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Biosynthetic studies of the tetramic acid antibiotic trichosetin

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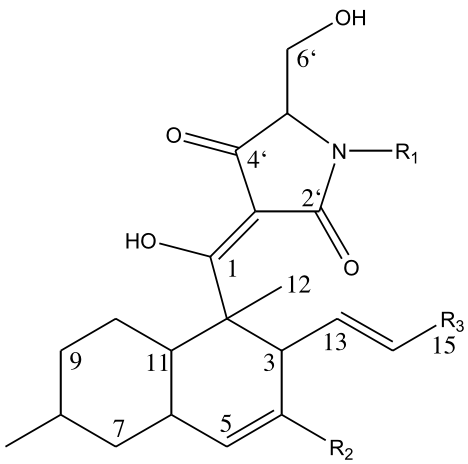
Abstract—A feeding experiment with ^{13}C-labeled precursors was conducted in the dual cultures of *Trichoderma harzianum* H14 and *Catharanthus roseus* callus to determine the biosynthetic origin of the carbon atoms in trichosetin. Results showed that trichosetin originated from two separate biogenetic units. One unit is an octaketide intermediate directly derived from eight intact acetate units joined in a head-to-tail fashion of a polyketide pathway. The other unit is serine. © 2002 Elsevier Science Ltd. All rights reserved.

1. Introduction

Trichoderma harzianum H14, when co-cultured with *Catharanthus roseus* callus on a hormone-free Murashige and Skoog medium, has been shown to produce trichosetin.[1] Trichosetin (**1**) exhibits a remarkable antimicrobial activity against Gram-positive bacteria, particularly *Staphylococcus aureus* and *Bacillus subtilis*. Structural elucidation revealed that it is a *N*-desmethyl homolog of equisetin (**2**), an antibiotic first isolated from *Fusarium equiseti*.[2] Another related compound is phomasetin (**3**) isolated from *Phoma* sp.[3] Equisetin and phomasetin are also toxic to Gram-positive bacteria and both of them have anti-HIV integrase activity.



(**1**): R_1=H, R_2=H, R_3=CH_3
(**2**): R_1=CH_3, R_2=H, R_3=CH_3
(**3**): R_1=CH_3, R_2=CH_3, R_3=$CHCHCH_3$

Aside from their diverse biological activities, investigation of this series of fungal metabolites is very interesting because they are consisted of two distinctive structural features. The first feature is the 2,4-pyrrolidinedione moiety which is the characteristic structural element of all tetramic acid antibiotics. In tenuazonic acid, erythroskyrine, malonomicin, streptolydigin and aflastatin A, C-2 and C-3 of this moiety were found to be derived from acetate, whereas C-4 and C-5 were from a certain amino acid, i.e. isoleucine, valine, serine, glutamate and alanine, respectively.[4–8] Carbons 4′, 5′ and 6′ of the analogous structure in equisetin and phomasetin were also proposed to originate from serine[3] based on the utility of this amino acid in the total synthesis of equisetin.[9] The second structural feature of interest is the bicyclic hydrocarbon moiety. The presence of a similar feature occurs in ilicicolin H where it was found to be derived through cyclization of a linear polyketide.[10]

In order to understand better the details of the formation of these two structural moieties, we investigated the biosynthesis of trichosetin by growing the dual culture of *T. harzianum* and *C. roseus* callus with stable isotope-labeled precursors. Labeled trichosetin was then isolated and the labeling pattern analyzed by ^{13}C NMR spectroscopy. This paper is the first report dealing with the biosynthesis of trichosetin.

2. Results and discussion

In order to elucidate the biosynthetic origin of the carbon atoms in trichosetin, the dual culture of *T. harzianum* and *C. roseus* callus was grown in a solid medium supplemented

Keywords: dual culture; *Catharanthus roseus*; *Trichoderma harzianum*; trichosetin; tetramic acid; polyketide; biosynthesis.

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Table 1. ^{13}C NMR assignments for trichosetin (**1**) and relative ^{13}C enrichments from ^{13}C-acetates, ^{13}C-serine, ^{13}C-glycine and ^{13}C-methionine

C- No.	Chemical shift (ppm)	[1-^{13}C] acetate	[2-^{13}C] acetate	[1,2-$^{13}C_2$] acetate, J_{C-C} (Hz)	^{13}C-serine	^{13}C-glycine	^{13}C-methionine
1	201.5	9.5		45.3			
2	50.0		8.3	45.7			
3	45.4	22.7		41.9			
4	132.3		20.2	Nd[a]			
5	131.2	27.6		Nd[a]			
6	39.8		23.0	33.1			
7	43.4	25.6		33.1			
8	34.8		21.4	32.7			
9	36.9	37.3		32.7			
10	29.2		27.8	33.9			
11	41.0	16.5		34.2			
12	13.7		3.4				29.8
13	127.6		24.4	41.9			
14	127.9	13.8		43.0			
15	18.6		17.7	43.0			
16	23.2		4.4				60.1
2′	180.9	7.3		62.8			
3′	100.7		8.2	63.2			
4′	192.9	2.0			3.8	1.9	
5′	64.5		4.4				
6′	61.9		6.8				1.9

Relative enrichments were normalized to the peak intensity of C-9, except for trichosetin labeled with [1-^{13}C] acetate, which was normalized to the peak intensity of C-2.

[a] Not determined due to signal overlapping.

with [1-^{13}C], [2-^{13}C] or [1,2-$^{13}C_2$] acetate. Labeled trichosetin was purified and the ^{13}C NMR spectra were then analyzed by comparison with the ^{13}C NMR spectrum of the unlabeled trichosetin. Chemical shift assignments were as previously reported,[1] as shown in Table 1. Trichosetin isolated from cultures supplemented with [1-^{13}C] acetate had enrichment for C-1, C-3, C-5, C-7, C-9, C-11, C-14, C-2′ and C-4′. Likewise, trichosetin biosynthesized from [2-^{13}C] acetate had enrichment for C-2, C-4, C-6, C-8, C-10, C-12, C-13, C-15, C-16, C-3′, C-5′ and C-6′. Thus, all the 21 carbon atoms of trichosetin were found to be labeled by acetate.

Supplementation with the doubly labeled [1,2-$^{13}C_2$] acetate afforded labeled trichosetin with the characteristic satellite peaks flanking the middle natural abundance signal in the ^{13}C NMR spectrum because of the incorporation of intact acetate units. The pair of methyl and carboxyl carbons of the intact acetate unit incorporated into trichosetin was readily identified by their similar J_{C-C} values (Table 1). The couplings found were C-1 and C-2, J_{C-C}=45 Hz; C-3 and C-13, J_{C-C}=41.9 Hz; C-4 and C-5, J_{C-C}=nd; C-6 and C-7, J_{C-C}=33.1 Hz; C-8 and C-9, J_{C-C}=32.7 Hz; C-10 and C-11, J_{C-C}=~34 Hz; C-14 and C-15, J_{C-C}=43.0 Hz; and, C-2′ and C-3′, J_{C-C}=~63 Hz. This result indicates that a total of eight intact acetate units, as shown in Fig. 1, were incorporated into trichosetin. These eight acetate units were joined in a head-to-tail fashion forming an octaketide unit very similar to ilicicolin H where it was found to be derived through cyclization of a linear polyketide. Cyclization of linear polyketide chains affording various metabolites with aromatic rings is well known, while the occurrence of reduced carbocycles in terpenes and sterol metabolites elaborated from linear terpene precursors is very common. Hence, the presence of this structural element in a fungal metabolite derived from a linear polyketide precursor is relatively novel.[10] The ^{13}C enrichment of the carbon atoms comprising this octaketide unit was relatively higher than in C-4′, C-5′, C-6′, C-12 and C-16. This was consistently observed when either [1-^{13}C]- or [2-^{13}C] acetate was used as precursor (Table 1 and Fig. 2). This incorporation pattern suggests that trichosetin was a product of mixed biosynthetic pathways, as has been observed in a number of tetramic acid antibiotics.[4–8]

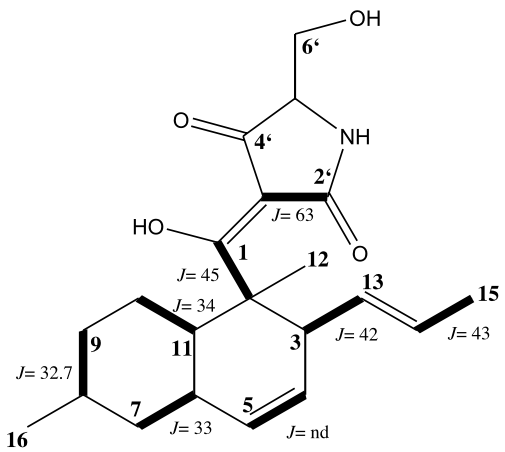

Figure 1. The eight intact acetate units incorporated into trichosetin as represented by thick lines with their corresponding coupling constants. Bold numbers represent carbon numbers.

The lower incorporation rate in C-4′, C-5′, C-6′, C-12 and C-16 suggests an indirect acetate origin for these carbon atoms. C-4′, C-5′ and C-6′ may be from an amino acid, similar to that of analogous carbons of other tetramic acid metabolites such as tenuazonic acid from isoleucine,[4] erythroskyrine from valine,[5] malonomicin from serine,[6] streptolydigin from glutamate,[7] and aflastatin A from alanine.[8] Based on the structure of trichosetin, the most plausible candidate would be serine. In fact, this amino acid was used in the total synthesis of the analogous compound

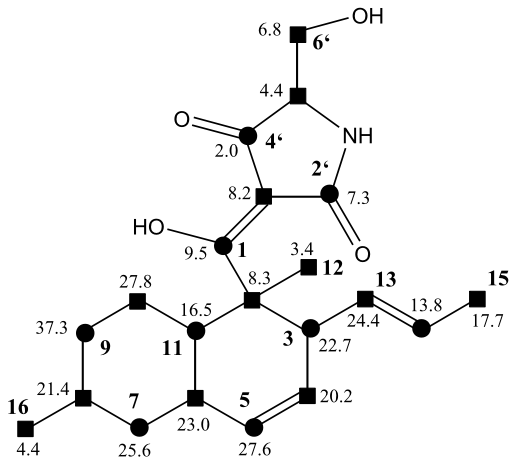

Figure 2. ^{13}C Enrichment pattern in trichosetin from the dual culture of *T. harzianum* and *C. roseus* callus labeled with [1-^{13}C] acetate (●) and [2-^{13}C] acetate (■). The degree of ^{13}C incorporation is shown opposite each carbon atom.

equisetin.[3] As expected, incorporation experiment using ^{13}C-serine as precursor showed an enrichment of C-4′ of trichosetin (Table 1) indicating that serine was actually the origin of C-4′, C-5′ and C-6′ of the tetramic acid moiety.

C-12 and C-16 methyl carbons, on the other hand, could be expected to be derived from the methyl group of methionine as this amino acid (in the form of *S*-adenosylmethionine, also called 'active methionine') was found to be an important biological methylating agent, such as in the synthesis of phosphatidylcholine from phosphatidylethanolamine and in the conversion of norepinephrine to epinephrine.[11] As expected, incorporation experiments using ^{13}C-methionine resulted in the enrichment of these two methyl carbons (Table 1). A particularly important observation was the enrichment of C-6′, although the degree of incorporation was not as high as those of C-12 and C-16. This implies that a C-2 amino acid, i.e. glycine is also a precursor of trichosetin contributing the C-4′ and C-5′ of the tetramic acid moiety. In this case, glycine may be formed from the transamination of glyoxylate,[12] which together with succinate were products of the cleavage of isocitrate catalyzed by the enzyme isocitrate lyase.[13] Glycine is converted to serine by hydroxymethyltransferase in a process that involves the transfer of the methyl group from methionine to the tetrahydrofolate (THF) pool.[11] This explains the ^{13}C enrichment of C-6′ by ^{13}C-methionine. Accordingly, incorporation experiment using ^{13}C-glycine showed that this amino acid was incorporated in the tetramic acid moiety of trichosetin (Table 1).

Similar to ilicicolin H,[10] the precise order of events leading to the formation of trichosetin was uncertain because the linear octaketide unit can link with serine prior to its cyclization to produce a tetramic acid intermediate with a linear side chain that can eventually cyclize. Another possibility is that the linear octaketide methylated at C-2 and C-8 can cyclize into a bicyclic hydrocarbon intermediate before it interacts with serine. Based on the present results, it can be concluded that acetate, serine and methionine are the precursors of trichosetin. The proposed scheme for

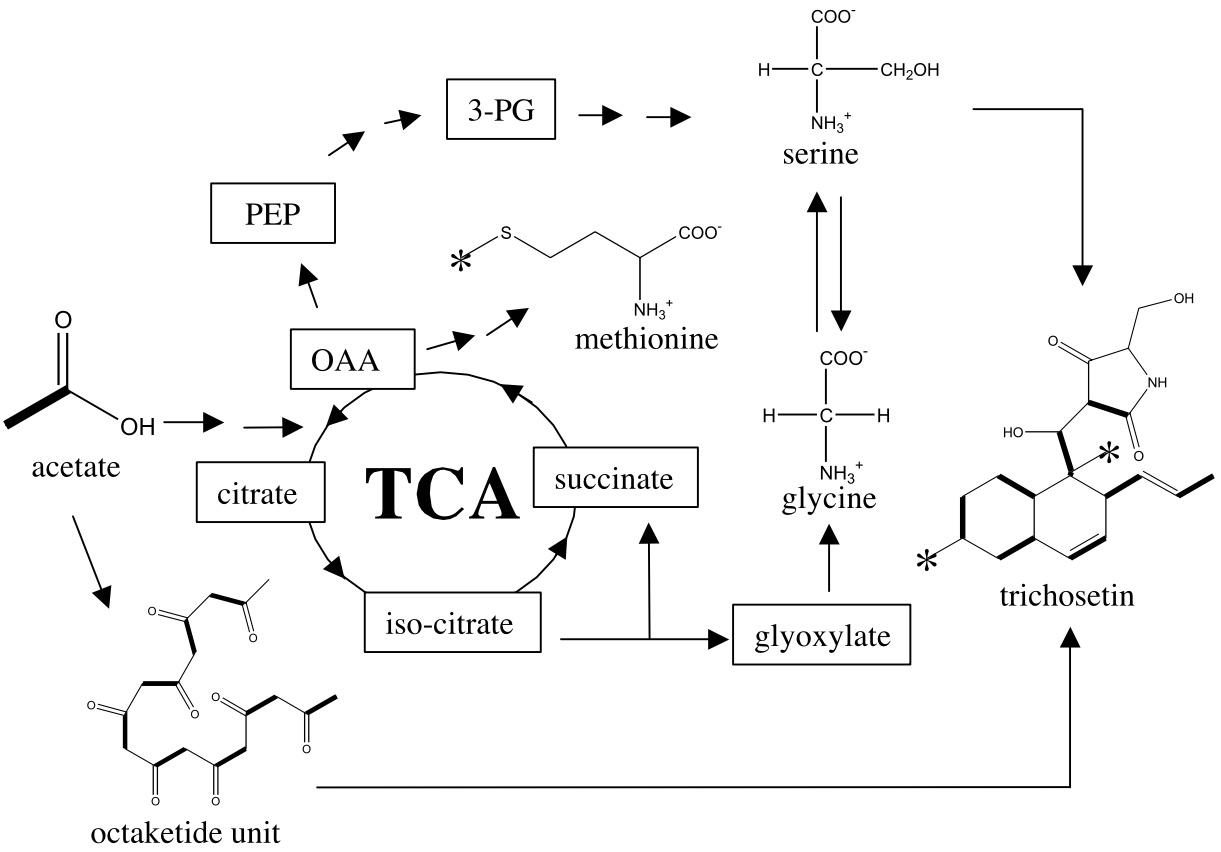

Figure 3. Proposed scheme for the biosynthesis of trichosetin.

trichosetin biosynthesis is shown in Fig. 3. It is highly probable that this pathway is also operational in the biosynthesis of equisetin and phomasetin.

3. Experimental

3.1. General

^{13}C NMR spectra were recorded on a JEOL 400 spectrometer in CD_3OD (99.8% atom enriched, Cambridge Isotope Laboratories, Inc., USA) at −80°C. The residual solvent signal (δ_C=49.9 ppm) was used as internal standard. All isotopically labeled compounds were 99% atom enriched and purchased from Isotec Co. (OH, USA).

3.2. Culture conditions

Isotopically labeled compounds were dissolved in water, filter-sterilized, and added into a hormone-free MS medium[14] containing 1% sucrose and 0.35% gellan gum (pH 5.8). Final concentration of [1-^{13}C] sodium acetate, [2-^{13}C] sodium acetate and [1,2-$^{13}C_2$] sodium acetate was 0.50 mg/ml, while 0.0625 mg/ml for DL-[1-^{13}C] serine, [1-^{13}C] glycine and L-[methyl-^{13}C] methionine. *C. roseus* callus (as an inducer of trichosetin production) was grown in MS medium with or without labeled precursor under continuous light at 27°C. After 14 days, the dual culture was established by inoculating spores of *T. harzianum* H14 onto the callus. The dual culture was harvested 10 days after fungal spore inoculation at which time the fungus has completely overgrown the callus.

3.3. Isolation of trichosetin

Isolation of trichosetin was carried out as previously reported.[1] Briefly, the infected calli and the spent medium were soaked in MeOH overnight. The filtrate was evaporated to dryness, the residue re-suspended in water and fractionated by partitioning between *n*-hexane–H_2O and EtOAc–H_2O. EtOAc fraction was chromatographed over Kieselgel 60 (70–230 mesh) eluted successively with toluene, toluene: acetone, acetone and MeOH. MeOH fraction was further purified by HPLC (ODS-3, Inertsil 10×250 mm^2, 64% MeCN–H_2O/0.1% HCOOH, 3 ml/min) to give trichosetin (t_R=37 min).

Acknowledgments

The 400 MHz NMR spectra were measured at the Analytical Center of Osaka University.

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