Isolation and Biological Activity of New Norhirsutanes from *Creolophus cirrhatus*

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Z. Naturforsch. **63c**, 203–206 (2008); received November 15/December 21, 2007

Five new norhirsutanes, named creolophins A–E, and complicatic acid were isolated from the culture broth of the rare tooth fungus *Creolophus cirrhatus* by solvent extraction, silica gel column chromatography and HPLC. In addition, neocreolophin, a complex dimerization product, was formed as an artefact during purification. The structures were elucidated by spectroscopic methods and are published in a separate paper. Two of the metabolites showed moderate antibacterial, antifungal and cytotoxic activities.

Key words: Creolophus cirrhatus, Creolophins A–E, Neocreolophin

Introduction

Sesquiterpenoids with a triquinane ring system belong to the more common secondary metabolites of basidiomycetes (Abraham, 2001). Some exhibit interesting biological activities (Lorenzen and Anke, 1998). For coriolin from *Coriolus consors* and its derivatives antibacterial, antitumour, and immunostimulatory effects have been described (Takeuchi *et al.*, 1969; Ishizuka *et al.*, 1972). Hypnophilin, pleurotellol, and pleurotellic acid isolated from *Pleurotellus hypnophilus* and complicatic acid, first described from *Stereum complicatum* (Melows *et al.*, 1973) and later from *Galerina cephalotricha*, inhibit the growth of some bacteria and fungi (Kupka *et al.*, 1981). In most cases the biological activities are due to the presence of a highly reactive Michael acceptor, *e.g.* an α-methylene ketone or lactone group. This paper describes the isolation, purification, and biological activity of five new linear triquinanes which we have named creolophins A–E, their presumed precursor complicatic acid and a dimer (neocreolophin) formed during purification. The elucidation of all structures (Fig. 1) is described in a separate paper (Opatz *et al.*, 2007).

Materials and Methods

Producing organism

Creolophus cirrhatus strain 02040 was isolated from a fruiting body collected in France. Herbarium specimen and mycelial cultures are deposited in the culture collection of the Institute for Biotechnology and Drug Research IBWF e. V., Kaiserslautern, Germany. For maintenance on agar slants the fungus was grown on YMG agar (g/l): yeast extract (4), glucose (10), malt extract (10); the pH value was adjusted to 5.5 before autoclaving. Solid media contained 2% of agar.

Fermentation and isolation of creolophins A–D

Creolophus cirrhatus was cultivated in BAF medium described previously by Singer (1986) with slight modifications. This medium contains (per liter H_2O): yeast extract (1 g), maltose (20 g), glucose (10 g), peptone (2 g), KH_2PO_4 (0.5 g), $MgSO_4 \cdot 7H_2O$ (1 g), $FeCl_3$ (10 mg), $ZnSO_4 \cdot 7H_2O$ (1.78 mg) and $CaCl_2$ (60 mg). Fermentation was carried out in 1 l batches in 2 l Erlenmeyer flasks at 22–24 °C on a rotary shaker (120 rpm). 250 ml of a well-grown culture in the same medium were used as inoculum. When the glucose was used up after 24 d, the culture fluid was separated from the mycelia by filtration. The culture fluid (630 ml) was extracted with an equal volume of ethyl acetate and the organic phase concentrated *in vacuo*. The crude extract (1 g) was applied onto a silica gel column (11 × 2.2 cm; Merck 60, 0.063 ~ 0.2 mm). Elution with ethyl acetate resulted in 550 mg of an intermediate product. Pre-

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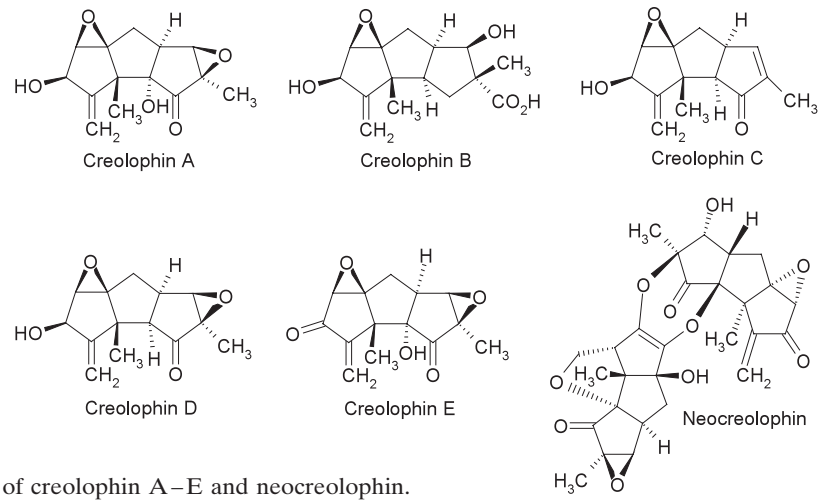

Fig. 1. Structures of creolophin A–E and neocreolophin.

parative HPLC (Merck Lichrosorb® RP18, 7 μm; column 25 × 250 mm; flow 20 ml/min; isocratic H_2O/MeCN 80:20) yielded 109 mg creolophin A and B in a 4.5:1 mixture (R_t = 11 min), 42 mg creolophin C (R_t = 15 min) and 136 mg creolophin D (R_t = 17 min).

Fermentation and isolation of neocreolophin and creolophin E

For the isolation of neocreolophin and creolophin E, *Creolophus cirrhatus* was grown in YMG medium in a 20 l fermenter (Biolafitte-C6). A well-grown culture (250 ml) in the same medium was used as inoculum. The fermentation was carried out with stirring (120 rpm) and aeration (3 l/min) at 22–24 °C. When the glucose was used up 13 d after inoculation, the culture fluid (15.5 l) was separated from the mycelia by filtration and extracted with an equal volume of ethyl acetate. The crude extract (4.8 g) obtained after concentration *in vacuo* was applied onto a silica gel column (17 × 2.2 cm; Merck 60, 0.063 ~ 0.2 mm). Elution with cyclohexane/ethyl acetate (1:1) yielded 1.3 g of an enriched product. Preparative HPLC (Macherey-Nagel, Nucleosil C 18, 7 μm; column 250 × 21 mm; flow 20 ml/min; gradient H_2O/MeCN 20:80 to 50:50 in 50 min) resulted in 401 mg of a product with the main components creolophin E and complicatic acid (R_t = 15.5 ~ 17.5 min). 150.6 mg complicatic acid (R_t = 6.1 min) and 129.8 mg of creolophin E (R_t = 6.9 min) were isolated by subsequent preparative HPLC (as above but isocratic H_2O/MeCN 60:40). Complicatic acid was identified by HPLC/MS (Kupka *et al.*, 1981). In an earlier attempt to purify creolophin E, small amounts of neocreolophin were detected as well. It turned out that this compound was an artefact formed by thermally induced dimerization of creolophin E. Thus, moderate heating of creolophin E (53.6 mg) to 50 °C *in vacuo* for 5 h yielded a mixture of compounds, from which 5.7 mg of neocreolophin (R_t = 11.9 min) were isolated through isocratic preparative HPLC as above.

Biological assays

The minimal inhibitory concentrations (MIC) were determined by the conventional serial dilution method with slight modifications (Ericsson and Sherris, 1971).

The suspension cell lines Colo-320 (DSMZ ACC 144), Jurkat (ATCC TIB 152), and L1210 (ATCC CCI 219) were grown in RPMI 1640 medium (GIBCO, BRL) and the monolayer cell lines MCF-7 (ATCC HTB-22) and MDA-MB-231 (ATCC HTB-26) in DMEM medium (GIBCO, BRL). The media were supplemented with 10% fetal calf serum (GIBCO, BLR), 65 μg/ml of penicillin G, and 100 μg/ml of streptomycin sulfate. The cells were maintained at 37 °C in 5% CO_2 and a humidity of 98%. $1 - 5 \cdot 10^5$ cells/ml were incubated in 96 microtiter well plates with or without the compounds. The viability of the suspension cell lines was measured with a XTT cell proliferation assay and the viability of the monolayer cell lines was measured by Giemsa staining after 72 h as described previously (Schoettler *et al.*, 2006).

Preparation of cysteine adducts

Creolophin E and neocreolophin (50 μg each) were incubated with equimolar amounts of L-cysteine in sterile water for 10 min and then assayed for antibacterial activity and cytotoxicity.

Results and Discussion

The minimal inhibitory concentrations of creolophin E and neocreolophin are shown in Table I. The creolophins A–D (A and B tested as 4.5:1 mixture) showed no antibacterial or antifungal activity at concentrations up to 50 μg/ml. While the antifungal activity of creolophin E – with the exception of the yeast *N. coryli* – is marginal, the antibiotic activity against Gram-positive (*C. insidiosum*, *B. subtilis*, *M. luteus*) and Gram-negative (*E. coli*) bacteria is quite pronounced. At higher concentrations some antibacterial activity could be observed by the dimeric neocreolophin.

The creolophins A–D showed no cytotoxic effects up to concentrations of 25 μg/ml. As shown in Table II, creolophin E and neocreolophin exhibit strong cytotoxic activities. Like complicatic acid, creolophin E and neocreolophin differ from the other creolophins by the presence of a highly reactive exomethylene ketone which readily reacts with nucleophiles. As has been observed for complicatic acid (Kupka *et al.*, 1981) addition of cysteine leads to a complete loss of antimicrobial and cytotoxic activities.

Table I. Antibacterial and antifungal activities of creolophin E and neocreolophin in the serial dilution assay.

	MIC [μg/ml][a]	
	Creolophin E	Neocreolophin
Fungi		
Absidia glauca (+)	50 z	> 50
Absidia glauca (–)	> 50	> 50
Ascochyta pisi	> 50	> 50
Aspergillus ochraceus	> 50	> 50
Fusarium fujikuroi	> 50	> 50
Fusarium oxysporum	> 50	> 50
Mucor miehei	50 z	> 50
Paecilomyces variotii	> 50	> 50
Penicillium islandicum	50 s	> 50
Penicillium notatum	> 50	> 50
Zygorhynchus moelleri	> 50	> 50
Nematospora coryli	5 s	> 50
Bacteria		
Enterobacter dissolvens	50 s	> 50
Escherichia coli K12	5 z	25 s
Pseudomonas fluoreszens	25 z	50 s
Arthrobacter citreus	10 s	50 s
Bacillus brevis	10 z	25 s
Bacillus subtilis	5 z	10 s
Corynebacterium insidiosum	2 s	50 z
Micrococcus luteus	5 z	25 s
Mycobacterium phlei	10 z	25 z

[a] z, fungicidal/bactericidal; s, fungi-/bacteriostatic.

Table II. Cytotoxic activity of creolophin E and neocreolophin.

Cell line	IC_{50} [μg/ml]	
	Creolophin E	Neocreolophin
Colo-320	1	5
Jurkat	0.75	2
L1210	1	5
MCF-7	7	15
MDA-MB-231	2	3

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