

Trichosetin, a Novel Tetramic Acid Antibiotic Produced in Dual Culture of *Trichoderma harzianum* and *Catharanthus roseus* Callus

Eufrocinio C. Marfori[§], Shin'ichiro Kajiyama, Ei-ichiro Fukusaki and Akio Kobayashi*

Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamada-oka, Suita-shi, Osaka 565-0871, Japan. Fax: +81-6-6879-7426. E-mail: kobayashi@bio.eng.osaka-u.ac.jp

* Author for correspondence and reprint requests

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Trichosetin, *Trichoderma harzianum*, *Catharanthus roseus*

The dual culture of *Trichoderma harzianum* and *Catharanthus roseus* callus produced an antimicrobial compound with a remarkable activity against the Gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis*. Structural elucidation revealed that this compound, which we have named trichosetin, is a novel tetramic acid (2,4-pyrrolidinedione) antibiotic and a homolog of the fungal metabolite equisetin. This compound however, was not produced in the individual culture of *T. harzianum* or *C. roseus* callus.

Introduction

Calli possess weaker physical and chemical barriers as compared to highly-differentiated intact plants. Hence, a fungus can easily infect a callus. Dual culture consisting of a fungus and a callus is a feasible approach to establishing any plant-fungus interactions which are otherwise impossible in the field. Upon fungal infection, new metabolites may be produced in an infected callus as a response of the callus to infection, or they may be produced by the fungus as it draws its nutrition from the callus. Dual culture of the fungus and callus, therefore, is a potential source of novel bioactive compounds.

We have previously reported the induction of the antifungal compound phytolaccoside B in the dual culture of *Phytolacca americana* callus and *Botrytis fabae* (Kobayashi *et al.*, 1995). As an extension of our work on the search for novel bioactive compounds induced in dual cultures, we found that the methanol extract from the dual culture of *Trichoderma harzianum* and *Catharanthus roseus* callus showed remarkable antimicrobial activity against the Gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis*. We subsequently iso-

lated the active compound and found it was a *N*-desmethyl homolog of equisetin, a tetramic acid antibiotic first isolated from *Fusarium equiseti* (Burmeister *et al.*, 1974). This paper is the first report of an equisetin-related compound produced by any species of *Trichoderma* or *Catharanthus*. The production, isolation, physico-chemical properties, structural elucidation and antimicrobial activity of this compound are discussed.

Results and Discussion

The crude MeOH extract from the dual culture of *T. harzianum* and *C. roseus* callus showed a very strong antimicrobial activity against the Gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis*, with an MIC of 31.3 µg/ml for both. It also showed moderate inhibitory activity against the yeast *Saccharomyces cerevisiae*, with an MIC of 500 µg/ml. We then proceeded to isolate the active compound under the guidance of MIC assay and bioautography. The methanol extract (3.93 g dry weight of residue) was fractionated into *n*-hexane, ethyl acetate and *n*-butanol, and the active compound was found in the ethyl acetate fraction. Open column chromatography of the ethyl acetate fraction afforded 7 fractions. The 7th fraction, which was eluted with methanol, showed the highest activity. Reversed phase HPLC of this fraction using an ODS-3 column and isocratic elution with

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64% CH₃CN/ 0.1% formic acid afforded compound **1** (5.7 mg) as the major active component.

Compound **1** was isolated as a pale orange amorphous solid. It was soluble in methanol, ethanol, acetone, chloroform, pyridine, and DMSO but insoluble in water. It appeared as a single spot on a TLC plate developed with toluene: acetone: methanol: acetic acid (7:3:0.5:0.5, v/v/v/v, R_f 0.38) when viewed under UV₂₅₄, or as a purple spot after spraying with vanillin-sulfuric acid followed by heating to 110 °C for 1 min. When developed with a solvent system containing no acetic acid, a tailing of this spot was observed suggesting its acidic nature. In LC-ESIMS, the molecular ion peak corresponding to [M+H]⁺ was observed at m/z 360. Its molecular formula was analyzed to be C₂₁H₃₀O₄N by HR-FABMS in the positive mode (found: 360.2164; calcd: 360.2175). It exhibited intense electronic transitions in MeOH at $\lambda_{\max}$ 204 (ϵ 15200), $\lambda_{\max}$ 252 (ϵ 5700) and $\lambda_{\max}$ 288 (ϵ 10400) which shifted to $\lambda_{\max}$ 248 (ϵ 10900) and $\lambda_{\max}$ 286 (ϵ 11300) in basic solution (10 mM NaOH-MeOH), or to $\lambda_{\max}$ 206 (ϵ 15000), $\lambda_{\max}$ 232 (ϵ 4900) and $\lambda_{\max}$ 288 (ϵ 10400) in acidic solution (20 mM HCl-MeOH). The IR absorption band at 3100–3600 cm^{−1} indicated the presence of an NH and/or OH group while the absorption band at 1658 cm^{−1} indicated the presence of a conjugated carbonyl group. While HR-FABMS clearly indicated the presence of 21 carbon atoms, the ¹³C-NMR taken at 25 °C showed only 18 carbon atoms. This problem was one of the difficulties encountered in the structural elucidation of this compound and was solved only when a measurement was taken at −80 °C in MeOH-*d*₄ which revealed the additional three carbon atoms (Fig. 1). Moreover, the ¹H-NMR spectrum showed abnormally broad peaks when measurement was taken at room temperature but was resolved into sharper peaks when measurement was taken at −80 °C. This peak broadening is probably due to a dynamic tautomerization that occurs at higher temperature. Henceforth, all NMR measurements were taken at −80 °C. The ¹H- and ¹³C-NMR assignments are listed in Table I. Interpretation of ¹H-¹H COSY, HMQC and HMBC data enabled us to elucidate two moieties, *i.e.* the hydrocarbon moiety and the conjugated carbonyl moiety. In the hydrocarbon moiety, HMBC correlation for H-12/C-2 revealed that the methyl carbon C-12 is connected to the

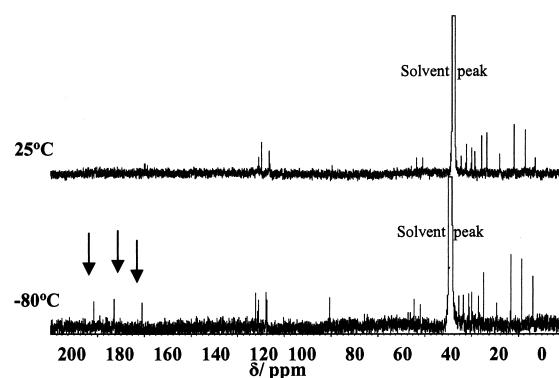


Fig. 1. ¹³C-NMR spectra of the active compound taken at 25 °C and −80 °C. The three additional peaks observed when measurement was taken at −80 °C are pointed by the arrows.

Table I. NMR assignments of compound **1** in MeOH-*d*₄ at −80 °C.

Position	δ_C , ppm	DEPT	δ_H , ppm
1	201.5	C ⁰	---
2	50.0	C ⁰	---
3	45.4	CH	3.43
4	132.3	CH	5.23
5	131.2	CH	5.44
6	39.8	CH	1.86
7	43.4	CH ₂	a: 0.86 b: 1.83
8	34.8	CH	1.49
9	36.9	CH ₂	a: 1.10 b: 1.78
10	29.2	CH ₂	a: 1.07 b: 2.02
11	41.0	CH	1.64
12	13.7	CH ₃	1.42
13	127.6	CH	5.38
14	127.9	CH	5.14
15	18.6	CH ₃	1.56
16	23.2	CH ₃	0.94
2'	180.9	C ⁰	---
3'	100.7	C ⁰	---
4'	192.9	C ⁰	---
5'	64.5	CH	3.76
6'	61.9	CH ₂	a: 3.79 b: 3.82

quaternary carbon C-2. C-2, in turn, was connected to C-11 and C-3 based on the HMBC correlations observed for H-11/C-2 and H-3/C-2, thus providing a ring closure and forming the bicyclic hydrocarbon moiety. In the conjugated carbonyl moiety, HMBC correlations observed for H-6'/C-4' and H-5'/C-2', and the chemical shifts of C-3'

(δ_C 100.7), C-2' (δ_C 180.9), C-4' (δ_C 192.9) and C-1 (δ_C 201.5) were suggestive of the presence of a tetramic acid skeleton (Sakuda *et al.*, 1996). Indeed, the pH-dependent UV spectrum and the rapid tautomerization of this compound were in good agreement with a tetramic acid (Stickings, 1959; Hayakawa *et al.*, 1991; Ono *et al.*, 1998; Steyn and Wessels, 1978; Saito and Yamaguchi, 1979; Nolte *et al.*, 1980; Philips *et al.*, 1989).

HMBC correlations observed for H₃-12/C-2 and H₃-12/C-1 connected the bicyclic hydrocarbon moiety to the tetramic acid moiety, thus the planar structure of compound **1** was completed as shown in Fig. 2. This compound is a *N*-desmethyl homolog of equisetin (a metabolite produced by *Fusarium* spp.) and was named as trichosetin. The relative stereochemistry of trichosetin, as depicted in Fig. 3, was inferred from a NOESY experiment. It showed NOE interactions between H₃-12, H-3 and H-6; H-6, H-7b and H-8 indicating a *syn* relationship among these protons. The other series of correlations observed for H-11, H-7a, H₃-16, H-9b, and H-10a suggested that all these protons were on the other side of the molecule. The optical rotation (-472°) and CD spectrum [0(323), $-8.8(305)$, $-10.8(262)$, $-4.4(236)$] were the same in sign as those of equisetin (Singh *et al.*, 1998) suggesting that the stereochemistry of trichosetin was the same as that of equisetin.

In the structural elucidation of equisetin (Philips *et al.*, 1989) and its homolog phomasetin (Singh *et al.*, 1998), assignments of the four olefinic carbons in the bicyclic hydrocarbon moiety were ambiguous due to overlapping signals. In trichosetin, these carbon atoms were assigned with more

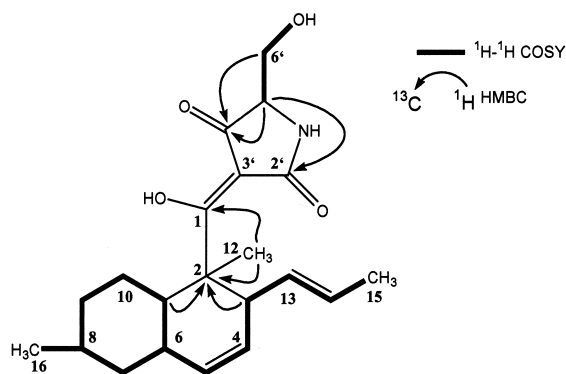


Fig. 2. Structure of trichosetin, as interpreted from ^1H - ^1H COSY (—) and HMBC (---) data.

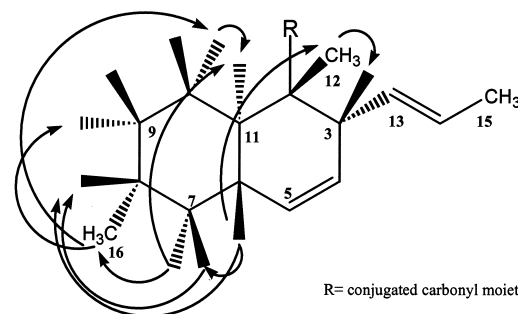


Fig. 3. Stereochemistry of trichosetin. Arrow connectors indicate the observed NOESY interactions.

certainty. This assignment was supported by the initial results of our labeling experiments (data not shown) wherein we observed alternate labeling pattern in these carbon atoms consistent with the proposed acetate origin of this compound. The full details of the labeling experiment which sought to determine the biosynthetic origin of all the carbon atoms in trichosetin will be published in a separate paper.

Microbial metabolites that contain a tetramic acid moiety are known to exhibit diverse biological activities (Singh *et al.*, 1998). Equisetin in particular has been shown to have strong antibiotic activity against certain Gram-positive bacteria (Burmeister *et al.*, 1974; Vesonder *et al.*, 1974). Recently, it was reported to be phytotoxic (Wheeler *et al.*, 1999) and an inhibitor of HIV-1 integrase (Singh *et al.*, 1998; Hazuda *et al.*, 1999). The antimicrobial activity of trichosetin is given in Table II and was found to have a similar inhibition spectrum as that of equisetin. Based on our preliminary experiments, this compound is also phytotoxic and has anti-HIV activity. Details of these assays will also be published elsewhere.

Since trichosetin is closely analogous to the fungal toxin equisetin, this compound might be considered as a metabolite that is produced by the fungus *T. harzianum* and not by *C. roseus* callus. It is noteworthy to mention, however, that this compound was induced only when *T. harzianum* was co-cultured with *C. roseus* callus, in which a "trigger" for production of trichosetin apparently exists. The mechanism of this induction is totally unknown. Investigation of the details of this induction mechanism is very interesting and will be the subject of future studies.

Table II. Antimicrobial activity of trichosetin and equisetin^a.

Test organism	MIC (nmol/ml)	
	Trichosetin ^b	Equisetin ^c
Gram-positive bacteria		
<i>Staphylococcus aureus</i> IFO 13276	4.34	
<i>Staphylococcus aureus</i> NRRL B-strains		1.34–2.68
<i>Bacillus subtilis</i> IFO 3134	4.34	
<i>Bacillus subtilis</i> NRRL B-strains		1.34–2.68
Gram-negative bacteria		
<i>Escherichia coli</i> IFO 3301	>300	
<i>Escherichia coli</i> NRRL B-210		>300
Yeasts		
<i>Saccharomyces cerevisiae</i> IFO 2375	139	
<i>Candida lipolytica</i> IFO 1658	>300	
<i>Candida albicans</i> NRRL Y-477		>300
<i>Rhodotorula rubra</i> NRRL Y-7222		>300
Molds		
<i>Aspergillus niger</i>	>300	
<i>Cladosporium herbarum</i>	>300	
<i>Mucor ramannianus</i>		>300
<i>Penicillium digitatum</i>		>300

^a Comparison of trichosetin and equisetin, as shown in this table, is intended only to relate their inhibition spectrum and not their strength since the assay procedure and microbial strains used by Burmeister *et al.* (1974) were different from those used in the present study.

^b Data of the present study. Reference antibiotics were tetracycline (MIC was 0.9 nmol/ml against *S. aureus* and *B. subtilis*) and cycloheximide (MIC against *S. cerevisiae* was 1.39 nmol/ml).

^c Data taken from Burmeister *et al.* (1974).

Experimental

Callus and microorganisms

Catharanthus roseus callus was initiated from flowers placed in MS agar medium (Murashige and Skoog, 1962) containing 3 mg/l benzylamino-purine and 0.5 mg/l 2,4-D. The initiated calli were subcultured at monthly intervals in a hormone-free MS agar medium. *T. harzianum* was isolated from an unidentified diseased plant (not *C. roseus*) and was maintained in PDA medium. All test microorganisms were obtained from the Institute of Fermentation, Osaka (IFO).

Production and purification

Spores of *T. harzianum* were inoculated onto a two week-old *C. roseus* callus grown in a hormone-free MS agar medium at 27 °C under light conditions. After two weeks, the callus was completely covered by the fungus and was harvested. Harvested biomass (521 g fr. wt.) was soaked in methanol overnight, then filtered. The filtrate was evaporated to dryness *in vacuo* below 40 °C and the residue (3.93 g) was dissolved in 100 ml water.

After extraction with *n*-hexane (100 ml, 3×), the aqueous layer was extracted with ethyl acetate (100 ml, 3×). The pooled ethyl acetate extract was evaporated to dryness *in vacuo* below 40 °C. The residue (110.7 mg) was then chromatographed over silica gel and eluted successively with toluene → toluene: acetone (9:1 → 8:2 → 7:3 → 6:4) → acetone → methanol. The concentrated methanol fraction (49.1 mg) was subjected to HPLC equipped with a photodiode array detector. Separation was achieved using an Inertsil ODS-3 (10 × 250 mm) column maintained at 40 °C in conjunction with a mobile phase of 64% acetonitrile/H₂O with 0.1% formic acid ran isocratically at a flow rate of 3 ml/min for 60 min. The eluent corresponding to the major peak with a retention time of 37 min was manually collected to give the active compound (5.7 mg).

Bioassays

Isolation of the antimicrobial compound was guided by an MIC assay as described by Kobayashi *et al.* (1994) and by TLC bioautography

following the method of Didry *et al.* (1990). Except as otherwise indicated, *Staphylococcus aureus* was used as the test organism. MIC is defined as the minimum concentration of the test compound that completely inhibited the growth of the test organism after 12 h incubation period. For bioautography, the test samples were loaded on a silica TLC plate and developed using toluene: acetone: methanol (7:3:0.5, v/v/v). MTT was used as the redox stain to detect the inhibition zones.

Physical and chemical analyses

NMR experiments were performed on a JEOL 400 MHz at 25 °C or at –80 °C using residual solvent signals as the internal standard. Mass spectra were obtained using a Shimadzu LCMS QP-8000. FAB-MS were recorded on a JEOL XR300 with a *m*-nitrobenzyl alcohol (NBA) matrix in the positive mode. IR and UV spectra were measured with a HORIBA FT-210 and Shimadzu UV-1600, respectively. Optical rotation and CD spectra were determined using JASCO P-1020 polarimeter and JASCO J-720S spectropolarimeter, respectively.

Physico-chemical properties of trichosetin

$[\alpha]_D^{26.8} = -471.6$ (c 0.1, MeOH); CD (MeOH) $\Delta\epsilon$ ($\lambda_{\max}$): 0 (323), –8.8 (305), –10.8 (262), –4.4 (236);

LC-MS m/z : 360 $[M+H]^+$; UV $\lambda_{\max}$ (MeOH) nm (ϵ): 204 (15,000), 252 (5,700), 288 (10,000); IR $\nu_{\max}$ (KBr) cm^{-1} : 3330, 3304, 2949, 2916, 2848, 1658, 1570, 1452; ^1H NMR (400 MHz, MeOH- d_4 , –80 °C): δ_{H} 3.43 (1H, appbrs, H-3), 5.23 (1H, dd, $J = 14.1, 5.0$ Hz, H-4), 5.44 (1H, brdd, $J = 14.1, 5.0$ Hz, H-5), 1.86 (1H, m, H-6), 0.86 (1H, appq, $J = 12$ Hz, H-7a), 1.83 (1H, m, H-7b), 1.49 (1H, m, H-8), 1.10 (1H, m, H-9a), 1.78 (1H, m, H-9b), 1.07 (1H, m, H-10a), 2.02 (1H, m, H-10b), 1.64 (1H, m, H-11), 1.42 (3H, brs, H-12), 5.38 (1H, brdd, $J = 10.6, 3.3$ Hz, H-13), 5.14 (1H, dd, $J = 10.6, 5.0$ Hz, H-14), 1.56 (3H, d, $J = 5.0$ Hz, H-15), 0.94 (3H, d, $J = 6.5$ Hz, H-16), 3.76 (1H, appbrs, H-5'), 3.79 (1H, dd, $J = 11.3, 2.6$ Hz, H-6'a), 3.82 (1H, brdd, $J = 11.3, 3.0$ Hz, H-6'b); ^{13}C NMR (400 MHz, MeOH- d_4 , –80 °C): δ_{C} 201.5 (C-1), 50.0 (C-2), 45.4 (C-3), 127.6 (C-4), 131.2 (C-5), 39.8 (C-6), 43.4 (C-7), 34.8 (C-8), 36.9 (C-9), 29.2 (C-10), 41.0 (C-11), 13.7 (C-12), 127.9 (C-13), 132.3 (C-14), 18.6 (C-15), 23.2 (C-16), 180.9 (C-2'), 100.7 (C-3'), 192.9 (C-4'), 64.5 (C-5'), 61.9 (C-6'). FAB-MS m/z : 360 $[M+H]^+$; HR-FABMS m/z : $[M+H]^+$: calcd. for $\text{C}_{21}\text{H}_{30}\text{O}_4\text{N}$, 360.2175; found, 360.2164.

Acknowledgements

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