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Microbial Hydroxylation and Simultaneous Formation of the 4''-*O*-Methylglucoside of the Tyrosine-Kinase Inhibitor CGP 62706

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Abstract. Two fungal strains of *Beauveria bassiana*, DSM 875 and DSM 1344, hydroxylated CGP 62706, an inhibitor of the EGF-receptor tyrosine kinase, in the C(4') position and subsequently formed the glucosylated metabolite with 5–11% and 7–15% yield, respectively. The reaction could be successfully scaled up to 3 l fermentation volume with strain DSM 875. The structure of the glycosylated compound was determined by micro-HPLC-MS and NMR after production by fermentation on the mg scale. In addition, the biotransformation also provides access to the free 4'-hydroxylated compound, as the glycoside can easily be hydrolyzed.

Introduction

The compound CGP 62706 (**1**) is an inhibitor of the EGF-receptor tyrosine kinase with potent and selective activity against the enzyme *in vitro* in both cell-free and cell-based assays [1][2]. The compound is rapidly metabolized in mice and rats after oral administration [3]. By micro-HPLC-MS, two hydroxylated derivatives of **1** could be detected. Tentatively, it was assumed that biotransformation had occurred either at the indole or at the chlorophenyl moiety, in either case in *para* position of an electron-donating NH-group. Since it was expected that at least one of these metabolites was the pharmacologically active principle, significant quantities of hydroxylated metabolites were requested for further evaluations.

Hydroxylated derivatives are often difficult to synthesize both by chemical means as well as by biotransformation with enzyme extracts from animal tissues. Therefore, we tried to find an efficient system for the microbiological hydroxylation of **1** (*Scheme*). Surprisingly, we did not identify a hydroxylated derivative, as expected, but the hydroxylated and subsequently glucosylated metabolite **2** as the main reaction product formed by *Beauveria bassiana* DSM 875. The structure of **2** was determined by micro-HPLC-MS and NMR after production by fermentation on the mg scale.

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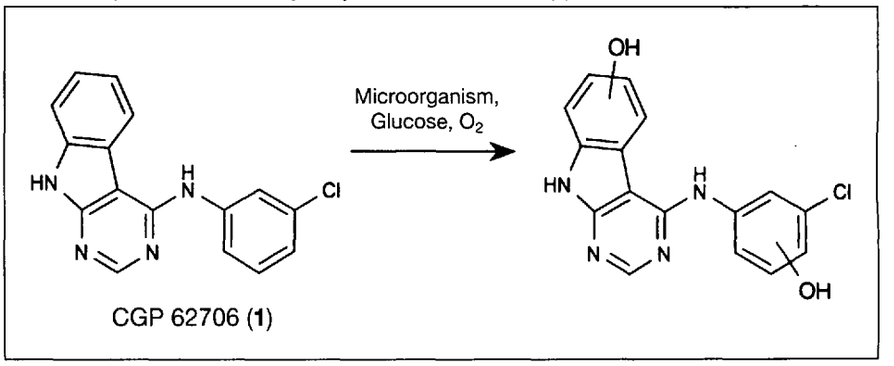
Materials and Methods

Biotransformation of CGP 62706 and Purification of Metabolite 2

Microorganisms were purchased from the *German Collection of Microorganisms and Cell Cultures* (DSM) in Braunschweig (D), the *American Type Culture Collection* (ATCC), Manassas, Virginia (USA), the *Centraalbureau voor Schimmelcultures* (CBS), Baarn (NL), and the *Agricultural Research Service Culture Collection* (NRRL), Peoria, Illinois (USA).

The microbial strains were grown for two passages of preculture in medium *NL148* (glucose 22 g/l, *Lab Lemco Oxoid* 4 g/l, peptone C 5 g/l, yeast extract 0.5 g/l, *Casitone* 3 g/l, NaCl 1.5 g/l, pH 6.7) at 28° and 220 rpm for 48–72 h. The inoculum size was always 5% (*v/v*). Main cultures were incubated under the same conditions using 100 ml *Erlenmeyer* flasks filled with 25 ml of medium. For a first series of organisms (17 strains), medium *NL148* was used. For a second series (11 strains) the *Streptomycetes* were cultured in medium *A* (glucose 20 g/l, soybean flour defatted 15 g/l, in *MV7* salt solution, pH 7), the fungi in medium *B* (glucose 20

Scheme. *Expected Microbial Hydroxylation of CGP 62706* (**1**)



g/l, soybean flour defatted 10 g/l, cornsteep 5 g/l, in *MV7* salt solution, pH 6). The composition of the salt solution *MV7* was: NH_4NO_3 2 g/l, $Na_2HPO_4 \times 2\ H_2O$ 1.75 g/l, KH_2PO_4 0.6 g/l, $MgSO_4 \times 7\ H_2O$ 0.2 g/l, $CaCl_2 \times 2\ H_2O$ 0.01 g/l, $FeSO_4 \times 7\ H_2O$ 0.001 g/l, trace-element solution 1 ml/l. The trace-element solution was composed of 20 mg/l of each of $Na_2MoO_4 \times 2\ H_2O$, $Na_2B_4O_7 \times 10\ H_2O$, $ZnSO_4 \times 7\ H_2O$, $MnSO_4 \times H_2O$, $CuSO_4 \times 5\ H_2O$. After two and four or five days of incubation, the glucose concentration and the pH value of the cultures were determined using glucose and pH-paper strips and were readjusted under sterile conditions with solutions of glucose (10%) and NaOH (1N). After two days of growth, CGP 62706 (**1**) was added in form of a concentrated solution in DMSO to a final concentration of 0.2 g/l. Two or three days and five days later, samples were taken and extracted with one volume of AcOEt. The organic phase was analysed by reversed-phase HPLC for newly occuring peaks under the following conditions: column *LiChroCart 125-4*, *LiChrosphere 100 RP-8*, 5 µm (*Merck*, Darmstadt); elution with a linear gradient from 20 to 66% MeCN in 3 mM H_3PO_4/KOH, pH 3, in 15 min; UV detection at 254 nm. For the detection of metabolites of **1**, the new peaks were then analyzed by HPLC-MS.

The metabolite **2** from strain DSM 875 was fermentatively produced on preparative scale as described above, but with orbital shaking at 180 rpm and using ten 1-l *Erlenmeyer* flasks filled with 200 ml of medium *NL148*, which had been prepared in *MV7* mineral-salt solution. The pH at the beginning was 6.5 and was not controlled during the cultivation. After five days of incubation in the presence of CGP 62706 (**1**), the mycelium was collected by centrifugation and washed once with half the initial volume of water. The aqueous phases were combined and extracted twice with an equivalent volume of AcOEt. After solvent removal under reduced pressure, the product was purified in two steps of preparative chromatography on silica gel, using a mixture of CH_2Cl_2 and MeOH in the ratio of 8:2 (*v/v*) as the eluent.

Micro-HPLC-MS and NMR Analysis

The liquid chromatographic separation was performed using a *Phoenix 40* syringe pump (*CE Instruments*, Milano) and a microbore column (150 mm × 1.0 mm) filled with *C18* reversed-phase material of 3 µm particle size (*Phenomenex*). Gradient mobile phase-programming was used with a flow rate of 45 µl/min. Eluent *A* was H_2O/MeCN 9:1 + 0.05% TFA.

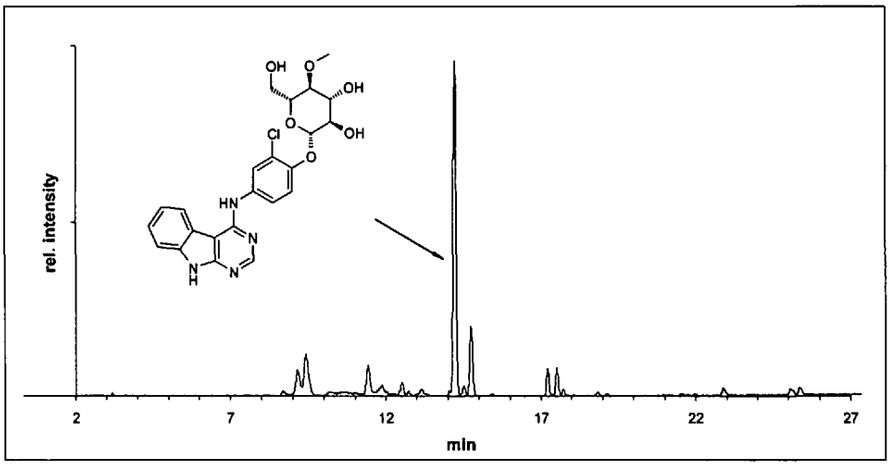


Fig. 1. *ES-TIC Chromatogram of an AcOEt extract after incubation of CGP 62706* (**1**) *with* Beauveria bassiana *DSM 875*

Eluent *B* was MeCN/H_2O 9:1 + 0.05% TFA. The mobile phase was held isocratic at 5% *B* for 5 min, followed by a linear gradient from 5% *B* to 95% *B* over 25 min and a 5 min hold at 95% *B*.

The column eluent was introduced directly into the ion source of a LCT time-of-flight mass spectrometer (*Micromass*, Manchester). The ionization technique employed was positive electrospray (ES). The cone voltage of the ion source was kept at a potential of 70 V.

Structure elucidation by NMR was performed in (D_6)DMSO using a *Bruker DPX 400* spectrometer.

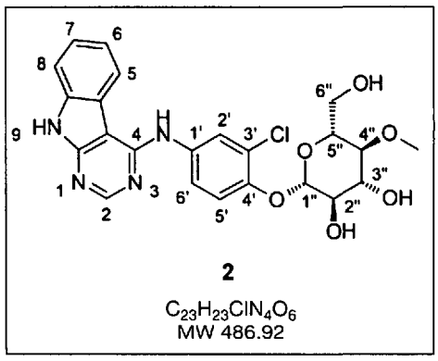


Fig. 2. *Glucoside* **2** *of CGP 62706 produced by* Beauveria bassiana *DSM 875*

Results

39 microorganisms, 1 *Comamonas*, 13 *Streptomycetes* and 25 filamentous fungi known for their ability to perform hydroxylations, were exposed to CGP 62706 (**1**). Seven of these strains had been described particularly to hydroxylate the indole nucleus of indole alkaloids [4]. 15 of the 39 tested microorganisms showed significant conversion of CGP 62706 (**1**). Micro-HPLC/MS analysis of the extracts of best-performing strains revealed that three strains (*Streptomyces sp.* DSM 40307, *Streptomyces sp.* DSM 40865, *Cunninghamella echinulata var. echinulata* ATCC 9244) produced hydroxylated metabolites of **1** (MW 310.74) giving rise to two small peaks which were not further investigated. Two fungal strains of *Beauveria bassiana*, DSM 875 (see *Fig. 1*) and DSM 1344, hydroxylated **1** in the C(4') position and subsequently glucosylated it forming metabolite **2** (MW 486.92) with 5–11 % and 7–15% yield, respectively (HPLC-peak area). The reaction could be successfully scaled up to 3 l fermentation volume with strain DSM 875. Since unreacted **1** was attached to the mycelium, it could be separated easily with the biomass by centrifugation (isolated yield: 90 mg).

A NOE between H–C(5') and H–C(1") indicated the substitution of CGP 62706 (**1**) in position 4', and not in the indole nucleus. The ^{1}H- and ^{13}C-shifts and the coupling constants of the glucose moiety confirmed the structure of the 4"-*O*-methylglucose (*Fig. 2*). The shifts were obtained from a HSQC and a COSY experiment in (D_6)DMSO (referenced to DMSO = 2.5 ppm for ^{1}H and 40 ppm for ^{13}C) and are listed below:

H–C(1"): 5.00; C(1"): 100.5; H–C(2"): 3.31; C(2"): 73.5; H–C(3"): 3.43; C(3"): 76.7; H–C(4"): 3.08; C(4"): 79.1; H–C(5"): 3.40; C(5"): 76.0; H–C(6"): 3.65/3.51; C(6"): 60.3; CH_3O–C(4"): 3.46; C: 60.0; HO–C(2"): 5.38; HO–C(3"): 5.25; HO–C(6"): 4.70. Coupling constants: $J_{H\text{-}C(1")\text{-}H\text{-}C(2")}$: 7.9 Hz.

Conclusions and Discussion

None of the strains under investigation, not even the indole-alkaloid-hydroxylating strains mentioned above, were able

to produce detectable amounts of a CGP 62706 metabolite hydroxylated at the indole moiety. The system with *Beauveria bassiana* offered the advantage that both hydroxylation and subsequent glucosylation at position 4' were achieved in a one-pot reaction. Furthermore, the glucosylated CGP 62706 derivative became easily accessible on a preparative scale, *i.e.*, up to 600 mg can be expected from biotransformation in a 20-l fermentor. In addition, this biotransformation provides also access to the free 4'-hydroxylated compound, as the glycoside can easily be hydrolyzed.

The simultaneous hydroxylation and formation of mono-*O*-methyl-β-glucosides of other structural types of aglycons has already been reported for the identical species *Beauveria bassiana* ATCC 7159 (= DSM 1344, formerly *Bassiana sulfurescens* or *Sporotrichum sulfurescens*) [5–7]. In contrast, *Rhizopus colinii* is reported to perform the formation of non-methylated β-glucosides [5].

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Structural Modification of the Immunosuppressants FK-506 and Ascomycin Using a Biological Approach

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Abstract. Bioconversion was utilized to conduct structural modification of the complex molecules FK-506 and ascomycin. Four interesting microbial reactions, regiospecific *O*-demethylation, glucosylation, hydroxylation, and phosphorylation, yielding various derivatives of the title compounds, were discovered from screening more than thousand microorganisms including bacteria, actinomycetes, and fungi. The isolated compounds were evaluated for a better understanding of structural and functional features responsible for their biological activity.

1. Introduction

FK-506 (**1**) is a relatively new macrolide immunosuppressant [1][2], reported to be 100 times more effective *in vitro* than cyclosporin [3], the extremely effective anti-rejection drug which revolutionized the field of organ-transplant surgery. The macrolide **1** is produced by *Streptomyces tsukubaensis* fermentation and was originally isolated by the *Fujisawa Pharmaceutical Company*. FR-900520 [4][5], a closely related macrolide, is produced by *Streptomyces hygroscopicus* subsp. *yakushimaensis* and was later shown to be identical with ascomycin (**2**) [6–10], an antifungal compound produced by *Streptomyces hygroscopicus* var. *ascomyceticus*, which was discovered by *Bristol-Myers & Co.* in 1960. FK-506 and ascomycin are useful in preventing host rejection of organ transplants, *e.g.*, bone marrow, liver, lung, kidney, and heart transplants. They inhibit interleukin production [11], mixed lymphocyte proliferation, and generation of cytotoxic T-cells [12][13]. Like cyclosporin, FK-506 and ascomycin have undesirable side effects, particularly CNS and renal toxicity [14]. FK-506 and ascomycin may offer some advantages over cyclosporin, but it is unlikely that they will become a safe replacement for cyclosporin in transplantation rejection. Thus, structural modification of **1** and **2** to generate new analogs with reduced side effects and increased bioavailability may provide utility not only in transplantation surgery, but also in other therapies.

The structure and absolute configuration of FK-506 was determined by chemical and spectroscopic techniques, including single-crystal X-ray analysis [2]. It is an unusual macrocyclic lactone possessing several functionalities including three methoxy groups at C(13), C(15), and C(31), two hydroxy groups at C(24) and C(32), one oxo group at C(22), two unsatura-

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