(+)-10α-Hydroxy-4-muurolen-3-one, a New Inhibitor of Leukotriene Biosynthesis from a *Favolaschia* Species. Comparison with Other Sesquiterpenes

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Z. Naturforsch. **51c,** 487–492 (1996); received February 14/March 25, 1996

Favolaschia Species, Basidiomycetes, Leukotriene Biosynthesis-Inhibitors, (+)-10α-Hydroxy-4-muurolen-3-one

A new inhibitor of leukotriene biosynthesis, (+)-10α-hydroxy-4-muurolen-3-one (**1**), was isolated from fermentations of *Favolaschia* sp. 87129. Its structure was established by spectroscopic methods. The compound exhibited no antifungal or antibacterial activities.

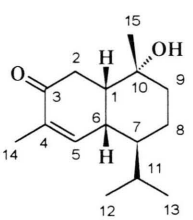
The effects of **1** on leukotriene biosynthesis were compared with (+)-T-cadinol, (-)-3-oxo-T-cadinol, and (+)-3α-hydroxy-T-cadinol, three related sesquiterpenes.

Introduction

The leukotrienes are potent biological mediators derived from arachidonic acid metabolism. They are generated via the 5-lipoxygenase pathway. Leukotriene B_4, a dihydroxy derivative, causes adhesion and chemotactic movement of leukocytes, enzyme release, and generation of superoxide in neutrophils. The sulfidopeptide leukotrienes C_4, D_4, and E_4, known as "slow reacting substance of anaphylaxis" (SRS-A) induce bronchoconstriction, stimulate mucus production and increase vascular permeability (Samuelsson *et al.*, 1987). Due to these effects the leukotrienes have been implicated as important mediators of inflammation and immediate hypersensitivity reactions in diseases like inflammatory bowel disease, psoriasis, and asthma (Ford-Hutchinson *et al.*, 1994; O'Byrne, 1994; Salmon and Garland, 1991).

During our screening for inhibitors of leukotriene biosynthesis, cultures of an ethiopian *Favolaschia* species were found to contain an active metabolite. This compound was isolated and elucidation of its structure revealed (+)-10α-hydroxy-4-muurolen-3-one (**1**), a new sesquiterpene of the cadinane type. In this paper the production, isolation, biological activities and structure elucidation of **1** will be reported. The effects of three other sesquiterpenes, (+)-T-cadinol (**2**), (-)-3-oxo-T-cadinol (**3**), and (+)-3α-hydroxy-T-cadinol (**4**), on leukotriene biosynthesis will be described.

Besides **1** *Favolaschia* sp. 87129 was found to produce strobilurins A and F, oudemansin A, 9-methoxystrobilurins A and K, as well as favolon, a new antifungal sterol (Zapf *et al.*, 1995a; Anke *et al.*, 1995).



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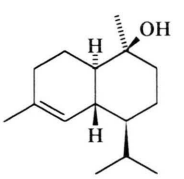

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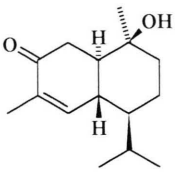

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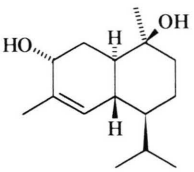

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Material and Methods

General experimental procedures

Spectral data were recorded on the following instruments: ^{1}H and ^{13}C NMR, Bruker AMXR-300; EI-MS, Finnigan MAT 95 Q; FT-IR, Bruker IFS 48; UV, Perkin-Elmer lambda 16; CD, Jobin Yvon CNRS Roussel-Jouan Dichrographe III. The optical rotation was recorded with a Perkin-Elmer 214 polarimeter. The melting point was determined with a Büchi 510 apparatus and is uncorrected. For TLC, aluminium foils coated with silica gel Merck 60 F_{254} were used.

Favolaschia sp. strain 87129

The small bright yellow fruiting bodies of *Favolaschia* sp. 87129 were found growing on wood in a forest close to Kolobo, Ethiopia. The specimen showed all characteristics of the genus (Singer, 1974), the species, however, could not be identified. Mycelial cultures were obtained from spore prints of fruiting bodies. The strain is deposited in the culture collection of the LB Biotechnologie, University of Kaiserslautern.

Favolaschia sp. 87129 was cultivated and maintained on YMG agar composed of (g/liter): yeast extract 4, malt extract 10, glucose 4, and agar 15, pH 5.5.

Fermentation

A well grown seed culture (500 ml) in YMG was used to inoculate 20 liters of YMG in a Biolafitte C-6 fermentation apparatus. The fermentor was incubated at 22°C with an aeration of 3 liters air/minute and agitation (120 rpm). After four days 10 liters of this culture were used as inoculum for 100 liters of the same medium in a Biostat U fermentation apparatus (Braun + Diessel, temperature: 27°C, aeration: 15 liters air/minute, stirrer speed: 150 rpm).

Isolation of (+)-10α-hydroxy-4-muurolen-3-one (**1**)

After seven days of fermentation, the culture fluid (90 liters) was separated from the mycelia and **1** was extracted from the broth by adsorption onto Mitsubishi Diaion HP21 resin. The resin was washed with H_2O and **1** was eluted from the column with 5 liters of acetone. After evaporation of the acetone the active compound was extracted from the residual aqueous phase with three times 600 ml of ethyl acetate. The crude product (5.35 g) obtained after removal of the solvent was further purified by chromatography on silica gel (Merck 60; elution with cyclohexane – EtOAc 1 : 1). 556 mg of an enriched product were obtained. Final purification was achieved by preparative HPLC (Merck LiChrosorb Diol 7 µm; column size: 2.5 x 25 cm; flow rate 5 ml/minute) using a cyclohexane – *tert*-butyl methyl ether (*t*-BME) gradient: 10% *t*-BME (30 minutes); 10~20% *t*-BME (10 minutes); 20% *t*-BME (10 minutes); 20~30% *t*-BME (10 minutes); 30% *t*-BME (15 minutes); 30~40% *t*-BME (20 minutes); 40% *t*-BME (85 minutes). **1** was eluted after 127 minutes.

Isolation of (+)-T-cadinol (**2**), *(-)-3-oxo-T-cadinol* (**3**), *and (+)-3α-hydroxy-T-cadinol* (**4**)

The compounds were isolated as described previously from the same sample of scented myrrh (the resin of the plant *Commiphora guidottii* Chiov. *Burseraceae*) obtained from the Somalian Frankincense and Gums Trading Branch that has been used in similar investigations (Claeson *et al.*, 1991). Their structures were confirmed by the comparison of their spectroscopic data with published data (Claeson *et al.*, 1991; Lin *et al.*, 1974).

Physico-chemical properties of (+)-10α-Hydroxy-4-muurolen-3-one (**1**)

Colorless crystals: MP 114.5 ~ 115.5°C; Rf 0.39 (toluene-acetone 7:3); $[\alpha]_D^{24}$ + 96.29° (*c* 0.18, $CHCl_3$); UV λ_{max}^{MeOH} nm (log ε) 240 (3.98); CD λ_{max}^{MeCN} nm (Δε) 212 (+4.51), 233 (+5.18), 338 (+0.62), 349 (+0.62); IR (KBr) cm^{-1} 3316, 2961, 2942, 2909, 2896, 1677, 1637, 1454, 1381, 1368, 1338, 1302, 1243, 1118; ^{1}H and ^{13}C NMR, Table 1;

EI-MS (direct inlet, 90°C) *m/z* (relative intensity%) 236.1764 (70, M^+, calcd. for $C_{15}H_{24}O_2$ 236.1776), 218 (17), 193 (71), 175 (100), 165 (26), 147 (10), 135 (29), 110 (26), 109 (67), 85 (25), 69 (20).

Tests for Biological Activities

Inhibition of Ca^{2+} induced leukotriene C_4 synthesis in RBL-1 cells (ATCC CRL 1378): The cells were grown in Dulbecco's modified Eagle medium containing 10% fetal calf serum at 37°C in a 5% CO_2 atmosphere. Cells were harvested by centrifugation (400 x *g*, 10 min), washed once with phosphate buffered saline (PBS), and resuspended in PBS with Ca^{2+}(0.9 mM), Mg^{2+} (0.5 mM) and 0.1% glucose at 4 x 10^6 cells per ml. 225 µl of the cell suspension were incubated with the test compounds dissolved in DMSO or ethanol (final concentration < 0.2% in a final volume of 250 µl) for 15 min. Then 2 µM of the calcium ionophor A23187 (Calbiochem) was added. After 10 min the reaction was terminated by addition of 250 µl of ice-cold PBS without Mg^{2+} and Ca^{2+} and centrifugation (1000 x *g*, 10 min). The concentration of leukotriene C_4 in the supernatant was determined by radioimmunoassay according to the manufacturer's instructions (Amersham – Buchler, Braunschweig).

Inhibition of Ca^{2+} induced leukotriene C_4 (LTC_4) and prostaglandin E_2 (PGE_2) synthesis in human peripheral blood leukocytes (PBL): Anticoagulated human venous blood was diluted with an equal volume of PBS buffer and centrifuged (800 x *g*, 25°C) for 10 min. The cell pellet was suspended to the original volume and the PBL were isolated by density gradient centrifugation by layering the diluted blood onto lymphocyte separation medium (Boehringer Mannheim). After centrifugation (400 x *g*) for 30 min the PBL at the interface were collected and washed once. The cell pellet was resuspended in PBS buffer with Ca^{2+}(0.9 mM) and Mg^{2+} (0.5 mM) to a titre of 1 x 10^7 cells/ml. 200 µl of the cell suspension were incubated with the test compounds dissolved in 0.2% DMSO for 25 min at 25°C. After 5 min at 37°C 25 µl A23187 solution (final concentration 0.25 µM) were added and the incubation continued for 10 min at 37°C. The reaction was terminated by addition of 250 µl of ice-cold PBS buffer and centrifugation (1000 x *g*, 10 min) at 4°C. The concentration of LTC_4 (Du Pont) and PGE_2 (Advanced Magnetics) was determined by radioimmunoassay according to the manufacturer's instructions.

5-Lipoxygenase-assay: The 5-lipoxygenase activity was determined in the cytosolic 13000 x *g* fraction of broken RBL-1 cells according to Hook *et al.* (1990) with modifications: The content of 5-hydroxyeicosatetraenoic acid was quantified by reversed phase HPLC using a 0.1% aqueous H_3PO_4/acetonitrile gradient.

Tests for cytotoxic activity on L1210, HL60, BHK21, and HeLaS3 cells were carried out as described previously (Zapf *et al.*, 1995b). RBL-1 cells were grown in D-MEM medium supplemented with 10% fetal calf serum and were treated as described in Zapf *et al.* (1995b).

The antimicrobial spectra were determined in the serial dilution assay.

Results and Discussion

Production of (+)-10α-hydroxy-4-muurolen-3-one (**1**)

Favolaschia sp. 87129 grew well in submerged cultures. During fermentation, the fungus consumed glucose and maltose simultaneously within seven days. Cultures were harvested when the free glucose in the medium was consumed.

Following the isolation procedure described above, 134 mg of **1** were obtained from 90 liters of culture filtrate.

Structural Elucidation

According to the HR-MS the antibiotic has the molecular formula $C_{15}H_{24}O_2$. A strong band at 1677 cm^{-1} in the IR spectrum (KBr) and signals at δ_C 198.78, 147.94, and 134.40 in the ^{13}C NMR spectrum indicate the presence of an α,β-unsaturated ketone. All NMR data are listed in Table I. The carbon resonances were assigned with the aid of a DEPT spectrum to three methylenes, four methyls and four methine groups. The oxygen-bearing carbon atom at δ_C 70.32 must be quarternary.

The ^1H NMR spectrum shows signals for one olefinic proton, two tertiary methyls, one isopropyl group, and nine additional protons in the aliphatic

Table I. ^1H (300 MHz) and ^{13}C (75.5 MHz) NMR chemical shifts of **1**.

Carbon No.	^{13}C chemical shifts (ppm)[a]		^1H chemical shifts (ppm)[b]	^1H chemical shifts (ppm)[c]
C-1	44.41		2.13 (1H, ddd, J = 13.8, 4.2, 4.2 Hz)	1.87
C-2	34.28	H_α	2.41 (1H, dd, J = 17.3, 13.8 Hz)	2.39
		H_β	2.61 (1H, dd, J = 17.3, 4.2 Hz)	2.85
C-3	198.78			
C-4	134.40			
C-5	147.94		6.77 (1H, dq, J = 5.9, 1.3 Hz)	6.44
C-6	36.75		2.28 (1H, ddd, J = 10.5, 5.9, 4.2 Hz)	1.95
C-7	42.24		~1.45 (1H, m)	~1.30
C-8	20.60	H_a	~1.59 (1H, m)	~1.34
		H_b	1.13 (1H, dm, J = 12.6 Hz)	0.89
C-9	33.52	H_a	~1.50 (1H, m)	~1.42
		H_b	~1.50 (1H, m)	~1.27
C-10	70.32		3.42 (OH, s, br)	3.11
C-11	26.35		1.82 (1H, dqq, J = 13.8, 6.9, 6.9 Hz)	1.69
C-12	20.40		0.86 (3H, d, J = 6.9 Hz)	0.80
C-13	14.84		0.83 (3H, d, J = 6.9 Hz)	0.72
C-14	14.94		1.72 (3H, d, J = 1.3 Hz)	1.94
C-15	26.63		1.25 (3H, s)	1.05

[a] Chemical shifts with reference to solvent $CDCl_3$ (δ_C 76.0 ppm).
[b] Chemical shifts with reference to $CDCl_3$ (δ_H 7.24 ppm).
[c] Chemical shifts with reference to solvent [D_6]benzene (δ_H 7.35 ppm).

region. The spin-spin connectivities between these signals were established by 2D ^1H-^1H COSY experiments. Thus, the olefinic proton at δ_H 6.77 exhibits vicinal coupling with the proton at δ_H 2.28, which is coupled to two other protons at δ_H 2.13 and 1.45. The proton at δ_H 2.13 is further connected to two geminal protons, which resonate at δ_H 2.41 and 2.61. In the ^1H-^1H COSY spectrum a multiplet at δ_H 1.45 shows cross peaks to the methine proton of an isopropyl group at δ_H 1.82 and to two geminal protons at δ_H 1.50 and 1.13 which form part of a CH_2CH_2-moiety. Since the signal at δ_H 1.45 is further coupled to the proton at δ_H 2.28, cadinane structure **1** can be proposed for the metabolite.

Structure **1** is supported by the EI-MS, in which consecutive loss of water and isopropyl leads to the base peak at m/z 175. Initial loss of the isopropyl group forms a prominent ion at m/z 193, which undergoes loss of CO to give fragment m/z 165.

The relative stereochemistry at the ring junction of **1** has been established to be *cis* by NOE experiments (Fig. 1). This is in accord with the angular coupling constant $^3J_{1\text{-H},6\text{-H}}$ = 4.2 Hz. The *trans*-diaxial position of 6-H and 7-H is indicated by the 10.5-Hz coupling between these protons. The methyl group at C-10 shows NOE correlations to

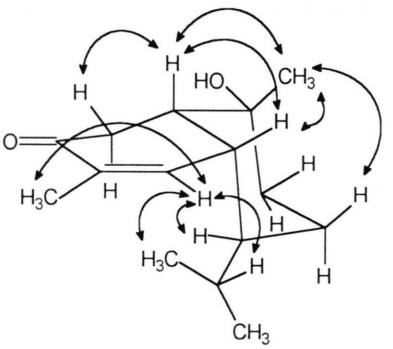

Fig. 1. Selected NOESY correlations for **1**.

1-H as well as to 6-H which confirms the *cis*-relationship between these substituents and leads to the relative configuration shown in formula **1**. According to Borg-Karlson *et al.* (1981) the new metabolite should be named (+)-10α-hydroxy-4-muurolen-3-one. Its absolute configuration could not be determined.

A stereomer of **1**, 10β-hydroxy-4-muurolen-3-one, has been isolated from the wood of *Taiwania cryptomerioides* (Kuo *et al.*, 1969).

Biological Properties

(+)-10α-hydroxy-4-muurolen-3-one (**1**), (+)-T-cadinol (**2**), (-)-3-oxo-T-cadinol (**3**), and (+)-3α-hydroxy-T-cadinol (**4**) showed no or only weak cytotoxic activities (Table II).

The inhibitory effects of the four tested sesquiterpenes on the leukotriene biosynthesis in RBL-1 cells are shown in Table III. (+)-T-Cadinol and (-)-3-oxo-T-cadinol showed the highest activity with IC_{50} values between 2 and 5 μg/ml (8.5 and 22.5 μM). For (+)-10α-hydroxy-4-muurolen-3-one and (+)-3α-hydroxy-T-cadinol the IC_{50} was determined between 5 and 10 μg/ml (21.2 and 42.4 μM) and > 10 μg/ml (> 42 μM) respectively.

Table II. Cytotoxic activities of (+)-10α-hydroxy-4-muurolen- 3-one (**1**), (+)-T-cadinol (**2**), (-)-3-oxo-T-cadinol (**3**), and (+)-3α-hydroxy-T-cadinol (**4**).

Cell line	IC_{50} [μg/ml]			
	1	**2**	**3**	**4**
RBL-1	>50	50	>50	>50
L1210	>50	n.t.	n.t.	n.t.
HL60	>50	50	n.t.	n.t.
BHK21	>50	25	n.t.	n.t.
HeLaS3	>50	25–50	n.t.	n.t.

n.t. = not tested

Table III. Inhibition of leukotriene C_4 biosynthesis in RBL-1 cells and human peripheral blood leukocytes by (+)-10α- hydroxy-4-muurolen-3-one (**1**), (+)-T-cadinol (**2**), (-)-3-oxo-T- cadinol (**3**), and (+)-3α-hydroxy-T-cadinol (**4**).

Compound	Inhibiton of leukotriene C_4 synthesis (%)					
	RBL-1 Concentration [μg/ml]			Human PBL Concentration [μg/ml]		
	2	5	10	2	5	10
1	–	27	67	21	29	35
2	46	63	80	26	64	77
3	53	68	82	–	24	49
4	–	–	35	–	8	17

– = no inhibition

The results of the inhibition of leukotriene biosynthesis in RBL-1 cells were compared with those obtained in human PBL (Table III). The data indicate that both the substitution at C-3 of **1** – **4** and the configuration (muurolen or cadinol derivatives) have only moderate influence on the inhibitory effects.

In order to approach to the cellular target, the influence on PGE_2 synthesis and 5-lipoxygenase was tested as described in the experimental section. For compounds **1** – **4** no inhibition of the cyclooxygenase pathway (PGE_2 synthesis in human PBL) could be detected at 10 μg/ml. At this concentration LTC_4 synthesis was significantly inhibited by **1** – **3** (Table III). The influence of **2** and **3** on the 5-lipoxygenase (5-LOX) of RBL-1 cells was tested in a cell free extract (Hook *et al.*, 1990). No inhibition could be observed at concentrations up to 50 μg/ml. This points to a reaction involved in the activation of 5-LOX (translocation to the membrane or transfer of arachidonic acid from 5-lipoxygenase-activating protein to the enzyme) or a later step in LTC_4 biosynthesis as possible target for **2** and **3**.

In the serial dilution assay no antimicrobial activity could be detected for **1** at 100 μg/ml using *Arthrobacter citreus, Bacillus brevis, Bacillus subtilis, Corynebacterium insidiosum, Micrococcus luteus, Mycobacterium phlei, Streptomyces* sp. ATCC 23836, *Acinetobacter calcoaceticus, Escherichia coli* K12, *Salmonella typhimurium* TA98, *Nadsonia fulvescens, Nematospora coryli, Rhodotorula glutinis, Saccharomyces cerevisiae* S 288 c, *Saccharomyces cerevisiae* is 1, *Fusarium oxysporum, Mucor miehei, Paecilomyces variotii, Penicillium notatum* and *Ustilago nuda* as test organims.

Acknowledgments

The financial support of the Deutsche Forschungsgemeinschaft ist gratefully acknowledged. A.W. was a fellow of the VCI. We thank W. Kinle, Boehringer Mannheim, for technical support.

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