H* was ionized; this equilibrium may be found at the higher pK_a of 8.5. In a basic medium, compound **5** underwent a hypsochromic shift to give a red colour (Fig. 2). The dipyrromethene part of structure **5** has a structural resemblance to tetrapyrrole pigments such as porphyrins. Coproporphyrin was used as a model compound to confirm the blue shift in a basic medium. The maximum absorption was found at *ca.* 550 nm in aq. solution (50% dioxane) at pH 2, while, in a basic solution (pH 9), the absorptivity was at a maximum at *ca.* 500 nm. The blue shift of coproporphyrin as a function of pH was consistent with that of compound **5**. Tetrapyrrole pigments are well known to complex with metal ions. Metabolite **5** was therefore examined to see whether it was also capable of metal complexation. Fig. 3 shows the electronic spectra measured at pH 7.9. On addition of zinc or cuprous ions, its spectrum dramatically changed to give a blue shift (Fig. 3), suggesting the occurrence of metal chelation. Fe^{2+} and Co^{2+} were also complexed with **5**. These findings provide additional evidence for the dipyrromethene moiety. Further investigation will be required to gain a better understanding of its electronic properties as a function of pH and the formula of the coordinated ligand on metal complexation.

The biosynthetic origin of the dipyrromethene structure

Table 1 ^{1}H and ^{13}C NMR Data for deoxychromoviridans **5** in [$^{2}H_6$]DMSO containing a trace of TFA and its tetraacetate in $CDCl_3$ [δ_H (multiplicity, *J*/Hz)].[a,b]

	Natural ([$^{2}H_6$]DMSO + TFA) δ (ppm)		Tetraacetate ($CDCl_3$) δ (ppm)	
Positions	^{1}H	^{13}C	^{1}H	^{13}C
1,1′	12.24 (br s)	(145[c])	—	—
2,2′	8.81 (br s)	131.2 (d)	7.93 (s)	124.1 (d)
3,3′	—	106.3 (s)	—	116.9 (s)
4,4′	—	124.7 (s)	—	127.9 (s)
5,5′	8.19 (br)	120.1 (d)	8.27 (d, *J* 7.6)	120.8 (d)
6,6′	7.32 (m)	121.4 (d)	7.14 (t, *J* 7.6)	124.6 (d)
7,7′	7.32 (m)	122.8 (d)	7.42 (t, *J* 8.0)	126.1 (d)
8,8′	7.60 (d, *J* 9.0)	112.7 (d)	8.53 (d, *J* 8.0)	116.8 (d)
9,9′	—	136.8 (s)	—	136.5 (s)
10,10′	12.67 (br)	(150[c])	14.32 (br)	—
11,11′	—	128.4 (s)	—	139.8 (s)
12,12′	—	142.2 (s)	—	134.6 (s)
13,13′	7.41 (s)	111.6 (d)	7.04 (s)	116.4 (d)
14,14′	—	147.7 (s)	—	148.2 (s)
15,15′	11.65 (br s)	(141[c])	—	—
16,16′	7.61 (br s)	127.1 (d)	7.42 (s)	122.9 (d)
17,17′	—	108.4 (s)	—	116.5 (s)
18,18′	—	125.8 (s)	—	129.8 (s)
19,19′	7.93 (d, *J* 7.8)	119.5 (d)	7.79 (d, *J* 8.0)	119.9 (d)
20,20′	7.18 (t, *J* 7.8)	120.5 (d)	7.32 (t, *J* 7.6)	124.0 (d)
21,21′	7.23 (t, *J* 7.8)	122.5 (d)	7.42 (t, *J* 8.0)	125.8 (d)
22,22′	7.49 (d, *J* 7.8)	112.3 (d)	8.45 (d, *J* 7.6)	116.9 (d)
23,23′	—	137.0 (s)	—	136.0 (s)
24	7.50 (s)	119.5 (d)	7.16 (s)	120.4 (d)
1,1′-Ac			2.62 (6H, s, 2Me)	168.44 (s) 24.0 (q)
15,15′-Ac			2.44 (6H, s, 2Me)	168.48 (s) 24.1 (q)

[a] In a basic medium, [$^{2}H_6$]DMSO solution containing one drop of saturated ammonia gas, the ^{1}H NMR signals became relatively broad, and **5** gradually decomposed, thus in a basic medium the ^{1}H and ^{13}C NMR resonances could not be assigned. [b] The ^{13}C–^{13}C, ^{1}H–^{13}C and ^{1}H–^{15}N coupling constants, obtained from feeding experiments with [2-^{13}C]- and [3-^{13}C]-tryptophans, [3-^{13}C]serine and $^{15}NH_4NO_3$, were as follows: $^{2}J_{C11C14}$ 4.3 Hz, $^{1}J_{C12C13}$ 57.5 Hz, $^{1}J_{H10N15}$ 84 Hz, $^{1}J_{H13C13}$ 175 Hz and $^{1}J_{H24C24}$ 157 Hz. [c] The values in parentheses denote the ^{15}N chemical shifts which were determined from the natural abundance ^{1}H–^{15}N HMQC spectrum (field gradient). The ^{1}H–^{15}N HMBC spectrum showed strong correlations between H24/N10(10′) and H13(13′)/N10(10′).

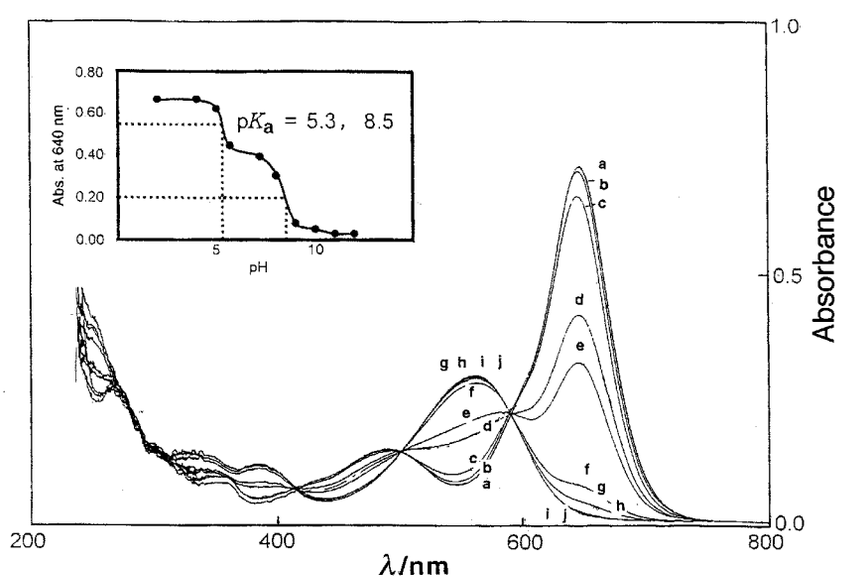


Fig. 2 Electronic spectra of deoxychromoviridans as a function of acidity. A stock solution in dioxane was diluted with an equal amount of aq. buffer solution, and then the electronic spectra were measured (final concentration of the pigment: 1.275×10^{-5} mol dm^{-3}). pH: a, 2.0; b, 3.0; c, 4.0; d, 7.2; e, 8.0; f, 9.0; g, 10.0; h, 11.0; i, 12.0.

was studied. Separate incorporation experiments of [3-^{13}C]- and [2-^{13}C]-tryptophan into **5** clarified the origins of the carbon atoms at the 12- and 13-positions and at the 11- and 14-positions, respectively. The results are depicted in Fig. 4. The ^{13}C–^{13}C couplings were observed due to the high incorporation rates of ^{13}C-labelled tryptophans: $^{1}J_{C12C13}$ 57.5 Hz and $^{2}J_{C11C14}$ 4.3 Hz (Table 1). These results demonstrated that a 1,2-shift rearrangement of the indole ring occurred and that the pyrrole ring was constructed by a condensation reaction between the side chains of tryptophan molecules, which indicate that the metabolite **5** was biosynthesized in a similar way to those of violacein and proviolacein.[2,5,6]

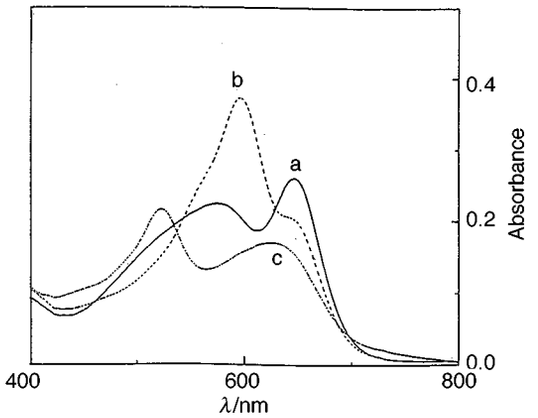


Fig. 3 Electronic spectra of metal (Zn^{2+} and Cu^{2+})-complexed compound **5** in 50% aq. dioxane solution (pH 7.9, pigment concentration: 1.324×10^{-5} mol dm^{-3}). a, no metal added; b, $ZnSO_4$ added, 1.0×10^{-3} mol dm^{-3}; c, $CuSO_4$ added, 1.0×10^{-3} mol dm^{-3}.

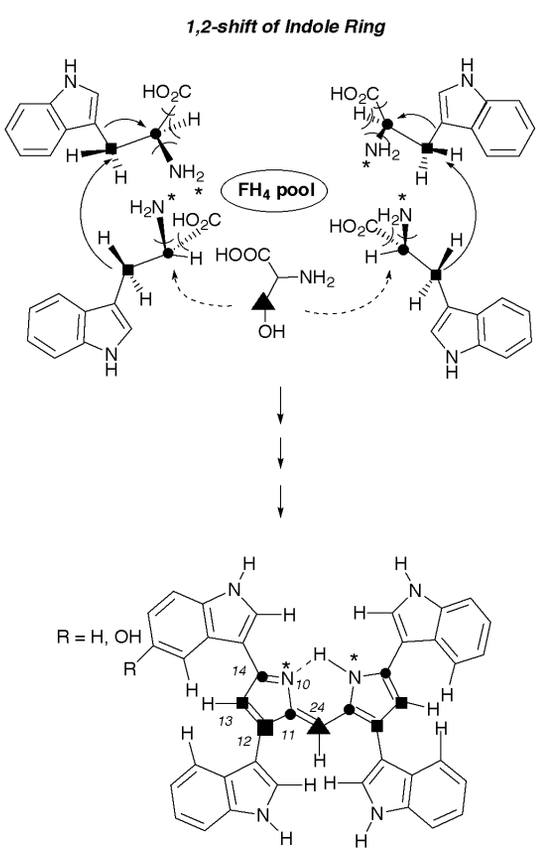


Fig. 4 Labelling patterns of compound **5** prepared by three feeding experiments with [2-^{13}C]tryptophan (■), [3-^{13}C]tryptophan (●) and a mixture of [3-^{13}C]serine (▲) and unlabelled tryptophan. The nitrogen atoms of the dipyrromethene moiety were labelled by incubating a mixture of unlabelled tryptophan and $^{15}NH_4NO_3$.

As a next step, we have tried to elucidate the biosynthetic origin of C_1 at the 24-position. A glance at the structure of **5** indicates that the carbon atom may be derived from the carboxy carbon of tryptophan, however, [1-^{13}C]tryptophan was not incorporated into **5**, demonstrating that a decarboxylation reaction proceeded during the biosynthesis as found for violacein.[3,5] As another candidate of the C_1 unit, serine was selected, because serine is well known to append a C_1 unit to a metabolic precursor.[8] By incubating a mixture of unlabelled tryptophan and [3-^{13}C]serine, the C_1 unit of **5** was proved to be derived from the serine; the signal at δ_C 119.5 ppm (24-position) was highly enriched and the direct ^{13}C–^{1}H coupling constant was determined to be 157 Hz from the 1D HMQC spectrum

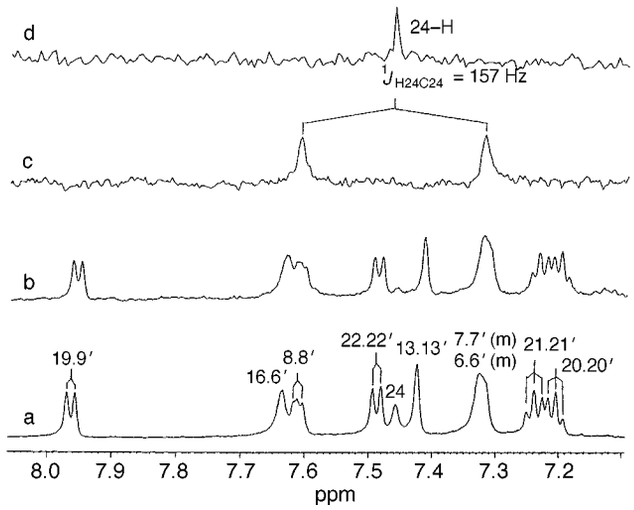


Fig. 5 ^{1}H NMR spectra of compound **5** measured in a neutral solution of [$^2H_6$]DMSO (600 MHz). a, Natural unlabelled compound **5**; b, **5** obtained from feeding a mixture of DL-[3-^{13}C]serine and unlabelled tryptophan, FH_4 being added to increase the ^{13}C-content (see Experimental section); c, 1D HMQC spectrum of b ($^1J_{H-C}$ 157 Hz, without decoupling by a garp cpdprg2, composite pulse-decoupling); d, 1D HMQC of b (with decoupling by a garp cpdprg2).

(Fig. 5), which was measured without a garp composite-pulse decoupling. The ^{13}C-content was estimated to be 39% by integration of the residual central peak of H-24. This finding demonstrated that serine hydroxymethyltransferase is responsible for constructing the dipyrromethene moiety, a tetrahydrofolate (FH_4) being converted into an N^5,N^{10}-methylenetetrahydrofolate by this enzyme.[8] Serine hydroxymethyltransferase catalyses a pyridoxal phosphate-dependent Cα–Cβ bond cleavage of the side chain of the amino acid to attach a C_1 unit to FH_4. Thus, the FH_4 cofactor is crucial for the production of chromoviridans analogues **5** and **6**. When the FH_4 cofactor was added to the cell-free system, only green pigments were produced.[1] This observation was further confirmed by the fact that the ^{13}C-incorporation ratio from [3-^{13}C]serine was increased to 85% by the addition of FH_4, while the incorporation was 39% when it was not supplemented. Addition of amethopterin (1 mM) to the cell-free system (supplemented with NADPH, 5 mM), an inhibitor of dihydrofolate reductase which catalyzes the formation of FH_4 from dihydrofolate (FH_2),[8] strongly inhibited the production of **5** to 3%. This remarkable decrease in the production of **5** by the inhibitor further supported the important role of the tetrahydrofolate pool for the biosynthesis of **5**.

On the other hand, NADPH is an essential cofactor for the production of violacein[1] and the monooxygenase of tryptophan hydroxylation is unambiguously involved in the biosynthesis of violacein.[7,9] The hydroxylation generally proceeds with the aid of molecular oxygen and 5,6,7,8-tetrahydrobiopterin, and the active form of the biopterin is produced from 7,8-dihydrobiopterin and NADPH; this reaction is also catalysed by a dihydrofolate reductase.[8] The formation of the tetrahydrobiopterin is a priming reaction for the hydroxylation.[8] The production of violacein was inhibited to 22%, when amethopterin (1 mM) was added to the cell-free system including NADPH (5 mM). Amethopterin inhibits the dihydrofolate reductase, thus the active form will be present in a lesser amount. This leads to less 5-hydroxytryptophan, a precursor of violacein pigment, thus the quantity of violacein produced would decrease. A mixture of 5-hydroxytryptophan and tryptophan was incubated in the absence of NADPH using the cell-free system, but no production of violacein and deoxyviolacein was observed. This finding suggested that the NADPH cofactor is also involved in a later stage of the violacein biosynthesis as well as with the initial hydroxylation of tryptophan. The pro-

Table 2 ^{1}H and ^{13}C NMR Data of chromoviridans **6** in [$^2H_6$]DMSO [δ_H (multiplicity, *J*/Hz)].

Position	δ_H	δ_C	Position	δ_H	δ_C
1	12.02 (br)	—	25	12.19 (br)	—
2	8.75 (br d)	130.57 (d)	26	8.82 (br d)	130.03 (d)
3	—	105.49 (s)	27	—	106.33 (s)
4	—	125.85 (s)[b]	28	—	124.79 (s)
5	7.32 (s)	104.15 (d)	29	8.15 (br)	120.12 (d)
6	9.22 (br s)	152.91 (s)	30	7.28 (m)	121.41 (d)
7	6.82 (dd, *J* 8.4, ~1)	112.76 (d)	31	7.28 (m)	122.81 (d)
8	7.40 (d, *J* 8.4)	113.45 (d)	32	7.60 (br)	112.86 (d)
9	—	131.23 (s)	33	—	136.99 (s)
10	12.66 (br)[a]	—	34	12.66 (br)[a]	—
11	—	142.46 (s)	35	—	141.71 (s)
12	—	128.35 (s)[b]	36	—	128.39 (s)[b]
13	7.28 (br m)	111.23 (d)	37	7.38 (s)	111.44 (d)
14	—	148.31 (s)	38	—	146.99 (s)
15	11.64 (br s)	—	39	11.66 (br)	—
16	7.60 (br)	127.65 (d)[b]	40	7.60 (br)	127.16 (d)[b]
17	—	108.35 (s)[b]	41	—	108.51 (s)[b]
18	—	125.87 (s)[b]	42	—	125.88 (s)[b]
19	7.91 (br)	119.33 (d)[b]	43	7.91 (br)	119.49 (d)[b]
20	7.22 (t, *J* 7.6)	120.48 (d)[b]	44	7.24 (t, *J* 7.6)	120.58 (d)[b]
21	7.18 (t, *J* 7.8)	122.48 (d)[b]	45	7.20 (t, *J* 8.0)	122.55 (d)[b]
22	7.48 (d, *J* 7.2)	112.27 (d)[b]	46	7.49 (d, *J* 7.2)	112.38 (d)[b]
23	—	136.83 (s)[b]	47	—	136.87 (s)[b]
24	7.45 (s)	119.15 (d)			

[a] The proton signals for the 10- and 34-positions became clear after adding a trace of TFA. The signals appeared to be equivalent to 1 × H by integration at the new chemical shifts of 12.66 and 12.68 ppm, respectively. [b] The following chemical shifts may be exchangeable: 12/36, 16/40, 17/41, 4/18/42, 19/43, 20/44, 21/45, 22/46 and 23/47.

duction of **3** was also inhibited, to 17%, by amethopterin (1 mM). The finding that the production of all the pigments was inhibited by amethopterin suggested that dihydrofolate reductase may be crucial to all the biosyntheses of **1**–**6**.

The structures and the biosynthetic routes to **5** and **6** are closely related to those of **1**, **2**, **3** and **4** except for the C_1-incorporation and oxygenation. In other words, two proviolacein skeletons are linked with an extra carbon from an FH_4 pool. If an oxygenation reaction takes place at the 11(11′)-position of **5**, then two molecules of **3** will be biosynthesized. However, conversion experiments of **5** into **3** and **3** into **5** failed in our cell-free system. This suggests that oxygenation and C_1-incorporation reactions at the 11-position might compete. Addition of NADPH to the cell-free system increased the quantity of violacein **2** produced, but decreased the amounts of **3** and **5**.[1] This observation suggests that the oxygenation reactions at the 11-position and at the 2-position of the indole ring might be catalysed by NADPH. However, the biotransformation of **3** into **1** was unsuccessful at a high concentration of NADPH (20 mM). The role of NADPH has yet to be fully understood and needs to be studied further in order to elucidate the details of the biosynthetic mechanism for the production of violacein. An elaborate mechanism of the metabolic regulation, which has yet to be solved by us, would appear to be involved in the biosynthesis of **1**, **2**, **3**, **4**, **5** and **6**. Studies on the biosynthetic relationship among these metabolites and further trials to isolate intermediates in the biosynthesis of violacein are in progress.

Experimental

UV spectra were measured on a Jasco Ubest-30 spectrophotometer, IR spectra with a Jasco IR-700 spectrophotometer, ^{1}H and ^{13}C NMR spectra on Bruker DPX 400 or DMX 600 spectrometers, and FAB mass spectra on a JEOL SX 100 mass spectrometer.

Isolation of compounds 5 and 6

Cell pellets prepared from a 6 dm^3 culture of *Chromobacterium violaceum* JCM 1249 (medium: nutrient broth) were sonicated in 100 ml of ammonia buffer (0.2 M, pH 8.5) containing 0.1% (w/v) Triton X-100 and 10 mM 2-mercaptoethanol to disrupt the cells and a crude enzyme solution was obtained after centrifugation at 10000*g* for 10 min. To the enzyme solution, adjusted to pH 8.5, was added 200 mg of L-tryptophan and the mixture was incubated at 25 °C for 24 h on a rotary shaker at 200 rpm, and then lyophilized. The powdered residue was dissolved in MeOH and subjected to column chromatography over Sephadex LH 20 using MeOH as eluent. The green pigments were strongly adsorbed on the column. After washing this column sufficiently, the green materials were eluted with MeOH containing a trace of TFA. Pure compound **5** was obtained by SiO_2 column chromatography using $CHCl_3$–MeOH (10 : 1) as eluent. Compound **5** was further purified by washing the pigment with benzene to give a yield of 2.5 mg. To obtain green metabolite **6**, mutant No. 26 was used. The washed cells from a 2 dm^3 culture of the mutant were suspended in 100 cm^3 of ammonia buffer (0.2 M, pH 8.5) including 50 mg of L-tryptophan and incubated under the same conditions described above. The green compounds were allowed to accumulate within the cells and then extracted with MeOH to give a solution containing several pigments including prodeoxyviolacein **3**, proviolacein **4** *etc.* The methanolic solution was concentrated into a small volume and left to stand for a few days at room temperature, resulting in the precipitation of green compounds **5** and **6**, while red pigments **3**, **4** and any other pigments were left in the supernatant. The precipitates were dissolved in acetone and subjected to column chromatography over Sephadex LH 20, as used in the purification of compound **5**, yielding 4 mg of pure compound **6** from 3 g of L-tryptophan.

Compound 5

Mp >230 °C (from acetone–hexane); λ_{max} (H_2O–MeOH, 25 : 75)/nm (pH 2) 641 (ε/dm^3 mol^{-1} cm^{-1} 59600), 489 (13400); (pH 12) 563 (27600), 387 (10580), 328 (12610); pK_a see Fig. 2; ν_{max} (KBr)/cm^{-1} (neutral) 3400, 3190, 1610, 1590, 1545, 1240, 1210, 1125, 1020, 740; δ_H (400 MHz; [$^2H_6$]DMSO) and δ_C (100 MHz) see Table 1; *m*/*z* (FAB, positive, a mixture of NBA and glycerol) 605.2517 (M^+ + H. $C_{41}H_{29}N_6$ requires *m*/*z*, 605.2454).

Tetraacetate of compound 5

Pigment **5** was acetylated using Ac_2O–Et_3N at room temperature for 30 min to give one spot on SiO_2 TLC [$CHCl_3$–MeOH (10:1)]. The product was insoluble in MeOH, but moderately soluble in $CHCl_3$ or DMSO. λ_{max} (H_2O–dioxane, 50:50)/nm (pH 2) 595 (ε/dm^3 mol^{-1} cm^{-1} 27400), 343 (23200); (pH 12) 545 (24980), 343 (21400), 300 (17500); pK_a 3.6; δ_H (400 MHz; $CDCl_3$) and δ_C (100 MHz) see Table 1; *m*/*z* (FAB, positive, a mixture of NBA and glycerol) 773.2848 (M^+ + H. $C_{49}H_{37}N_6O_4$ requires 773.2878).

Compound 6

Mp >230 °C (from acetone–hexane); λ_{max} (H_2O–MeOH, 25:75)/nm (pH 2) 644 (53000), 490 (18400); (pH 12) 562 (27800), 388 (16600), 338 (19300); pK_{a_1} 5.4, pK_{a_2} 8.7, ν_{max} (KBr)/cm^{-1} (neutral) 3440, 1610, 1590, 1550, 1245, 1200, 1135, 1025, 740; δ_H (400 MHz; [$^2H_6$]DMSO) and δ_C (100 MHz) see Table 2; *m*/*z* (FAB, positive, a mixture of NBA and glycerol) 621.2440 (M^+ + H. $C_{41}H_{29}N_6O$ requires 621.2402).

Preparation of the ^{13}C-labelled compound 5

L-[2-^{13}C] or L-[3-^{13}C]Tryptophan was prepared according to the literature,[10] and was subjected to optical resolution with L-aminoacylase from Sigma. [^{13}C]Formaldehyde (20% aq. solution, 99 atom%) and diethyl [2-^{13}C]acetamidomalonate (99 atom%) starting materials were purchased from Isotech Inc. DL-[1-^{13}C]Tryptophan (99 atom%) and DL-[3-^{13}C]serine (99 atom%) were available from Cambridge Isotope Laboratories, Inc. and Isotech Inc., respectively. To 20 cm^3 of the crude cell-free extract (no NADPH added), prepared by the method described above, 4 mg of each labelled tryptophan was added, then incubated, and compound **5** was purified as described above in a yield of *ca*. 0.3 mg. For the serine feeding experiment, a mixture of natural tryptophan (4 mg) and the labelled serine (4.25 mg) was incubated in 20 cm^3 of the cell-free extract to produce compound **5**. The ^{13}C-content increased remarkably when tetrahydrofolate (10.8 mg) was added, compared to the experiment where no cofactor was added: 39 atom% at 24 position in the absence and 85 atom% in the presence of tetrahydrofolate. The ^{13}C-content was estimated by the comparison of the integration of the central peak of the resonance for H-24 with that of the H-13 signal. The nitrogen label in the dipyrromethene moiety was introduced by incubating a mixture of natural tryptophan (4 mg) and 99% $^{15}NH_4NO_3$ (56 mg), yielding *ca.* 0.2 mg **5** with a content of about 20% ^{15}N atomic excess, which was estimated by integration of the satellite peaks.

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