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Enhancement of a pentacyclic tyrosine kinase inhibitor production in *Cladosporium* cf. *cladosporioides* by Cladosporol

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Abstract The binaphthyl derivative cladosporol **3** was supplied from 60 to 200 mg l^{-1} to shaken cultures of *Cladosporium* cf. *cladosporioides*. Compared to blank, fungal biomass was not affected by adding cladosporol till 100 mg l^{-1}: it rather increased at higher ratios between 150 and 200 mg l^{-1}. The production of the major pentacyclic metabolite **1**, a cytokine production and tyrosine kinase inhibitor, was enhanced tenfold when cladosporol was supplied at the highest ratio (200 mg l^{-1}) to shaken growing cultures of the fungus. The bioconversion of cladosporol to cladosporol D through reductive cleavage of the epoxide group was also observed. Interest in this kind of metabolites lies in their potential activity vs DNA topoisomerase I.

Introduction

In a model of antitumor strategy, novel chemotherapeutics are represented by inhibitors of topoisomerases, a class of nuclear enzymes involved in transcription and information processing by catalyzing topological change in DNA structure (Liu 1994).

Among these antitumor drugs, several small molecules with specific cell activity and reduced side effects are now progressing to clinical trials. Nevertheless, the large number of cancer types still demands new compounds to be tested in the wide range of assays designed to unravel the biochemical mechanisms aberrantly activated in the different tumor cells.

A series of pentacyclic compounds, including the major metabolite **1** with cytokine production and tyrosine kinase inhibitory properties, has been recently isolated from cultures of *Cladosporium* cf. *cladosporioides* (Wrigley et al. 2001). These metabolites are novel reduced benzo[j]fluoranthen-ones, related in some aspects to bulgarein **2** and bulgarhodin, the first of which induces mammalian topoisomerase I-mediated DNA cleavage in vitro (Edwards and Lockett 1976; Fujii et al. 1993). In a screening program devoted to find new promising topoisomerase I inhibitor compounds, we investigated the possibility of biotransforming the binaphthyl derivative cladosporol **3** (Sakagami et al. 1995), by supplying it to the culture of *C. cladosporioides*. This paper reports both the production enhancement of **1** by supplying cladosporol **3** to shake cultures of *C. cladosporioides* and the accompanying biotransformation of cladosporol **3** to cladosporol D **4** (see Scheme 1).

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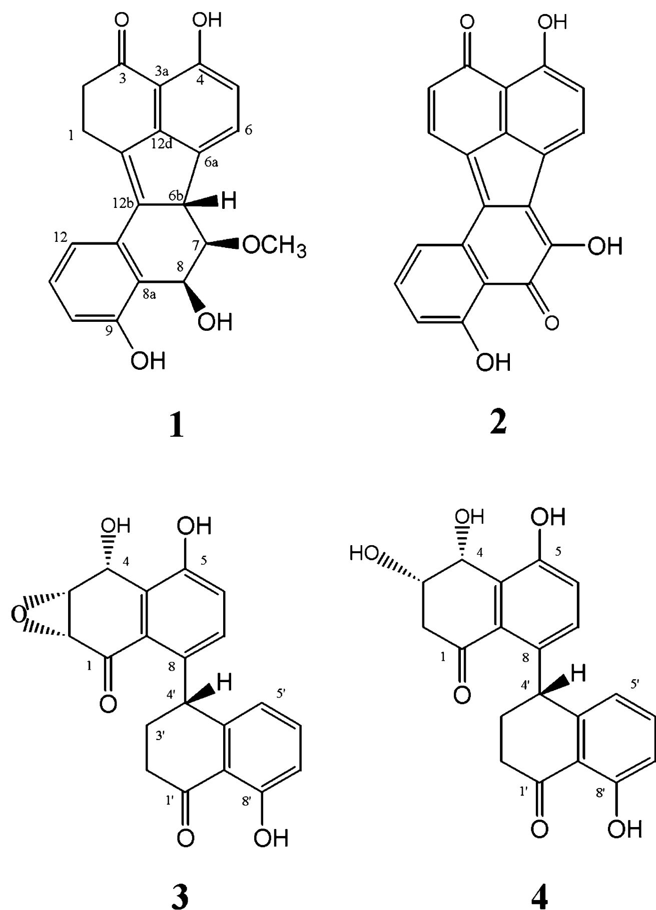
Materials and methods

Cladosporium cf. *cladosporioides* was kindly received as monocytogenetic strain Culture Collection N° CBUK20700 from Cubist (formerly TerraGen and Xenova).

The master fungal strain was preserved as mycelium plugs maintained in 10% aqueous glycerol at 4°C. Stock cultures were grown at 24°C on potato dextrose agar.

After preliminary screening on several media either in surface or submerged culture, *C. cladosporioides* large-scale cultures were made in Roux flasks (750 ml) containing 100 ml SSA (sucrose, soybean flour, KH_2PO_4, $MgSO_4·7\ H_2O$, $Mg_3(PO_4)_2·8\ H_2O$, agar at 50, 15, 0.5, 0.5, 0.5, 20 g l^{-1}, respectively). Flasks were inoculated with 5 ml of a shaken 4-day preculture in MYG broth (malt extract, yeast extract, glucose at 20, 2, 20 g l^{-1}, respectively), maintained at 24°C in the dark and extracted twice with AcOEt/MeOH (100:1) after 14-day growth. The



Scheme 1 Structures of the Bynaphthyl compound **1**, Bulgarein **2**, Cladosporol **3** and Cladosporol D **4**

organic fraction was dried with Na_2SO_4 and evaporated in a Rotavapor.

Broth cultures were made in baffled Erlenmeyer flasks (300 ml) containing 100 ml CSB (corn steep, glucose, $NaNO_3$, K_2HPO_4, KH_2PO_4, $MgSO_4{\cdot}7\ H_2O$, KCl, $FeSO_4{\cdot}7\ H_2O$ at 10, 30, 2, 1, 0.5, 0.5, 0.5, 0.02 g l^{-1}, respectively, plus 0.1% antifoam A), inoculated as previously described and placed on a rotary shaker at 180 rpm (24°C, dark). They served for verifying biotransformation of cladosporol **3**. This compound was purified from crude extracts of *Cladosporium tenuissimum* ITT21, cultured as already described (Nasini et al. 2004). It was dissolved in DMSO and supplied at 6, 10, 15 and 20 mg/flask to *C. cladosporioides* cultures after 4 days from inoculation. Solvent did not exceed 0.1% (v/v). Each cladosporol concentration was tested twice on 20 shake flasks. Controls were inoculated flasks containing only solvent or uninoculated flasks containing cladosporol. Production of metabolites and conversion of cladosporol **3** were monitored every 24 h on withdrawn samples (10 ml) by extraction and chromatography, as described below. Cultures were harvested and extracted around 96 h after cladosporol addition, when it had disappeared completely and production of **1** reached its maximum. Mycelia were separated from culture broth by centrifugation (3,500×*g* for 15 min), dried in an oven under vacuum at 40°C, and both separately extracted twice with AcOEt/MeOH (100:1). The organic solvents were evaporated in a Rotavapor. The crude extracts were checked for metabolite production by means of thin-layer chromatography (TLC) on Silica gel plates in CH_2Cl_2/MeOH 15:1 with pure samples as references.

Separation and purification of the metabolites were performed on silica gel (0.04–0.06 mm) by column chromatography with a stepwise elution with CH_2Cl_2:MeOH from 200:1 to 4:1. Further purification was performed on preparative plates (PLC) with CH_2Cl_2:MeOH (15:1) as eluent.

The purified compounds were checked for their identity by NMR, MS, and consistency with other spectroscopic data reported for reference compounds (Nasini et al. 2004; Wrigley et al. 2001).

Results

The pentacyclic active metabolite **1** has been demonstrated to be a tyrosine kinase inhibitor (Wrigley et al. 2001). In several agar and liquid media tested, *C. cladosporioides* was able to form high biomass and sporulate, but the amount of this metabolite and related compounds occurred in a low amount in CSB (6 mg l^{-1}) and was only just detected in SSA, having the same composition previously utilized for bulgarein production by *Heteroconium* sp. in a jar fermenter (Fujii et al. 1993), plus agar. Its appreciable de novo production in surface culture is here first reported: the highest yield of **1** was obtained at the end of exponential growth phase (40 mg l^{-1}, corresponding to ten Roux flasks).

Bulgarein **2** was shown to induce mammalian topoisomerase I–mediated DNA cleavage in vitro with activity comparable to that of camptothecin, but with a different mechanism of stabilizing this reversible enzyme–DNA cleavable complex (Fujii et al. 1993), and thus far, representing a new class of potentially active agents. Unfortunately the natural occurrence of **2** is restricted to the winter fruit bodies of the wood decay ascomycete *Bulgaria inquinans*, because batch cultures of the producing fungi, *B. inquinans*, and *Heteroconium* sp., the latter received from Kiowa Ltd. (Fujii et al. 1993), were unsuccessful in producing the desired metabolite.

Cladosporol **3** is a known 1,3-β-glucan inhibitor produced by several *Cladosporium* strains (Sakagami et al. 1995; Moricca et al. 2001). Considerable amount of **3** was produced by *C. tenuissimum*, hyperparasite of rust pathogens, together with a family of related antifungal compounds, partly responsible for the mycoparasitic properties of the producing strain (Assante et al. 2004): from batch cultures more than 200 mg l^{-1} could be typically collected. Its structural relation to metabolites **1**, **2**, and other active perylenequinone compounds led us to perform biotransformation trials using *C. cladosporioides* as a microbial regiospecific biocatalyst. It was supplied as a cosubstrate at varying amounts from 6 to 20 mg-flask to growing *C. cladosporioides* in CSB medium. The compound, added from 0 to 100 mg l^{-1}, did not inhibit the growth of the

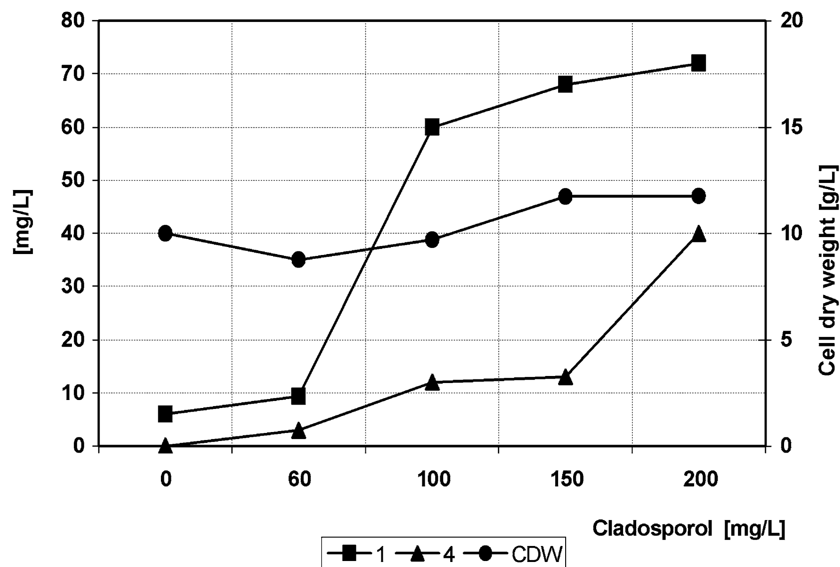


Fig. 1 Enhancement of compound 1 by adding cladosporol 3 and biotransformation of 3 to 4 by *C. cladosporioides* (CDW cell dry weight). 146×99 mm (300×300 DPI)

fungus, the biomass production reaching an average of the final cell dry weight (CDW) of 10.2±1.1 g l^{-1} after 96 h, and still increasing slowly at highest ratio of supply. Metabolite **1** production was monitored daily and reached its maximum 96 h after cladosporol supply. Longer cultivation periods were inadequate because fungal mycelium underwent lysis. At this time of the process, the total yield of **1** increased from 6 mg l^{-1} with no cladosporol added to 72 mg l^{-1} with 200 mg l^{-1} cladosporol added. Cladosporol **3** at 6% increased the total amount of metabolite **1** by about 50% compared to blank. When supplied from 10 to 15%, production of **1** underwent a sudden increase and was enhanced ten times with respect to controls, whereas at the highest concentration of 20%, production of **1** was increased further. No cladosporol was recovered at the end of all experiments, its consumption being complete in the same 4-day period. Its exhaustion was accompanied by formation of cladosporol D **4** at different degrees. This bioconversion occurred at 5%, when **3** was added at the lowest amount (60 mg l^{-1}), remained around 10% at **3** supplied from 100 to 150 mg l^{-1}, and reached the 20% when **3** was supplied at the highest concentration tested (200 mg l^{-1}). Noninoculated medium supplemented with cladosporol **3** served as chemical blanks where no conversion or degradation could be recorded over the whole period. Results are summarized in Fig. 1. Whereas cladosporol D **4** was always and only recovered from broth, metabolite **1** was extracted either from broth (approximately 60%) and from mycelium (40%) at every cladosporol **3** concentration used.

Discussion

Binaphthyl systems, occurring in cladosporol **3**, are also produced by many fungal species, i.e., *Daldinia* spp. and other xylariaceaus taxa, via the poliketide pathway. An intramolecular phenol oxidation results in the formation of polyaromatic metabolites. One of the simplest natural compounds, the 4,9−dihydroxyperylene−3,10−quinone, shows a coupling between C1 and C5′ (for enumeration, see skeleton of **3** Scheme 1), and is considered the parent compound of several bioactive-extended quinone pigments, like cercosporins, phleichromes, elsinochromes, calphostins, and cladochromes (Weiss et al. 1987; Arnone et al. 1989). The biological activity of the aforementioned compounds is mainly due to their photodynamic properties, which in the cladochromes and calphostins have been also related to their capability to inhibit the protein kinase C isoenzyme family (Tamaoki and Nakano 1990). Inasmuch as a modified skeleton of the binaphthyl system occurs also in the derivative **1**, in bulgarein **2** and bulgarhodin (Edwards and Lockett 1976), and in other few compounds, i.e., hypoxylonols (Koyama et al. 2002), hortein (Brauers et al. 2001), and daldinones (Quang et al. 2002), it is reasonable to assume that their formation occurs by a *para*–*meta* coupling between C1 and C3′ in the two aromatic diphenyl rings.

The bioconversion of cladosporol **3** to cladosporol D **4** involves the reductive cleavage of the epoxide, albeit at low rate, possibly catalyzed by xenobiotic−induced unspecific catabolic hydrolases and is a quite common biotransformation finalized to detoxify a xenobiotic. The enhancement of the production of compound **1** by *C. cladosporioides* could be seen as an accumulation of a dead-end product or a defence substance, as a result of the fungus–cladosporol interactions. However, the biochemical pathway should be investigated further, eventually feeding the fungus with ^{13}C-cladosporol or other binaphthyl derivatives to clarify the biosynthetic steps involved. New research will also consider the possibility of introducing some structural chemical modifications in metabolite **1**, i.e., by oxidation or dehydrogenation reactions, to obtain new derivatives to test for biological activity.

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