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 glucosylated 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.

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 Streptomyces 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...
g/l, soybean flour defatted 10 g/l, corns- 
tea 5 g/l in MV7 salt solution, pH 6). The 
composition of the salt solution MV7 was: 
\( \text{NH}_4\text{NO}_3 2 \text{g/l} , \text{Na}_2\text{HPO}_4 \times 2 \text{H}_2\text{O} 1.75 \text{g/l} , \text{K}_2\text{HPO}_4 0.6 \text{g/l} , \text{MgSO}_4 \times 7 \text{H}_2\text{O} 0.2 \text{g/l} , \text{CaCl}_2 \times 2 \text{H}_2\text{O} 0.01 \text{g/l} , \text{FeSO}_4 \times 7 \text{H}_2\text{O} 0.001 \text{g/l} , \) trace-element solution 1 ml/l. The trace- 
element solution was composed of 20 mg/l of each of \( \text{Na}_2\text{MoO}_4 \times 2 \text{H}_2\text{O} , \text{Na}_2\text{B}_4\text{O}_7 \times 10\text{H}_2\text{O} , \text{ZnSO}_4 \times 7\text{H}_2\text{O} , \text{MnSO}_4 \times 7\text{H}_2\text{O} , \text{CuSO}_4 \times 5\text{H}_2\text{O} . \) 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 
derefer to sterile conditions with solutions of 
glucose (10%) and NaOH (1%). 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 occurring peaks under the fol-
lowing conditions: column LiChroCart 
125-4, LiChrosphere 100 RP-8, 5 \( \mu \)m 
(Merck, Darmstadt); elution with a linear 
gradient from 20 to 66% MeCN in 3 \text{mm}
\( \text{H}_2\text{PO}_4/\text{KOH} , \text{pH} 3 \), in 15 min; UV detection 
at 254 nm. For the detection of metab-
olites of 1, the new peaks were then ana-
lyzed by HPLC-MS.

The metabolite 2 from strain DSM 875 
was fermentatively produced on prepara-
tive scale as described above, but with 
orbital shaking at 180 rpm and using ten 1-
1 L Erlenmeyer flasks filled with 200 ml of 
nedium NLI48, 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. Af-
solvent removal under reduced pres-
nure, the product was purified in two steps 
of preparative chromatography on silica 
gel, using a mixture of \( \text{CH}_2\text{Cl}_2 \) and MeOH 
in the ratio of 8:2 (vlv) as the eluent.

**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 eluant was introduced di-
rectly into the ion source of a LCT time-of-
flight mass spectrometer (Micromass, 
Manchester). The ionization technique em-
ployed 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 
Streptomyces and 25 filamentous fungi 
known for their ability to perform hydrox-
ylations, were exposed to CGP 62706 (1).

Seven of these strains had been described 
particularly to hydroxylate the indole nu-
cleus 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, Cunning-
hamella echinulata var. echinulata ATCC 
29244) 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, 
dominated by an in vitro screening 
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 
attracted to the mycelium, it could be sep-
parated easily with the biomass by centri-
figation (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 1H and 13C-shifts and the 
couple constants of the glucose moiety 
confirmed the structure of the 4'-O-meth-
ylglycerol (Fig. 2). The shifts were ob-
tained from an HSQC and a COSY experi-
ment in (D\_6)DMSO (referred to DMSO 
= 2.5 ppm for 1H and 40 ppm for 13C) and 
are listed below:

\[
\begin{align*}
H\text–C(1') & : 5.00; C\text–C(1') : 100.5; H\text–C(2') : 
3.31; C\text–C(2') : 73.5; H\text–C(3') : 3.43; C\text–C(3') : 
76.7; H\text–C(4') : 3.08; C\text–C(4') : 79.1; H\text–C(5') : 
3.40; C\text–C(5') : 76.0; H\text–C(6') : 3.65/3.51; 
C\text–C(6') : 60.3; C\text–C(6') : 3.46; C : 60.0; 
H\text–C(2') : 5.38; H\text–C(2') : 5.25; H\text–C(3') : 
4.70. \text{Coupling constants: } J_{H-C(1')-1'-(C(2'))} = 7.9 \text{ Hz.}
\end{align*}
\]

**Conclusions and Discussion**

None of the strains under investiga-
tion, not even the indole-alkaloid-hydrox-
ylating strains mentioned above, were able
to produce detectable amounts of a CGP 62706 metabolite hydroxylated at the indole moiety. The system with \textit{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, \textit{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-\beta-glucosides of other structural types of aglycons has already been reported for the identical species \textit{Beauveria bassiana} ATCC 7159 (= DSM 1344, formerly \textit{Bassalia sulphurescens} or \textit{Sporotrichum sulphurescens}) [5-7]. In contrast, \textit{Rhizopus colinus} is reported to perform the formation of non-methylated \beta-glucosides [5].

We gratefully thank Mrs. A. Vargas and N. Cardozo Lima-Hofmann for technical assistance, Dr. K. Laumen for help purifying the glucoside, and Dr. P. Traxler for supplying CGP 62706 (1).

\begin{itemize}
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\end{itemize}

1. Introduction

FK-506 (1) is a relatively new macrolide immunosuppressant [1][2], reported to be 100 times more effective \textit{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 \textit{Streptomyces tsukubaensis} fermentation and was originally isolated by the Fujisawa Pharmaceutical Company. FR-900520 [4][5], a closely related macrolide, is produced by \textit{Streptomyces hygroscopicus} subsp. \textit{yakushimae}nis and was later shown to be identical with ascomycin (2) [6–10], an antifungal compound produced by \textit{Streptomyces hygroscopicus} var. \textit{ascomycetius}, which was discovered by Bristol-Myers & Co. in 1960. FK-506 and ascomycin are useful in preventing host rejection of organ transplants, \textit{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 unsaturation...