J Ind Microbiol Biotechnol (2012) 39:1719–1724
DOI 10.1007/s10295-012-1165-2

SHORT COMMUNICATION

# Microbial transformation of the sesquiterpene lactone tagitinin C by the fungus *Aspergillus terreus*

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Received: 7 February 2012 / Accepted: 16 June 2012 / Published online: 11 July 2012
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**Abstract** The biotransformation of the sesquiterpene lactone tagitinin C by the fungus *Aspergillus terreus* MT 5.3 yielded a rare derivative that was elucidated by spectrometric methods. The fungus led to the formation of a different product through an unusual epoxidation reaction between C4 and C5, formation of a C3,C10 ether bridge, and a methoxylation of the C1 of tagitinin C. The chemical structure of the product, namely 1$\beta$-methoxy-3$\alpha$-hydroxy-3,10$\beta$-4,5$\alpha$-diepoxy-8$\beta$-isobutyroyloxygermacr-11(13)-en-6$\alpha$,12-olide, is the same as that of a derivative that was recently isolated from the flowers of a Brazilian population of Mexican sunflower (*Tithonia diversifolia*), which is the source of the substrate tagitinin C. The in vitro cytotoxic activity of the substrate and the biotransformed product were evaluated in HL-60 cells using an MTT assay, and both compounds were found to be cytotoxic. We show that soil fungi may be useful in the biotransformation of sesquiterpene lactones, thereby leading to unusual changes in their chemical structures that may preserve or alter their biological activities, and may also mimic plant biosynthetic pathways for production of secondary metabolites.

**Keywords** Sesquiterpene lactones · Microbial transformation · *Aspergillus terreus* · Cytotoxic activity

**Abbreviations**

| | |
|---|---|
| DAD | Diode array detector |
| HPLC | High-performance liquid chromatography |
| HR-ESIMS | High-resolution electrospray ionisation mass spectrometry |
| MeCN | Acetonitrile |
| MeOH | Methanol |
| NMR | Nuclear magnetic resonance |
| STL | Sesquiterpene lactone(s) |
| TLC | Thin-layer chromatography |
| UV | Ultraviolet |

## Introduction

Sesquiterpene lactones (STLs) are a large group of natural products found mainly in plants of the family Asteraceae. They are considered chemical markers within the family and have ecological as well as economic value [10, 37]. Moreover, STLs exhibit an array of interesting biological activities such as antimicrobial [5], cytostatic [25], and antifeedant activities [30]. They are the main active constituents of many medicinal plants of Asteraceae worldwide [32].

Tagitinin C (**1**, Fig. 1) is an important STL occurring in the glandular trichomes of the leaves and inflorescences of Mexican sunflower (*Tithonia diversifolia* Hemsl. A. Gray, Asteraceae) [2]. It shows cytotoxic [19], anti-inflammatory [33], and antifeedant activities [2], and thus is a promising compound for further research into its mechanisms of action in different targets. *T. diversifolia*, the source of **1**, is used in folk medicine in many countries [8, 14] and has

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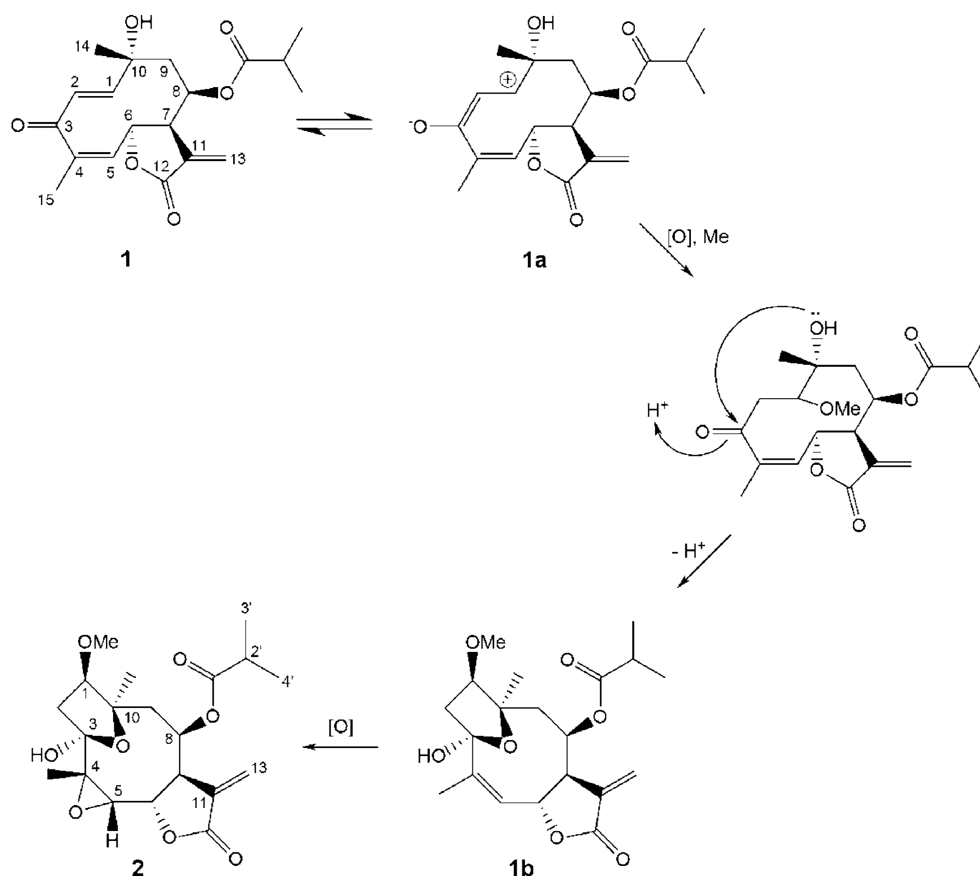


**Fig. 1** Proposal of biotransformation of **1** into **2** by *A. terreus* MT 5.3 (adapted from Spring et al. [38])

anti-inflammatory and antimalarial properties [8]. However, there remains concern regarding the ingestion of STLs because of their toxicity [35]. An interesting approach is to obtain STL derivatives that may have lower toxic effects or improved pharmacological activities, or both. Obtaining STL analogues, however, based on classic synthetic methods is not always a straightforward procedure, so the biotransformation of biologically active STLs is an attractive alternative providing new derivatives whose effects should be investigated [20].

The biotransformation of organic compounds by microorganisms is considered an economically and ecologically viable technology. In addition, it is a useful tool for the structural modification of bioactive natural products and the study of natural product metabolism [15, 16]. Moreover, some microorganisms can transform drugs in a similar way to mammals, and the utilization of microbial systems as models which mimic the metabolism of drugs in humans has received considerable attention [7, 16].

Fungal biotransformations of naturally occurring STLs have been carried out to obtain modifications that enhance activity and/or decrease toxicity [21, 24], develop structure–activity relationships [1, 4], and establish in vitro models to predict mammalian metabolites [3, 39]. A considerable number of biotransformations of STLs have been described using fungi [29]. *Aspergillus niger* (ATCC 16888), for example, transformed costunolide into four main products, 1$\beta$-hydroxyarbusculin, colartin, 11,13-dihydrosantamarine, and 11,13-dihydroreynosin [20]. In the same study, *A. ochraceous* (CECT 2069) transformed deoxyvulgarin into two products, vulgarin and dihydrodouglanin [20]. The microbial transformation of sclareolide by *A. niger* (ATCC 10549) yielded four oxidized metabolites identified as 1$\beta$-hydroxysclareolide, 3$\beta$-hydroxysclareolide, 1$\alpha$,3$\beta$-dihydroxysclareolide, and 1$\beta$,3$\beta$-dihydroxysclareolide [31]. The STL artemisitene was metabolized by *A. niger* (NRRL 599) to yield 11-*epi*-artemisinin, 9$\beta$-hydroxydeoxy-11-*epi*-artemisinin, and 9$\beta$-hydroxy-11-*epi*-artemisinin [27]. Microbial transformation of the germacranolide parthenolide using *A. niger* (NRRL 599) and *A. ochraceous* (NRRL 2295) yielded 11$\beta$H-dihydroparthenolide [11]. *A. terreus* (IFO6123) transformed dehydrocostuslactone into two derivatives, 11$\alpha$,13-dihydrodehydrocostuslactone and 16-(1-methyl-1-propenyl)eremantholide [13]. Cultures of two strains of *A. niger* (AS 3.1858 and VKM F-1119) transformed one of the most important STLs, artemisinin, into four hydroxylated products, 3$\beta$-hydrodeoxyartemisinin, 1$\alpha$-hydroxyartemisinin, 5$\beta$-hydroxyartemisinin, and 7$\beta$-hydroxyartemisinin [28, 39]. According to the literature, the main enzymatic reactions of STLs that are able to be catalyzed by *Aspergillus*

species are hydrogenation, hydroxylation, reduction, and acetylation.

Thus, with the aims of obtaining new, potentially bioactive compounds, and giving insights into the new metabolic reactions of STLs, we carried out the biotransformation of tagitinin C (**1**, Fig. 1) by the fungus *Aspergillus terreus* MT 5.3.

## Materials and methods

### Isolation of tagitinin C (**1**)

Leaves of *Tithonia diversifolia* (400 g) were collected by B. A. Rocha, in May 2007, in Ribeirão Preto, Brazil. The material was identified by F. B. Da Costa, and a voucher specimen was deposited in the SPFR Herbarium, Department of Biology, Ribeirão Preto, SP, University of São Paulo, under the number FBC #126. Air-dried entire leaves were rinsed for a few seconds with dichloromethane, for dissolution of the glandular trichomes [2], thereby yielding 5 g of a yellow crude extract after filtration with common filter paper and solvent evaporation under reduced pressure. The dry residue was re-suspended in MeOH/$H_2O$ (7:3, v/v) and extracted with *n*-hexane followed by dichloromethane. After solvent evaporation, the dichloromethane layer was fractionated over silica gel 60H (Merck, Brazil, cat. no. 7736) by vacuum liquid chromatography [9] using increasing amounts of ethyl acetate in *n*-hexane, thereby yielding nine fractions of 250 ml each. The tagitinin C-rich fractions (3 and 4) (1.5 g, confirmed by infrared spectroscopy, TLC, and reversed-phase HPLC analysis, as well as comparison with an authentic sample [2]) were purified by centrifugal chromatography (silica gel $PF_{254}$, Merck, Brazil, cat. no. 7749; 4 mm thickness; *n*-hexane/diethyl ether/ethyl acetate 6:3:1 v/v as eluent; flow rate 2 ml/min; UV lamp 254 nm). For final purification, preparative TLC was used (silica gel $PF_{254}$, Merck, Brazil, cat. no. 7730; 1 mm thickness; *n*-hexane/ethyl acetate/chloroform 5:3:2 v/v, and 2 % acetic acid as eluent). The isolated compound (**1**, 200 mg) was analyzed by HPLC and $^1$H NMR spectroscopy in order to check its purity before the biotransformation procedure. The $^1$H NMR spectral data of compounds (**1** and **2**) were in accordance with those reported [2].

### Biotransformation procedure and analysis

Screening was performed on a small scale (30 ml of fermentation medium in 125-ml Erlenmeyer flasks) to select efficient conditions for the biotransformation of **1**. The trials for screening procedures were performed as previously described by Krishna-Kumari et al. [17]. *A. terreus* MT 5.3 was cultivated in malt extract medium and incubated at 30 °C for 7 days to obtain spores. After that, the spores were harvested using a 2 % Tween 80 aqueous solution and counted in a Neubauer hemocytometer. The pre-fermentative medium was inoculated with $1 \times 10^7$ spores/ml and incubated with agitation (150 rpm) at 30 °C for 48 h. The resulting mycelium was harvested, rinsed with sterilized $H_2O$, and transferred to fresh Czapek medium (initial pH 6.0). The culture was then incubated under the same conditions for 10 days. After 24 h, 0.1 mg/ml of **1**, which had been previously dissolved in dimethyl sulfoxide (3 mg dissolved in 300 µl), was added to the culture medium. Control flasks contained culture medium with the fungus but without **1**, culture medium with the fungus and dimethyl sulfoxide and without **1**, culture medium with **1** and without the fungus, or only the culture medium. A time course study was carried out as follows. One Erlenmeyer flask was taken every 24 h; the product was extracted with dichloromethane, and then analyzed by TLC to check the degree of transformation of compound **1**. TLC was carried out on silica gel $GF_{254}$ (Merck, Brazil, cat. no. 7730) plates (0.25 mm thickness, 20 × 20 cm), and the spots were visualized after spraying the plates with vanillin/sulfuric acid (1 % $H_2SO_4$ in ethanol).

Thirty milligrams of **1** was used for the preparative-scale incubation with *A. terreus* MT 5.3 for 120 h, at 150 rpm, and 30 °C. The culture broth was filtered using filter paper and extracted with dichloromethane. The organic layer was dried over anhydrous $Na_2SO_4$, and the solvent was removed under a vacuum. The resulting residue (45 mg) was subjected to separation by HPLC in a Shimadzu SCL 10Avp liquid chromatograph with an SPD-M10Avp photodiode array detector (Japan) using a C-18 column (Shimadzu, Japan, ODS Shim-pack 5 µm, 4.6 × 250 mm; flow rate of 1 ml/min; DAD detection). The main metabolite (**2**, 4 mg) was purified after repeated injections according to the following gradient: 0.1 min 20 % MeCN, 35 min 60 % MeCN, 38 min 100 % MeCN, 43 min 100 % MeCN, 48 min 20 % MeCN, and 53 min 20 % MeCN. Compounds **1** and **2** were observed at retention times of 24 and 29 min, respectively (UV detection set at 210 nm). Comparison of the HPLC profiles of the controls with those biotransformed ensured that **1** had been converted to **2** through microbial catalysis.

The structures of compounds **1** and **2** were determined by HR-ESIMS, 1D- and 2D-NMR techniques, and comparison of the spectroscopic data with an authentic sample and published data [2]. NMR spectra were recorded using a Bruker (Germany) DPX 500 spectrometer (500 MHz for $^1$H). Samples were dissolved in deuterated chloroform with tetramethylsilane as internal reference. Deuterated solvents were purchased from Aldrich (USA). High-resolution mass

spectra were recorded on a UltrOTOFq-ESI-TOF mass spectrometer (Bruker Daltonics, USA).

Microorganism

*Aspergillus terreus* MT 5.3 was isolated from soil in Mato Grosso do Sul State, Brazil, and identified by C. M. S. Motta from "Coleção de Culturas–Micoteca URM–Departamento de Micologia/CCB-UFP", Av. Prof. Nelson Chaves s/no., Cidade Universitária, 50670-420, Recife, Brazil. The fungus was maintained by periodic transfers on PDA at 8 °C.

The microorganism *A. terreus* MT 5.3 was deposited in the collection "Coleção de Culturas Tropicais" (Tropical Culture Collection) of André Tosello Foundation (www.fat.org.br), under accession number CCT-7640.

Cytotoxicity assay

Human promyelocytic leukemia cells (HL-60) were obtained from the American Type Culture Collection (ATCC). Cell viability was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, as described by Mossman [23]. The MTT (Sigma, USA) solution at a final concentration of 500 µg/ml was added to the culture medium 4 h before the end of the treatment, and the reaction was stopped by the addition of 100 µl of dimethyl sulfoxide to the cell culture. For cell treatment we used compounds **1** and **2**, at concentrations of 5, 10, 25, 50, 100, 200, 250, 300, 400, and 500 µg/ml. Non-treated culture cells were used as a negative control, and cyclophosphamide was used as a positive control. Statistical analysis was performed by Student's *t* test; and Dunnett's test was used for multiple comparisons. A $p$ value less than 0.05 was considered statistically significant. Results are expressed as the mean ± SEM.

## Results and discussion

The transformation of **1** by *A. terreus* MT 5.3 yielded the unusual STL **2** as the main compound. This was identified as 1-methoxy-3-hydroxy-3,10$\beta$-4,5$\alpha$-diepoxy-8$\beta$-isobutyroyloxygermacr-11(13)-en-6$\alpha$,12-olide. The $^{1}$H NMR spectral data of **1** and **2** are shown in Table 1. Although several researchers worldwide have chemically investigated *T. diversifolia*, compound **2** was isolated only from a Brazilian population [2]. As already mentioned, it should be emphasized that the main enzymatic reactions that *Aspergillus* promotes in STLs are hydrogenation, hydroxylation, reduction, and acetylation [6, 11, 15, 17, 20, 27, 29, 31, 39, 40]. Interestingly, we have uncovered an unusual epoxidation between C4 and C5; a methoxy group was added at C1, and a C1,C10 ether bridge was formed in the 10-membered ring of **1**. Nevertheless, the exocyclic double bond at C11–C13 was preserved. Compound **2** was recently isolated from a rinse extract of the inflorescences of Brazilian *T. diversifolia* [2]. Although its chemical structure is already known, this is the first time that **2** has been obtained as a microbial biotransformation product. An interesting observation is that the *Aspergillus* strain used in our work was able to catalyze chemical reactions that mimicked those which occur inside the glandular trichomes from the inflorescences of *T. diversifolia*. Glandular trichomes of Asteraceae species are the places where the biosynthesis of STLs usually occurs, and possibly where **1** is converted to **2**. Thus, this fungus can be used as an alternative means of gaining **2** from **1**, therefore confirming the potential of biotransformation of natural compounds by microorganisms, as well as their ability to mimic plant biosynthetic pathways [34].

**Table 1** $^{1}$H NMR spectral data of compounds **1** and **2** (500 MHz, $CDCl_3$, $J$ in Hz)

| H | 1 | 2 |
|---|---|---|
| 1 | 6.93 d (16.9) | 3.99 dd (6.7, 10.4) |
| 2a | 6.26 d (16.9) | 2.57 dd (6.7, 12.5) |
| 2b | – | 1,93 dd (10.7, 12.6) |
| 5 | 5.88 d (9.0) | 3.33 s |
| 6 | 5.41 d (9.0) | 5.13 dd (4.65) |
| 7 | 3.55 m | 4.33 dddd |
| 8 | 5.33 ddd (3.2, 6.0, 10.0) | 5.53 ddd (4.2; 7.1, 11.0) |
| 9a | 2.40 dd (6.0, 13.8) | 1.74 dd (11.1, 14.7) |
| 9b | 2.00 dd (10.0, 13.8) | 1.66 dd (4.8, 14.7) |
| 13a | 6.36 d (1.7) | 6.29 dd (2.8) |
| 13b | 5.81 d (1.8) | 5.62 dd (2.3) |
| 14 | 1.53 s | 1.50 s |
| 15 | 1.95 s | 1.44 s |
| 2′ | 2.44 sept | 2.41 sept |
| 3′ | 1.06 d (7.1) | 1.05 d (7.0) |
| 4′ | 1.03 d (7.1) | 1.04 d (7.0) |
| –OMe | – | 3.35 s |

Spring et al. [38] reported the isolation of a closely related analogue of **1** from *Viguiera quinqueremis* (Asteraceae), and proposed its conversion to the hemiketal form, such as **1b** depicted in Fig. 1. The structural differences between the previously reported analogue and **1** are the nature of the C8 side-chain ester (methylbutyrate instead of isobutyrate) and the presence of a hydroxyl group at C15. The authors also discussed a spontaneous methoxylation of the **1a** analogue at C1 in the presence of MeOH, thereby leading to the formation of an analogue of **1b** (Fig. 1) [38]. Thus, it could be argued in our work that **2** is a compound produced by *A. terreus* starting from **1b**, an artifact that had

originated from **1** in the presence of MeOH. Nevertheless, a spontaneous interconversion where **1** was converted to **1b** would never occur in methods such as ours, because MeOH was not used in any step of the experimental procedure, either for isolation, analysis, or structure elucidation of **1**. Compound **2** is therefore a natural product obtained by biotransformation of **1**. In addition, the crude extract from the leaves of *T. diversifolia* used in this work had been screened for the presence of **2** by HPLC–DAD analysis, and it was not detected even as a trace.

According to Barrero et al. [6], epoxidation and hydrogenation reactions of the exocyclic double bond at C11–C13 seem to be the most common processes in the microbial transformation of STLs by filamentous fungi. In order to explain the formation of the epoxide group between C4 and C5 in the biotransformed molecule **2** as well as the methoxylation of C1, we followed the proposal of Onken and Berger [26]. The authors state that terpenoids are preferentially dissolved in the cell membranes of fungi, thereby inducing changes in properties of the membrane and causing toxicity. Fungi react against these effects and co-metabolize compounds to others that are more water soluble, thus showing that the enzyme systems involved in this process of detoxification are comparable to those of other eukaryotic cells. In the first step, monooxygenases of cytochrome P450 catalyze the oxyfunctionalization of the molecules [12, 22]. Then, in the second step, more water-soluble products are formed by the action of hydrolases or conjugation by glutathione S-transferases [6, 12]. Thus, the biotransformation of **1** leading to **2** might be part of a detoxification process created by the fungus.

The cytotoxicity bioassay was designed to observe whether **1** and **2** could elicit cell death at the selected concentrations. Both compounds showed cytotoxic effects on HL-60 cells lines comparable to that of the positive control, which was statistically significant when compared with the negative control ($p < 0.05$). The results indicated that **1** and **2** have cytotoxic activity against HL-60 cells at the lowest tested doses. Similar results were found while using peripheral blood mononuclear cells, where **1** and **2** also presented cytotoxic effects, thus indicating non-selective toxicity. It has previously been shown that **1** has cytotoxic activity against HL-60 cells [19], but its activity has not yet been compared with that of the derivative **2**. In the same work, compound **1b** was isolated from *T. diversifolia* as a natural compound and was found to be approximately 10 times more active than **1**. In our work, compound **2**, a C4–C5 epoxy derivative of **1b**, had a statistically similar activity to **1**.

Kupchan et al. [18] demonstrated that the in vitro cytotoxicity of STLs against human carcinoma was dependent on the presence of the α-methylene-γ-lactone moiety. Moreover, evaluation of the cytotoxic activities of further derivatives indicated which functional groups contributed to the activity. So-called active functional groups have therefore been proposed for several STLs. It has been verified, for example, that an extra α,β-unsaturated moiety contributed to enhancing the activity of STLs with the α-methylene-γ-lactone group, whereas the compounds containing only this additional unsaturation did not present significant activity [18]. In another study, it was confirmed that among other structural and electronic factors, the α,β-unsaturated carbonyl structure elements (cyclopentenone and α-methylene-γ-lactone) are correlated with cytotoxicity [36]. In our work, compound **1**, containing three α,β-unsaturated moieties, had statistically similar activity to **2**, with only one α,β-unsaturated moiety. This suggested therefore that its extra unsaturations were not important to the effect observed in HL-60 cells.

In summary, we have observed that in the presence of compound **1**, enzymes from *A. terreus* MT 5.3 were able to generate **2** in the same way as the enzymes located in the glandular trichomes of *T. diversifolia*. This fungus can thus be used as an alternative source of **2** because it mimics plant biosynthetic processes. The absence of two α,β-unsaturations in **2** did not improve or decrease its cytotoxic activity in the HL-60 cells when compared with **1**. Finally, we suggest that the mammalian metabolism of **1** after oral consumption of preparations containing *T. diversifolia* leaves should be researched in future, because this STL may form toxic compounds when metabolized.

**Acknowledgments** The authors acknowledge Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP–Bioprospecta/Biota–process # 04/07935-6), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for financial support, and Prof. N. A. J. C. Furtado (FCFRP-USP) for technical support.

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