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OR-1 – a Mixture of Esters of Glyceric Acid Produced by *Penicillium funiculosum* and Its Antitrypsin Activity

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ABSTRACT. A mixture of related metabolites (denoted OR-1) was isolated from the fermentation broth of *Penicillium funiculosum* together with mitorubrinic acid. Structurally OR-1 is glyceric acid esterified with C_{14}–C_{18} fatty acids. Steady-state studies revealed that OR-1 behaved like an uncompetitive trypsin inhibitor with K_i 17.6 μmol/L.

Various strains of *Penicillium funiculosum* synthesized an array of metabolites, *e.g.*, SQ 30,957 (Singh *et al.* 1986), funiculosin (Ando *et al.* 1969), the antiinflammatory agent 11-deacetoxywortmanin (Wiesenger *et al.* 1974), pigments mitorubrinic acid, mitorubrin, mitorubrinol (Locci *et al.* 1967), or the aromatase inhibitor TAN-931 (Takafumi *et al.* 1991).

Proteinase inhibitors are very effective in suppressing carcinogenesis in many different *in vivo* and *in vitro* systems (Kennedy 1994). While several types of proteinase inhibitors are able to prevent the carcinogenic process, the majority of them inhibit chymotrypsin or chymotrypsin-like proteinases (Kennedy 1998). Here we report the production, isolation and identification of metabolite OR-1 produced by *P. funiculosum* and the study of its antitrypsin activity.

MATERIALS AND METHODS

Strain and cultivation. The producing organism, *Penicillium funiculosum* strain 992, was derived from *Penicillium funiculosum* CCM F-8080 (*Czech Collection of Microorganisms*, Brno) by active selection after UV radiation.

The production strain was maintained on Cd_i medium composed of (in g/L) sucrose 30, $NaNO_3$ 3, KH_2PO_4 1, KCl 0.5, $FeSO_4 \cdot 7H_2O$ 0.01, $MgSO_4 \cdot 7H_2O$ 0.5, agar 20 (pH 6.3). The strain was incubated at 28 °C for 10 d.

Spore suspension from one slant was transferred into 500-mL flasks containing 100 mL of Cd_i medium without agar at pH 6.2, which was cultivated on a rotary shaker (frequency 50 Hz, 28 °C, 2 d).

The production medium consisted of (in g/L) glucose 80, $(NH_4)_2HPO_4$ 2, KH_2PO_4 1, $MgSO_4 \cdot 7H_2O$ 0.5, L-phenylalanine 1; pH was adjusted to 6.2. This medium in a 500-mL flask was seeded with 10 % of inoculum and cultivated on a rotary shaker (frequency 50 Hz, 28 °C, 8 d).

OR-1 was isolated from the culture medium after 8 d of cultivation. Medium (5 L) was extracted with ethyl acetate (2 L), the organic layer was concentrated *in vacuo* and the residue (4 g) was re-extracted into *n*-heptane. This solution was concentrated and the residue was chromatographed on a column packed with silica gel (Silpearl, *Kavalier*, Czechia) eluted with chloroform–methanol (9 : 1). The separation was monitored by TLC on Silufol UV-366 plates (*Kavalier*) in chloroform–methanol (9 : 1); detection at 366 nm. Fractions containing OR-1 (R_F 0.95) were combined and evaporated to dryness. The 100 mg of orange oily mixture named OR-1 was thus prepared.

Spectral data of OR-1. IR (KBr, $\tilde{\nu}$, cm^{-1}): 3007, 2924, 2853, 1746, 1456, 1353, 1163, 1029, 721, 574. ^{1}H-NMR ($CDCl_3$, δ, ppm): 0.81 (6H, m), 1.26 (40H, br s), 1.60 (4H, br s), 2.02 (4H, m), 2.29 (3H, m), 2.77 (1H, t), 4.14 (1H, dd, 12.0, 6.0 Hz), 4.30 (1H, dd, 12.0, 4.5 Hz), 5.26 (1H, dd, 6.0, 4.5 Hz), 5.36 (4H, m). ^{13}C-NMR ($CDCl_3$, δ, ppm): 14.09, 14.14 (CH_3), 22.60–34.2 (CH_2), 62.12 (CH_2), 66.91 (CH), 127.92–130.2 (CH), 172.86, 173.27, 173.30 (C).

Effect of OR-1 on trypsin activity was determined according to Gaertner *et al.* (1992). Trypsin (EC 3.4.21.4) and the substrate N^{α}-benzoyl-DL-arginine-4-nitroanilide (BAPNA) were purchased from *Sigma* (St. Louis, USA). Stock solutions of the inhibitor were prepared in dimethyl sulfoxide.

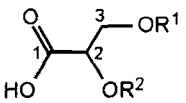
Antifungal activity was determined by the agar dilution method. The extract of culture broth (5 mL of culture broth extracted with 2 mL ethyl acetate) after a 8-d cultivation was tested for their antimicrobial acti-

vity. Paper discs (Whatman no. 2; 7 mm diameter) containing the test substance were placed on a plate with Sabouraud agar medium. After incubation (25 °C, 3 d) the diameters of inhibition zones were measured.

RESULTS AND DISCUSSION

During the experiments with production of mitorubrinic acid (Proksa *et al.* 1997) by *P. funiculosum* formation of an orange lipophilic metabolite OR-1 on a medium containing L-phenylalanine was observed. IR spectra of OR-1 purified by column chromatography contained absorption bands characteristic for aliphatic esters. According to NMR (^1H and ^{13}C, COSY, HETCOR and SINEPT experiments) OR-1 contained glyceric acid (Table I) esterified with fatty acids, which were identified by gas chromatography (Shimizu *et al.* 1989) after methanolysis (Table II). Backed by these data we assume that OR-1 represents a mixture of glyceric acid esterified with C_{14}–C_{18} fatty acids.

Table I. NMR data of glyceric acid portion of OR-1

Formula	Position	C δ, ppm	H δ, ppm	J_{H-H} Hz
 R^1, R^2 = acyl of C_{14}–C_{18}	1	173.3 C	–	–
	2	68.9 CH	5.26 dd	4.5, 6.0
	3	64.7 CH_2	4.30 dd	12.0, 4.5
			4.14 dd	12.0, 6.0

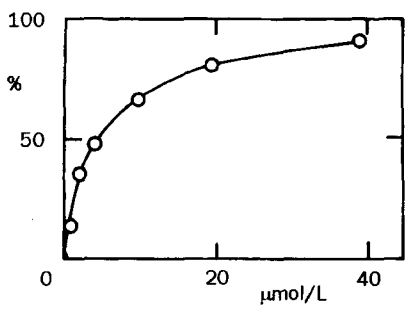


Fig. 1. Inhibition (%) of trypsin activity with OR-1 at different concentration of the inhibitor (μmol/L) in Tris-HCl buffer (pH 7.6, 25 °C); concentration of trypsin was 25 mg/L; concentration of substrate (BAPNA) was 1 mmol/L.

Table II. Fatty acids present in the structure of OR-1[a]

No.	t_r min	Concentration %	Fatty acid
1	3.88	0.37	14:0
2	4.93	0.61	14:1
3	6.36	23.90	16:0
4	7.11	0.96	16:1
5	8.32	0.68	17:0
6	9.29	0.23	17:1
7	11.07	7.23	18:0
8	12.18	37.02	18:1
9	14.55	29.0	18:2

[a]Determined by GC after methanolysis; GC column packed with 15 % DEGJ, carrier gas N_2, 90 kPa, 186 °C; t_r – retention time.

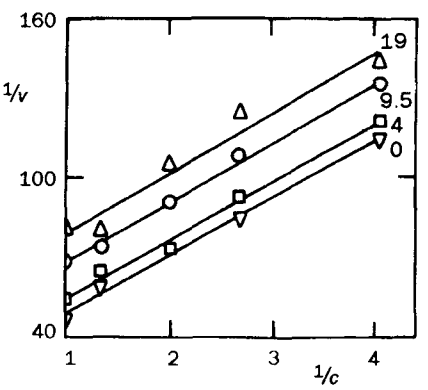


Fig. 2. Lineweaver–Burk plot of inhibition of trypsin by OR-1 (0, 4, 9.5, 19 – μmol/L, concentration of OR-1); concentration of trypsin was 25 mg/L; pH 7.6; 25 °C; $1/c$ – reciprocal BAPNA concentration (L/mmol); $1/v$ – reciprocal reaction velocity (min/ΔA_{410}).

The average molar mass 630 g/mol of OR-1 was used in the experiments with inhibition of trypsin activity. This inhibition was concentration-dependent (Fig. 1) with IC_{50} = 2.68 mg/L (4.25 μmol/L). Generally most trypsin inhibitors are proteins in nature except the free fatty acids reported by Wang *et al.* (1975). They described oleic, linoleic and linolenic acids isolated from soybean after fermentation by *Rhizopus oligosporus* as the first non-peptidic trypsin inhibitors. However, not only free fatty acids diminish the activity of trypsin, but also their esters with glyceric acid as confirmed in our experiments.

It is evident from the Lineweaver–Burk plot that the inhibition of trypsin activity by OR-1 has an uncompetitive character (Fig. 2) with the inhibition constant K_i 17.6 μmol/L.

No antifungal activity of ethyl acetate extract against *Cryptococcus neoformans* CCY 17-1-6, *Trichosporon cutaneum* CCY 30-5-10, *Torulopsis glabrata* and *Candida albicans* CCY 29-3-32 was detected at concentration of 20 μL per disc.

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