Biosynthetic Study of Chaetoglobosin A: Origins of the Oxygen and Hydrogen Atoms, and Indirect Evidence for Biological Diels–Alder Reaction

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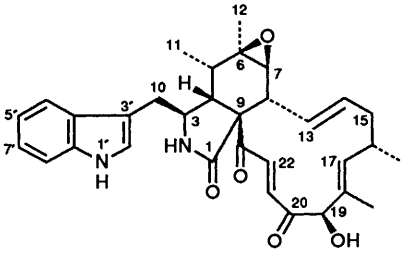
The biosynthetic origins of the oxygen and hydrogen atoms in the mycotoxin chaetoglobosin A **1** have been investigated by the incorporation of sodium [1-^{13}C,$^{18}O_2$]-, [1-^{13}C,$^{2}H_3$]-acetate and $^{18}O_2$ gas into the mycotoxin by using the chaetoglobosin-producing strain *Chaetomium subaffine*. Considering the results of the *P*-450 inhibitor experiment, the results obtained from the above experiments support the biogenetic pathway proposed. Attempts at direct conversion of ^{14}C- or ^{13}C-labelled prochaetoglobosin I **2** using whole cells were unsuccessful. Formation of the diastereoisomer **10** of **2** in the retro-Diels–Alder reaction of compound **2** has provided indirect evidence that the plausible precursor hexaene **6** is able to cyclize *via* [4 + 2]cycloaddition in the biosynthesis of chaetoglobosin A **1**.

In the preceding paper[1] we reported on the structures of the plausible biosynthetic intermediates, prochaetoglobosins I **2**, II **3**, III **4** and IV **5**, of chaetoglobosin A **1**. Here we describe the origin of oxygen and hydrogen atoms in compound **1** and discuss the biosynthetic pathway of chaetoglobosin A **1** in its later stages.



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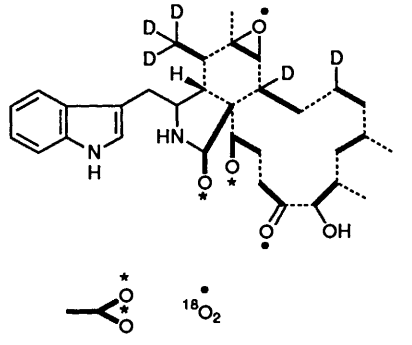
Results and Discussion

Previous studies show that compound **1** is biosynthesized *via* the coupling between one molecule of tryptophan and the polyketide from one molecule of starter acetate, eight molecules of malonate and three C_1 units from methionine.[2] Establishing the source of oxygen in the biosynthesis of secondary metabolites provides valuable information on the biosynthetic process.[3] Also, the fate of hydrogen atoms on acetate provides data of the process used for the construction of the molecular skeleton.[3] Sodium [1-^{13}C,$^{18}O_2$]acetate was administered to a culture of *Chaetomium subaffine*. In the ^{13}C NMR spectrum of this labelled product **1**,[4] isotopically shifted signals were observed for C-1 and C-23 as shown in Table 1. Also, chaetoglobosin A **1** obtained by fermentation under [$^{18}O_2$]oxygen gas showed upfield shifted signals for C-6, C-7 and C-20 in its ^{13}C NMR spectrum (Table 1). These results clearly show that the oxygen atoms at both C-1 and C-23 are derived from acetate and those at the epoxide and carbonyl (C-20) are introduced by oxidation. In these incorporation experiments, the origin of an oxygen atom at C-19 could not be detected due to signal broadening. Previously, Vederas *et al.* had studied the biosynthetic origin of oxygen atoms in cytochalasin **B 8**[5] which is considered to be biosynthetically analogous to compound **1**. A similar result was obtained in that the oxygen atoms located at C-1 and C-23 also originated from acetate.

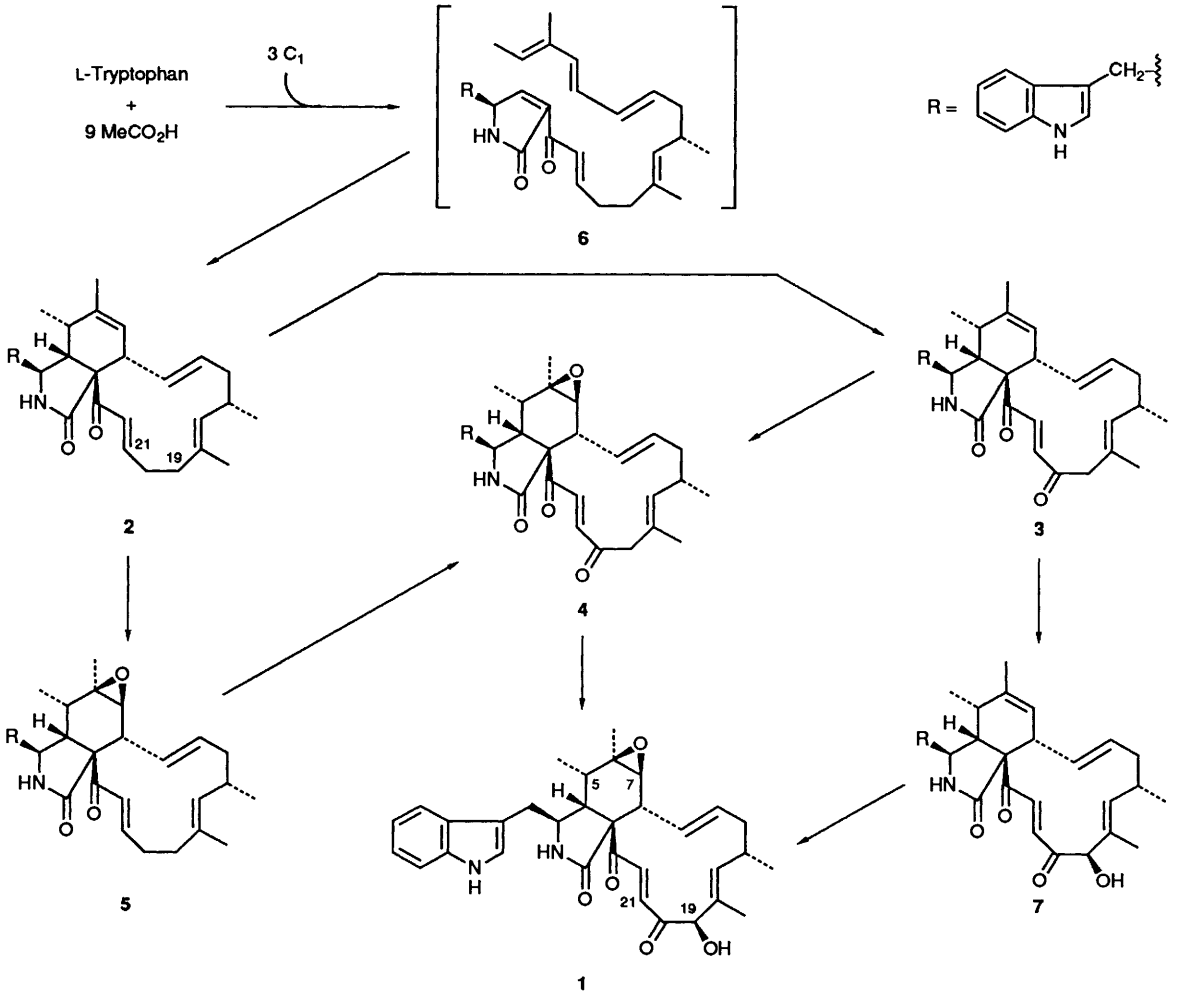
Next, we examined the incorporation with deuterium-labelled acetate. When Probst and Tamm examined the fate of hydrogen atoms in 19-*O*-acetyl-**1** by using [2-^{13}C,$^{2}H_3$]acetate, the labels were found only in the methyl of the starter unit and of the acetyl group.[2a] The use of [2-^{13}C,$^{2}H_3$]acetate is disadvantageous for the detection of isotopically shifted signals due to its coupling with deuterium and to the line broadening of the ^{13}C signal caused by quadrature relaxation. In order to observe the low degree of deuterium retention, we used [1-^{13}C,$^{2}H_3$]acetate. In the analysis of the ^{13}C NMR spectrum of compound **1** fed by [1-^{13}C,$^{2}H_3$]acetate, isotopically shifted signals were seen for C-5, C-13 and C-15 as shown in Table 1. This information indicates the retention of deuteriums at C-11, C-8 and C-14 since the observed shifts are induced by the



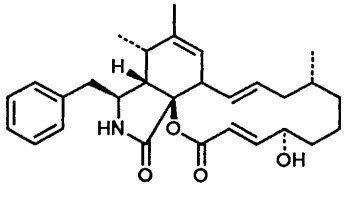
Labelling of chaetoglobosin A **1** by sodium [1-^{13}C,$^{18}O_2$]- and [1-^{13}C,$^{2}H_3$]-acetate and [$^{18}O_2$]oxygen gas

β-isotope effect.[3] This result was also confirmed in ^{2}H NMR spectroscopy due to the observation of three signals at δ 1.2, 2.1 and 5.3. The low level of enrichment at C-8 and C-14 suggests that a significant loss of deuterium occurred during incorporation. The retention of deuterium is limited to the first four units of the nonaketides. Moreover, the presence of deuterium at C-14 precludes the anionic carbon–carbon-bond formation proposed,[6a] in which the carbonyl group was located at C-14.

In the biosynthetic study of betaenone B,[7] we prepared the isotopically labelled putative intermediates by fermentation in the presence of an inhibitor when simple labelled precursors, *i.e.* acetate or methionine, were admistered to the micro-organism. Successful conversion from these labelled compounds into betaenone B confirmed the intermediacy of the accumulated compound obtained from inhibitor experiments.[7] Using 1 mmol of dm^{-3} metyrapone[1] as *P*-450 inhibitor, we prepared the ^{14}C-labelled prochaetoglobosin I **2** by feeding with [1-^{14}C]-acetate. Re-incorporation of this compound resulted in



Scheme 1 The proposed biosynthetic pathway of chaetoglobosin A **1**



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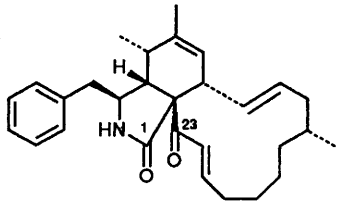

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insufficiently low conversion (0.24%). To examine the direct transformation of compound **2**, ^{13}C-labelled **2** was also prepared in a similar way by using [1,2-$^{13}C_2$]acetate. The ^{13}C NMR spectrum of labelled **2** showed high enrichments due to the observation of very low intensity natural-abundance peaks which were accompanied by not only doublet satellites arising from coupling of internal acetate units but also by double-doublet satellites caused by adjacent acetate units* (Fig. 1).

Table 1 Results of incorporation experiments in *Ch. subaffine*. ^{13}C Chemical shifts of chaetoglobosin A **1** in ^{13}C NMR spectra (125 MHz; $CDCl_3$)

	δ_C	[^{18}O]NaOAc[a]	$^{18}O_2$[a]	[^2H]NaOAc[a]
C-1	173.2	0.031 (70:30)[b]		
C-5	36.3			0.081, 0.161, 0.243
C-11	13.2			(27:7:15:51)[b,c]
C-6	58.0		0.043 (88:12)[b]	
C-7	62.4		0.036 (86:14)[b]	
C-8	48.8			(90:10)[b]
C-13	128.3			0.095
C-14	133.1			(94:6)[b]
C-15	41.7			0.124
C-16	32.0			(100:0)[b]
C-19	81.7			
C-20	201.4		0.046 (91:9)[b]	
C-22	136.1			(100:0)[b]
C-23	197.1	0.038 (66:34)[b]		

[a] The isotope shifts are given as Δ (ppm) upfield from the natural-abundance signal. [^{18}O]- and [^2H]-NaOAc mean sodium [1-^{13}C,$^{18}O_2$]-and [1-^{13}C,$^2H_3$]-acetate, respectively. [b] The values in parentheses are the ratio between the signal intensity of the natural-abundance peak to that of the shifted peak. [c] CH_3:CH_2D:CHD_2:CD_3.

* The coupling constants ($^1J_{CC}$) of compound **2** are as follows; $J_{5,11}$ (37.5 Hz), $J_{5,6}$ (39.1), $J_{6,7}$ (68.3), $J_{7,8}$ (41.1), $J_{8,13}$ (45.0), $J_{13,14}$ (73.4), $J_{14,15}$ (43.4), $J_{15,16}$ (36.5), $J_{16,17}$ (44.3), $J_{17,18}$ (73.9), $J_{18,19}$ (40.6), $J_{19,20}$ (34.3), $J_{20,21}$ (41.6), $J_{21,22}$ (68.0), $J_{22,23}$ (53.8), $J_{23,9}$ (54.1) and $J_{9,1}$ (48.5).

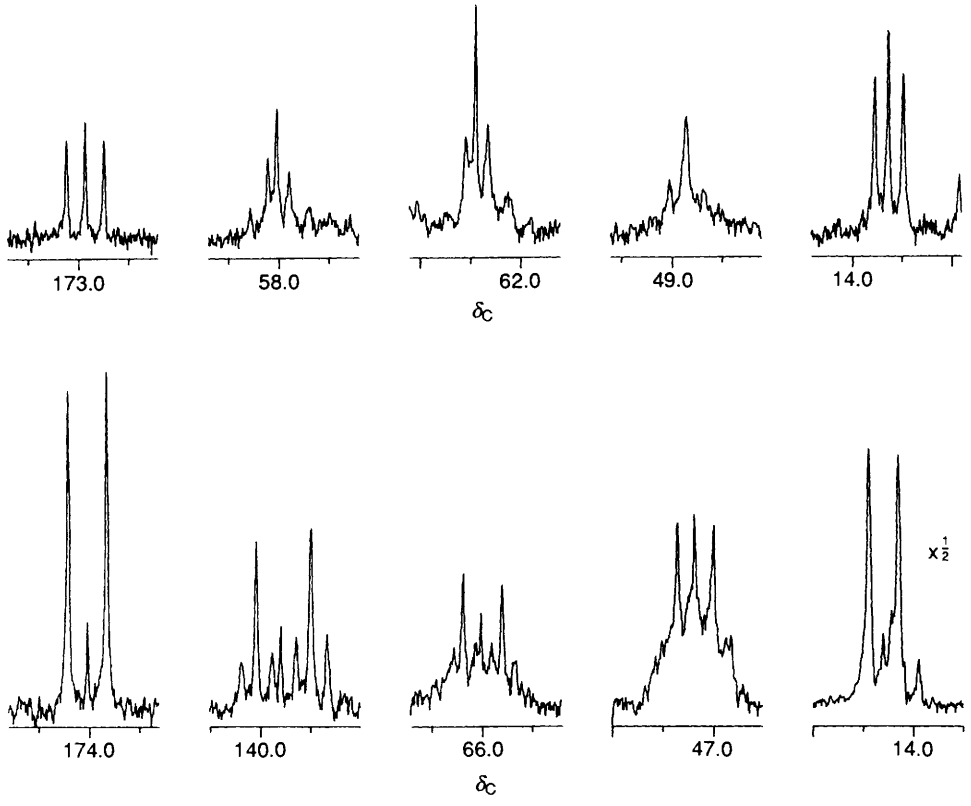


Fig. 1 ^{13}C NMR spectra obtained from the feeding experiment of sodium [1,2-$^{13}C_2$]acetate: chaetoglobosin A **1** (upper); prochaetoglobosin I **2** (lower). The signals from left to right are C-1, C-6, C-9, C-8 and C-11, respectively.

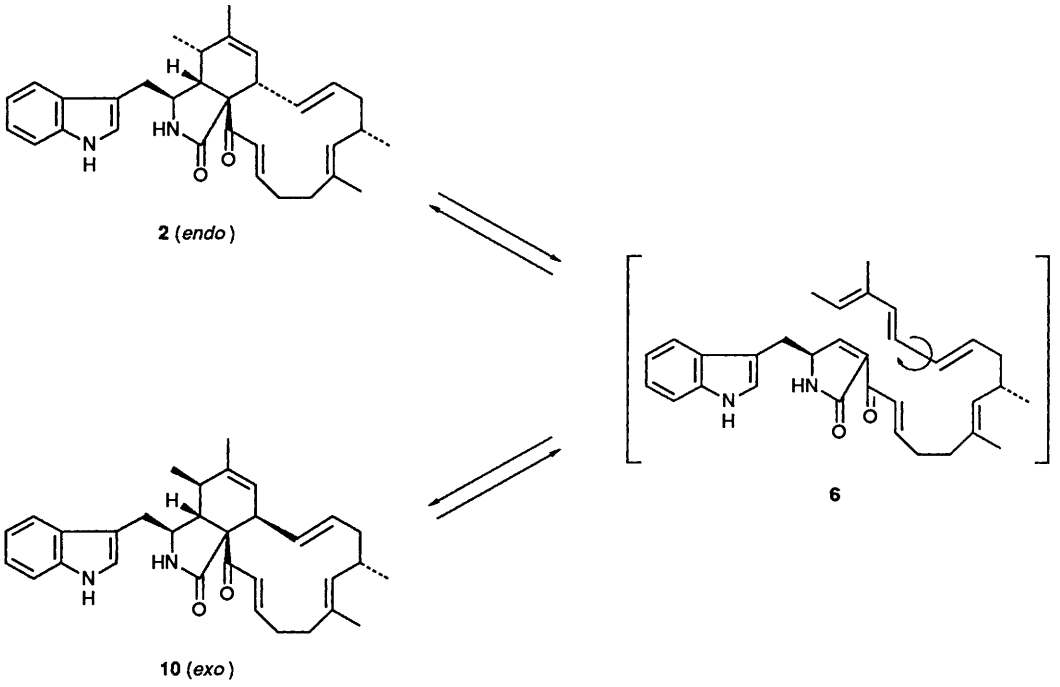
Even at distantly located units, *e.g.* C-8 and C-9, further coupling complicated the signal patterns. These observations indicate (falsely) that the polyketide chain is uniformly labelled when, in fact, it is not. Interestingly, enrichments of compounds **1** and **2** in this experiment were quite different (Fig. 1). This result suggests that the inhibitor blocks the oxidation of **2** to **1** almost completely at an early stage after feeding of the labelled precursor. Unfortunately, feeding of this heavily labelled precursor **2** did not result in any significant incorporation.

Since treatment of the fungi with the specific inhibitor of mono-oxygenase cytochrome *P*-450 resulted in accumulation of the partially oxidized metabolites **2**, **3**, **4** and **5**, we assume that the plausible intermediate **2** is modified by stepwise oxidation in the introduction of oxygen atoms at three extra sites to produce compound **1**. Hence, we propose the biosynthetic pathway of chaetoglobosin A **1** as depicted in Scheme 1 (see above). Although the possibility still remains that the oxidative modification at C-19 and C-20 occurs prior to cyclization and that chaetoglobosin J **7**[8a] is then formed by the cycloaddition, the negative incorporation results mentioned previously can be explained by assuming that the substrate did not reach the enzyme responsible for oxidation for some reason, such as the permeability of the cell membranes. The occurrence of non-oxidized derivatives, proxiphomin **9**[9] in cytochalasins and prochaetoglobosin I **2** in chaetoglobosins, suggests that these compounds are the first intermediates in post-cyclization, and that a variety of modifications produce a number of analogues.[6a,8] In order to confirm this hypothesis, we are now investigating the transformation of compounds **2** and **4** in microsomal fraction of *Ch. subaffine*.

The skeleton of cytochalasins is thought to be constructed *via* an intramolecular Diels–Alder reaction due mainly to the stereochemistry of the six-membered ring and the difficulty of explaining this by normal anion-based cyclization.[6,10] Until now, no experimental evidence for this has been obtained. We demonstrated the occurrence of the retro-Diels–Alder reaction for the purposes of obtaining the putative intermediate hexaene **6** from accumulated prochaetoglobosin I **2** in an isotopically labelled form. In the total synthesis of cytochalasins, intramolecular Diels–Alder reaction did not proceed completely, in that there yet remained some starting material.[11] Therefore, we expect that a reasonable amount of hexaene can be produced once the six-membered ring of intermediate **2** is thermally opened.

The pyrolysis of compound **2** was undertaken in a sealed tube at 180 °C. A new, less polar compound **10** was detected on TLC after 5 h in nearly equal amounts with the starting material. Prolonged reaction times resulted in the degradation of both substrate **2** and product **10**. The product **10** did not possess a conjugated triene moiety as expected but was assumed to be closely related to substrate **2** based on its UV and MS spectra. From its molecular formula (the same as that of substrate **2**) compound **10** was assumed to be a diastereoisomer of compound **2**. The COSY spectrum of compound **10** gave essentially the same correlation peaks as did that of substrate **2**. However, the chemical shifts of the signals of 4-H, 5-H, 8-H and 13-H in compound **10** differ by more than 0.5 ppm from those in compound **2**. This indicates that the major structural changes in compound **10** are concentrated in the cyclohexene ring. In addition, relatively small coupling constants $J_{8,13}$ (6.1 Hz), $J_{14,15a}$ (7.2 Hz) and $J_{14,15b}$ (7.2 Hz) in the macrocyclic part of compound **10** suggest that the skeleton of this part of the molecule does not exist in the 'zig-zag form', the typical conformation for all chaetoglobosins from fungi.[1,8] On the basis of NOE experiments on compound **10**, the stereochemistry was determined to be as shown in Fig. 2.

In synthetic studies of the structurally related cytochalasans,[12] it has been found that the pyrrol-2(5*H*)-one system such as that in hexaene **6** is thermally unstable and results in irreversible tautomerization to its enol which is unable to



Scheme 2 The retro-Diels–Alder reaction of prochaetoglobosin I **2**

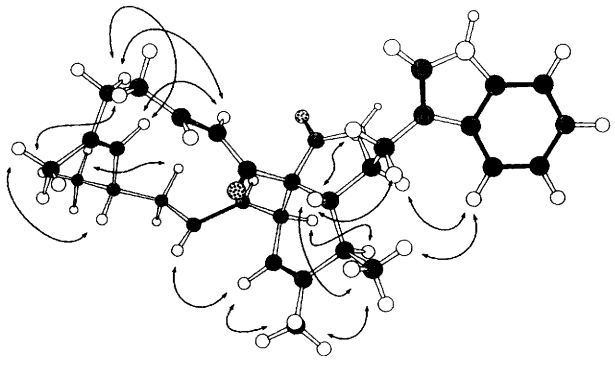


Fig. 2 NOEs of the product **10** in the retro-Diels–Alder reaction

produce the cycloadduct. Introduction of the *N*-acyl group on the pyrrolone is necessary for the [4 + 2]cycloaddition in order to increase anti-aromatic character and prevent the enolization. The occurrence of product **10** demonstrates that the Diels–Alder reaction actually takes place *via* hexaene **6** although the *N*-acyl group is absent. Moreover, these results indicate that the *endo*- and *exo*-transition states do not possess a sufficient energy difference, and this results in the adducts **2** and **10** in similar amounts (Scheme 2). This speculation is consistent with the result that no selectivity was observed in the thermal cyclization of the tetraene which is a synthetic precursor of the 13-membered macrocycle proxiphomin **9**.[13] The lack of stereoselectivity in this reaction indicates that the enzyme which is responsible for [4 + 2]cycloaddition stabilizes the *endo* transition state and results exclusively in the production of compound **2**.

In conclusion, we propose the biosynthetic pathway of chaetoglobosin A **1** which involves the cyclization of the hexaene and the following successive oxidations on the basis of the data from the inhibitor experiment and the incorporations. In addition, we have succeeded chemically in reproducing the putative Diels–Alder reaction which is presumably occurred in fungi. Verification of the involvement of this reaction *in vivo* is in progress.

Experimental

For general direction see ref. 1. Sodium [1-^{13}C,$^{18}O_2$]- (99 atom-% ^{13}C), [1-^{13}C,$^{2}H_3$]- (99 atom-% ^{13}C, 98 atom-% ^{2}H), [1,2-$^{13}C_2$]acetates (99 atom-% ^{13}C) were purchased from Cambridge Isotope Lab., $^{18}O_2$ gas (50.2 atom-%) was from MSD Isotopes, and sodium [1-^{14}C]acetate (1 mCi; 58.0 mCi/mmol) was from New England Nuclear. Circulation pump for $^{18}O_2$ gas incorporation was a Watson–Marlow 601S/R. ^{2}H NMR spectra were measured on a Bruker AM-500 instrument in the non-locked condition.

Feeding Experiments with Sodium [1-^{13}C,$^{18}O_2$]-, [1-^{13}C,$^{2}H_3$]-*Acetate.*—*Chaetomium subaffine* Sergejeva IFO8361 (ATCC22132) was inoculated in cultures in two 500 cm^3 flasks containing 2% potato-sucrose medium (150 cm^3). On the fourth day after inoculation, an aqueous solution (4.2 cm^3) of [1-^{13}C,$^{18}O_2$]acetate (100 mg) filtered through a sterilized microfilter (0.2 μm) was equally distributed between two 500 cm^3 flasks. After further incubation (10 days), mycelium was collected by filtration and extracted with acetone. This extract was concentrated under reduced pressure and the resultant aqueous layer was extracted with EtOAc. After being dried over Na_2SO_4, the extract was concentrated under reduced pressure to afford an oily residue, which was chromatographed on a silica gel column [(i) $CHCl_3$–acetone (100:0 to 50:50); then (ii) toluene–EtOAc (3:1)] to yield chaetoglobosin A **1** (6.9 mg). Similarly, the feeding experiment with [1-^{13}C,$^{2}H_3$]acetate (100 mg) afforded compound **1** (2.5 mg).

Fermentation of Ch. subaffine *under* $^{18}O_2$ *Gas.*—The fungus was inoculated in the culture in a 1 dm^3 flask prepared from water (7.5 cm^3) and polished rice (75 g). The flask was attached to a condenser to keep moisture in the culture and was then connected with the fermentation apparatus described by Simpson.[14] First the burette was charged with $^{16}O_2$ gas (~1 dm^3) and the atmosphere was circulated by rotary pump (1.2 dm^3/min). After the consumption of oxygen gas had reached ~150 cm^3/day (7 days), the burette was charged with $^{18}O_2$ gas (1 dm^3). When labelled gas had been consumed (*ca.* 4 days), the burette was filled with nitrogen gas and incubated for a further 3 days. The work-up reported[1] gave compound **1** (40.6 mg).

Preparations of the Isotopically Labelled Prochaetoglobosins I **2**, *II* **3**, *III* **4** *and IV* **5** *by feeding with Sodium* [1,2-$^{13}C_2$]-, [1-^{14}C]-*Acetate*.—Aqueous solutions of labelled precursors were added in equal portions, under sterile conditions, to the cultures (2% PS medium) after 4 days. The following are the quantities of precursors fed, the amounts of the *P*-450 inhibitor metyrapone, and the volume of culture used. [1,2-$^{13}C_2$]Acetate (700 mg), 340 mg in dimethyl sulfoxide (DMSO) (20 cm^3) to 10 × (150 cm^3); [1-^{14}C]acetate (350 μCi; 200 mg), 136 mg in DMSO (8 cm^3), to 4 × 150 (cm^3). This resulted in the final concentration of the inhibitor being 1 mmol dm^{-3}. After incubation (14 days), work-up as described before [1] gave compounds **2** (5.8 mg), **3** (1.0 mg), **4** (1.3 mg) and **1** (2.5 mg) on feeding with [1,2-$^{13}C_2$]acetate. The mycelial extract from feeding with [1-^{14}C]acetate was roughly separated on a silica gel column as above [1] to give crude fraction A containing compounds **2**, **3** and **4**, and fraction **B** containing compound **5**. These fractions were further chromatographed on PLC [C_6H_6–EtOAc (4:1) for fr. A and C_6H_6–$CHCl_3$–acetone (2:5:3) for fr. **B**] to afford compounds **2** (3.5 mg; 865 640 dpm, 53.7 μCi/mmol), **3** (0.6 mg; 17 766 dpm, 6.6 μCi/mmol), **4** (1.9 mg; 96 300 dpm, 11.7 μCi/mmol) and **5** (1.9 mg; 388 603 dpm, 45.9 μCi/mmol).

Feeding Experiments with ^{13}C-, ^{14}C-*Labelled Prochaetoglobosin I* **2**.—To two flasks containing 2% PS medium an EtOH solution (4 cm^3) of ^{14}C-labelled **2** (0.39 mg; 96 182 dpm) and non-labelled **2** (2.0 mg) was administered in equal portions on the sixth day after inoculation. Work-up was carried out as described above to yield compound **1** (3.5 mg; 221.6 dpm; 0.24% incorporation). In the case of ^{13}C-labelled **2** an aliquot (5.8 mg) of the sample was dissolved in EtOH (6 cm^3) and fed to three flasks. Similar work-up gave compound **1** (3.6 mg) together with recovered substrate **2** (1.3 mg).

Retro-Diels–Alder Reaction of Prochaetoglobosin I **2**.—Prochaetoglobosin I **2** (5.0 mg, 0.010 mmol) was dissolved in anhydrous toluene (15 cm^3). This solution was heated in a sealed tube at 180 °C for 5 h under argon. After cooling to room temperature, the reaction solution was concentrated under reduced pressure and the residue was chromatographed by PLC [C_6H_6–EtOAc (1:1)] to give 5,8-di-*epi*-prochaetoglobosin I **10** (2.3 mg) and recovered substrate **2** (2.0 mg).

Compound **10**: crystals, m.p. 206–208 °C (from MeOH); $[\alpha]_D$ −81.9 × 10^{-1} deg cm^2 g^{-1} (*c* 0.32 in MeOH); ν_{max}(NaCl)/cm^{-1} 3250, 2900, 1680, 1080 and 730; λ_{max}(MeOH)/nm ($\log_{10}$ ε) 222.0 (4.28) and 291.0 (3.26); δ_H(500 MHz; C_6D_6) (primed locants = indole nucleus) 7.58 (1 H, d, *J* 7.7, 4′-H), 7.07–7.25 (3 H, m, 5′-, 6′- and 7′-H), 6.93 (1 H, m, 21-H), 6.83 (1 H, d, *J* 15.4, 22-H), 6.46 (1 H, s, 2′-H), 5.92 (1 H, dd, *J* 15.4, 6.1, 13-H), 5.67 (1 H, br s, 7-H), 5.53 (1 H, ddd, *J* 15.3, 7.2, 7.2, 14-H), 5.43 (1 H, br s, 2-H), 4.71 (1 H, d, *J* 9.9, 17-H), 3.52 (1 H, br s, 8-H), 3.21 (1 H, m, 3-H), 2.83 (1 H, dd, *J* 13.9, 5.0, 10-H_a), 2.65 (1 H, dd, *J* 13.9, 9.2, 10-H_b), 2.58 (1 H, dd, *J* 6.3, 4.1, 4-H), 2.45 (1 H, m, 16-H), 2.05–2.18 (2 H, m, 15-H_a, 20-H_a), 1.91–2.00 (2 H, m, 20-H_b, 19-H_a), 1.77–1.87 (2 H, m, 15-H_b, 19-H_b), 1.72 (1 H, m, 5-H), 1.56 (3 H, s, 12-H_3), 1.40 (3 H, s, 18-Me), 0.93 (3 H, d, *J* 6.9, 16-Me) and 0.91 (3 H, d, *J* 6.9, 11-H_3); δ_C(125 MHz; C_6D_6), 196.9 (C=O), 176.2 (C=O), 145.9 (CH), 138.9 (C), 137.3 (C), 132.7 (CH), 131.5 (CH), 130.2 (C), 130.1 (CH), 129.9 (CH), 128.1 (C), 124.2 (CH), 123.5 (CH), 122.8 (CH), 120.2 (CH), 119.5 (CH), 112.5 (C), 112.11 (CH), 63.8 (C), 58.5 (CH), 50.7 (CH), 41.4 (CH_2), 41.4 (CH), 37.6 (CH_2), 37.0 (CH), 33.4 (CH), 33.2 (CH_2), 30.0 (CH_2), 22.1 (CH_3), 21.7 (CH_3), 18.4 (CH_3) and 17.2 (CH_3); *m/z* 482.2918 (M^+, $C_{32}H_{38}N_2O_2$ requires M, 482.2935); *m/z* 482 (M^+), 352, 131, 130 and 41.

Compound **2**: δ_H(500 MHz; C_6D_6) 7.58 (1 H, d, *J* 7.6, 4′-H), 7.40 (1 H, d, *J* 15.6, 22-H), 7.05–7.25 (3 H, m, 5′- 6′- and 7′-H), 6.96 (1 H, ddd, *J* 15.6, 6.8, 5.9, 21-H), 6.53 (1 H, ddd, *J* 15.1, 10.2, 1.9, 13-H), 5.44 (1 H, br s, 7-H), 5.36 (1 H, br s, 2-H), 5.17 (1 H, ddd, *J* 15.1, 10.5, 2.9, 14-H), 5.04 (1 H, d, *J* 8.9, 17-H), 3.23 (1 H, dd, *J* 4.5, 4.5, 4-H), 3.09 (1 H, m, 3-H), 2.82 (1 H, dd, *J* 14.2, 3.7, 10-H_a), 2.76 (1 H, br d, *J* 10.2, 8-H), 2.42 (1 H, dd, *J* 14.2, 9.8, 10H_b), 2.32–2.44 (2 H, m, 5- and 16-H), 2.03–2.31 (4 H, m, 15-, 19- and 20-H_a, 20-H_b), 1.97 (1 H, m, 15-H_b), 1.84–1.92 (1 H, m, 19-H_b), 1.54 (3 H, s, 12-H_3), 1.39 (3 H, s, 18-Me), 1.02 (3 H, d, *J* 7.2, 11-H_3) and 0.91 (3 H, d, *J* 6.7, 16-Me).

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