Benesudon, a New Antibiotic Fungal Metabolite from Cultures of *Mollisia benesuada* (Tul.) Phill

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A novel metabolite, benesudon, possessing antibacterial, antifungal, cytotoxic and phytotoxic activities, was isolated from submerged cultures of the ascomycete *Mollisia benesuada*. Benesudon contains a reactive α-methyleneketone moiety which is believed to be responsible for its antibiotic activities, it reacts with cysteine and the reaction products are devoid of biological activities. Benesudon is the first secondary metabolite described from *Mollisia benesuada*, and its structure was determined by spectroscopic techniques.

Mollisia benesuada is not a common fungus[1], and no secondary metabolites have so far been described from this species. In a screening of fungal cultures obtained from fruit bodies of ascomycetes for antimicrobial activity, extracts of the culture filtrate of *M. benesuada* strongly inhibited the growth of bacteria and fungi. In addition, the extract also exhibited cytotoxic and phytotoxic activities. Thin layer chromatography followed by extraction of the zone containing the antimicrobial activity revealed that only one compound was responsible for all activities. This report describes the producing organism, the production of the active principle benesudon, the determination of its structure by spectroscopic techniques, and its biological activities.

Materials and Methods

General

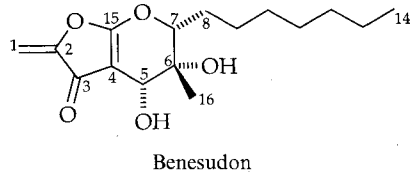
Materials used for preparative HPLC were obtained from Jasco. Preparative HPLC was performed with a Jasco MD910 equipped with a diode array detector. Analytical HPLC was carried out on Hewlett-Packard HP 1090 Type II equipped with a diode array detector. UV spectra were obtained with a Perkin Elmer λ_{16}, and IR spectra with a Bruker IFS 48. The optical rotation was measured with a Perkin Elmer 1541 polarimeter with a cell path of 10 cm. Mass spectra were recorded with a Jeol JMS-SX102 spectrometer, and ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded at 320 K in CD_3OD with a Bruker ARX 500 spectrometer with an inverse 5 mm probe equipped with a shielded gradient coil. The solvent signals at 3.31 ppm in the ^{1}H NMR spectrum and at 49.15 ppm in the ^{13}C NMR spectrum were taken as reference. COSY, HMQC and HMBC experiments were performed with gradient enhancements using sine shaped gradient pulses, and for the 2D heteronuclear correlation spectroscopy the refocusing delays were optimised for $^1J_{CH}=145$ Hz and $^2J_{CH}=10$ Hz. The raw data were transformed and the spectra were evaluated with the standard Bruker UXNMR software (rev. 941001).

Producing Organism

Mycelial cultures of *M. benesuada* were obtained from ascopores. The fruiting bodies (apothecia) of strain A26-93, growing on a dead branch of *Alnus*, were collected in the vicinity of Kaiserslautern, Germany, in autumn 1993. A voucher specimen of the fungus is deposited in the herbarium of the department Biotechnology, University of Kaiserslautern. The culture is kept on YMG agar containing (g/liter): glucose 4, malt extract 10, yeast extract 4, agar 20.

Fermentation

From a well grown agar culture (YMG agar) six agar pieces each measuring one cm^2 were cut out and used to inoculate a 5-liter Erlenmeyer flask containing 2 liters of corn meal medium composed of g/liter: glucose 20, corn meal 10, KH_2PO_4 1.5, KCl 0.5, $Mg_2SO_4 \cdot 7H_2O$



Benesudon

0.5, $NaNO_3$ 0.5. Alternatively, 5% of a well grown submerge culture were used as inoculum. The pH 6.3 was adjusted with HCl. The flasks were incubated on a rotary shaker (120 rpm) at 22°C. Fermentations were stopped after 380 hours, when the content of the active compound, measured by HPLC analysis, had reached a plateau. During fermentations, aliquots of the culture fluid (50 ml) were extracted twice with ethyl acetate. The combined extracts were dried with Na_2SO_4 and concentrated *in vacuo* (40°C). The oily residue was dissolved in 1 ml of methanol and this solution was used to determine the antimicrobial activities against *Nematospora coryli*, *Paecilomyces variotii* and *Mucor miehei*. For HPLC analysis 10 μl of the methanolic solution were used.

Isolation of Benesudon

The culture fluid from four 5-liter flasks (8 liters) was applied onto Mitsubishi HP 21 resin. The column (4 × 40 cm) was washed with 2 liters of water. Bioactivity was followed with *Paecilomyces variotii* as test organism. The bioactive compound was eluted with 2 liters of acetone. The acetone was evaporated *in vacuo* to an aqueous residue, which was extracted twice with ethyl acetate. After evaporation of the solvent from the combined ethyl acetate extracts, 780 mg of an oily crude product were obtained. Chromatography on silica gel 60 (25~40 μm; column size 150 × 25 mm) in cylohexane-EtOAc (3:1) yielded 175 mg of intermediate product. From this intermediate product, 51 mg of benesudon were obtained by HPLC (LiChrosorb, 7 μm; 250 × 25 mm; flow rate 5 ml/minute) with a water-methanol gradient (0~40 minutes: 0~30%; 40~90 minutes: 30~100%) followed by HPLC on LiChrogel PS1 (7 μm; 250 × 25 mm; flow rate 5 ml/minute) in 2-propanol. The retention time for benesudon in this system was 26 minutes.

Benesudon

Benesudon was obtained as a colourless oil. $[\alpha]_D^{22}$ −129° (*c* 0.7 in $CHCl_3$). UV (MeOH) λ_{max} (ε): 234 (9,300) and 305 (3,600). IR (KBr): 3400, 2930, 1695, 1595, 1470, 1305 and 1045 cm^{-1}. ^1H NMR (500 MHz), δ, multiplicity, *J* (Hz): 5.51, d, $J_{1a\sim1b'}$=3.0, 1-Ha; 5.24, d, $J_{1a\sim1b}$=3.0, 1-Hb; 4.46, ddd, $J_{5\sim7}$=1.5, $J_{7\sim8a}$=11.4, $J_{7\sim8b}$=2, 7-H; 4.23, d, $J_{5\sim7}$=1.5, 5-H; 2.03, dddd, $J_{7\sim8a}$=11.4, $J_{8a\sim8b}$=15.1, $J_{8a\sim9a}$=9.1, $J_{8a\sim9b}$=4.9, 8-Ha; 1.76, dddd, $J_{7\sim8b}$=2.2, $J_{8a\sim8b}$=15.1, $J_{8b\sim9a}$=6.2, $J_{8b\sim9b}$=9.7, 8-Hb; 1.57, m, 9-Ha; 1.43, m, 9-Hb; 1.36, s, 16-H_3; 1.32, m, 10-H_2, 11-H_2, 12-H_2 and 13-H_2; 0.90, t, $J_{13\sim14}$=7.1, 14-H_3. ^{13}C NMR (125 MHz), δ: 183.0 C-3; 179.5 C-15; 155.2 C-2; 97.1 C-1; 94.5 C-4; 93.1 C-7; 72.8 C-6; 67.2 C-5; 33.0 C-12; 30.4 C-8; 30.2 C-10; 30.2 C-11; 27.9 C-9; 23.7 C-13; 20.7 C-16; 14.4 C-14. MS (EI, 70 eV), *m/z*: 296 (M^+, 1%), 278 (2%), 222 (9%), 220 (15%), 205 (8%), 156 (33%), 96 (21%), 71 (100%). MS (CI, NH_3) *m/z*: 314 ($M+NH_4^+$, 31%), 297 ($M+H^+$, 100%).

Biological Assays

Antimicrobial activity was determined in the serial dilution assay or the plate diffusion assay as described previously[2]. Tests for cytotoxicity towards L 1210 cells (ATCC CCL 219), RBL-1 cells (ATCC CRL 1378), BHK 21 cells (ATCC CCL 10) and B16-F1 cells (ATCC CRL 6323) was determined as reported by ZAPF *et al.*[3]. Inhibition of growth of germinated seeds of *Setaria italica* and *Lepidium sativum* was tested as described by ANKE *et al.*[2]. Nematicidal activity was measured as described by STADLER *et al.*[4].

Results and Discussion

Taxonomy of the Producing Organism

The identification of species of genera belonging to the Helotiales, of which *Mollisia* (Fr.) Karst. is one of the largest, is difficult, as they are poorly described in the literature. The fruiting bodies of strain A26-93, collected on a dead twig of *Alnus incana*, belongs to the above mentioned group of ascomycetes. The apothecia, which are often distorted, are erumpent, normally between 0.5 and 1.5 mm in diameter, and grow in clusters. The receptaculum, as the hymenum, is light grey, towards the base more or less brown. The asci are iodine positive, 55~70 × 5.5~7 μm. The paraphyses are filiform, septate and are often branched in the upper half. The collection fits best into *Mollisia benesuada* (Tul.) Phill. (syn. *Pyrenopeziza benesuada*) (Tul.) Gremmen sensu[5], a member of the Dermataceae. *M. benesuada* seems to be a rare but widely spread species in temperate regions. It has been reported from France[6], Germany[7], Great Britain[8] and the U.S.A.[9]. Besides on *A. incana* it is also found on *A. glutinosa*, *Quercus* and *Salix caprea*[1].

Fermentation

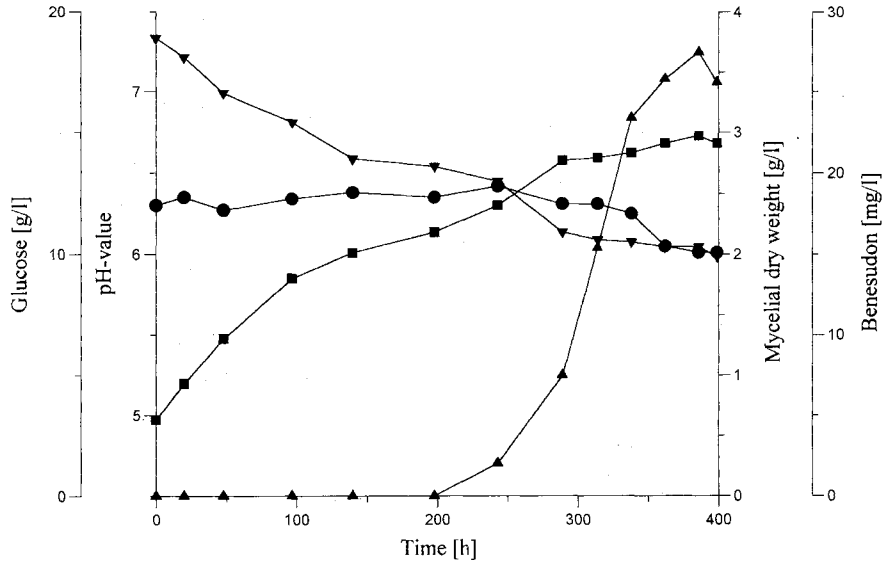
A typical fermentation diagram of *Mollisia benesuada* in corn meal medium is decipted in Fig. 1. Benesudon was only produced in flask cultures but not in 20-liter fermenters. The production was lower in YMG medium than in corn meal medium. The fermentation was terminated after 380 hours when mycelial dry weight and the concentration of benesudon started to decrease. The mycelia did not contain antibiotic activity and were discarded. The active compound was isolated by activity-guided fractionation, as described in materials and methods section.

Structure Determination

Although the molecular ion of benesudon in the EI mass spectrum was small, the CI spectrum clearly showed that its molecular weight is 296, which is consistent with the elemental composition $C_{16}H_{24}O_5$ (unsaturation

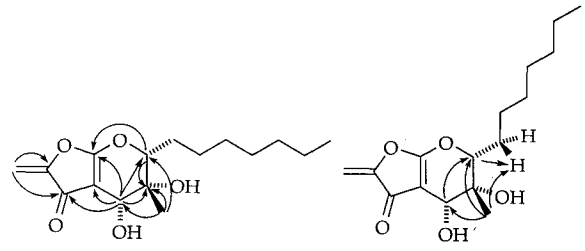
Fig. 1. Time course of *Mollisia benesuada* A26-93 fermentation in 2 liters scale and production of benesudon.

● pH-value, ■ mycelial dry weight, ▼ content of glucose, ▲ content of benesudon.



index 5). NMR measurements were initially made in $CDCl_3$ and C_6D_6 at room temperature, but it was not possible to obtain the resolution required for the unambiguous assignment of all signals. Satisfactory resolution was obtained in CD_3OD at a slightly elevated temperature (50°C). The ^1H and ^{13}C NMR data are given in materials and methods, and the correlations observed in the HMBC and NOESY spectra are summarised in Fig. 2. Benesudon contains several unusual structural features, and it was difficult from only the ^{13}C NMR data to determine the number of carbonyl groups and double bonds. However, the presence of an exomethylene function was suggested by the triplet at 97 ppm with the coupling constant 165 Hz (C-1). 1-H_2 give HMBC correlations to two carbons, C-2 and C-3, indicating that C-2 is bonded to a heteroatom. The C-6 methyl protons (16-H_3), which appear as a singlet in the ^1H NMR spectrum, give HMBC correlations to the three oxygenated carbons C-5, C-6 and C-7, and the heptyl group is attached to C-7. 7-H consequently gives HMBC correlations to C-6 and C-8, but also to C-15 which according to its chemical shift must be an enolic carbon. The HMBC correlations from 5-H to C-3, C-4 and C-15 close the dihydropyran ring, and the structure of benesudon can only be as shown in Fig. 2. The relative stereochemistry suggested for benesudon is supported by the correlations observed in the NOESY spectrum. The correlation between 5-H and 7-H, and those between 16-H_3 and 5-H as well as 7-H suggest that these protons are positioned on the same side of the ring with the

Fig. 2. Pertinent HMBC (left) and NOESY (right) correlations observed with benesudon.



5-hydroxy group and the 7-heptyl group equatorial. Both 7-H and 16-H_3 give strong NOESY correlations to 8-Hb, but not to 8-Ha, and as $J_{7\sim8a}$ is large (11.4 Hz) while $J_{7\sim8b}$ is small the dihedral angle between 7-H and 8-Ha must be close to 180°. The most stable conformation of benesudon in methanol should therefore be as depicted to the right in Fig. 2. Benesudon shares some of its functionalities with the *Penicillium* metabolites carolic acid, dihydrocarolic acid and terrestric acid, and the ^{13}C NMR data are in agreement with those previously reported[10].

Biological Properties

The antimicrobial spectrum of benesudon is shown in Table 1. Gram-negative and -positive bacteria are equally sensitive, the MICs ranging from 2.5 to 10 μg/ml in nutrient broth. The highest antifungal activity was observed against *Paecilomyces variotii* at 1 μg/ml. Benesudon showed cytotoxic activity against all cell lines

Table 1. Antimicrobial activity of benesudon in the serial dilution assay.

Organism	MIC (μg/ml)	Incubation temperature
Bacteria (Difco Nutrient Broth):		
Bacillus brevis	5	37°C
Bacillus subtilis	2.5	37°C
Escherichia coli	2.5	37°C
Salmonella typhimurium TA98	5	37°C
Sarcina lutea	10	37°C
Streptomyces bikiniensis DSM 40581	10	37°C
Yeasts (YMG medium):		
Nematospora coryli	5	27°C
Rhodutorula glutinis	10	27°C
Fungi (YMG medium):		
Mucor miehei	2.5	37°C
Penicillium notatum	2.5	27°C
Paecilomyces variotii	1	37°C

Size of inoculum: 1×10^5 cells or spores/ml. Activity was evaluated after 24 hours of incubation.

tested. Proliferation of the cells was reduced to 10% between 1 and 2 μg/ml. The compound was phytotoxic against *Setaria italica* and *Lepidium sativum*. The germination of seeds and growth of seedlings was inhibited at 10 μg/disk. *Oryza sativa* was slightly less senstive, 50% inhibition or germination was observed at 20 μg/ml. Nematicidal activity was observed against *Caenorhabditis elegans* at 50 μg/ml while *Meloidogyne incognita* was insensitive up to 100 μg/ml. Upon incubation with L-cysteine, benesudon immediatly lost its biological activities and ninhydrin positive adducts were detected on TLC plates, suggesting that the activity of the compound is due to the reactivity of the α-methyl-eneketone moiety.

Several metabolites with varying biological activities have been reported from *Mollisia* species. Mollisin (8-(dichloroacetyl)-5-hydroxy-2,7-dimethyl-1,4-naphthoquinone) with activity towards phytopathogenic fungi has been isolated from *Mollisia caesia* and *Mollisia fellens*[11]. In co-culture with the phytopathogenic fungus *Heterobasidion annosum*, *Mollisia* spec. produced higher amounts of mollisin. The authors suggested that the fungus may protect plants from fungal superinfections[11]. It remains to be investigated if this also applies to benesudon, *e.g.* whether *Mollisia benesuada* can protect the trees it grows on. Several compounds produced by *Mollisia ventosa*, KS-504a, KS-504b, KS-504d and KS-504e, are inhibitors of the Ca^{2+} and calmodulin-dependent phosphodiesterase[12].

Table 2. Cytotoxicity of benesudon towards various cell lines.

Cell line	IC_{90} (μg/ml)	Medium[3]
L1210 (ATCC CLL 219)	2	F12-medium
RBL (ATCC CRL 1378)	1	RPMI
BHK21 (ATCC CLL 10)	2	DMEM
B16-F1 (ATCC CRL 6323)	1	DMEM

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