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# Novel Analgesic Triglycerides from Cultures of *Agaricus macrosporus* and Other Basidiomycetes as Selective Inhibitors of Neurolysin

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**Abstract** The agaricoglycerides are a new class of fungal secondary metabolites that constitute esters of chlorinated 4-hydroxy benzoic acid and glycerol. They are produced in cultures of the edible mushroom, *Agaricus macrosporus*, and several other basidiomycetes of the genera *Agaricus*, *Hypholoma*, *Psathyrella* and *Stropharia*. The main active principle, agaricoglyceride A, showed strong activities against neurolysin, a protease involved in the regulation of dynorphin and neurotensin metabolism ($IC_{50}$=200 nM), and even exhibited moderate analgesic *in vivo* activities in an *in vivo* model. Agaricoglyceride monoacetates ($IC_{50}$=50 nM) showed even stronger *in vitro* activities. Several further co-metabolites with weaker or lacking bioactivities were also obtained and characterized. Among those were further agaricoglyceride derivatives, as well as further chlorinated phenol derivatives such as the new compound, agaricic ester. The characteristics of the producer organisms, the isolation of bioactive metabolites from cultures of *A. macrosporus*, their biological activities, and preliminary results on their occurrence in basidiomycetes, are described.

**Keywords** agaricoglycerides, pain, fermentation, fungi, *in vivo* activity

## Introduction

Neurolysin (EC 3.4.24.16) is a zinc metalloprotease that inactivates particular biologically active peptides, such as neurotensin and dynorphin A, by specific cleavage [1]. Whereas the kappa-opioid receptor agonist dynorphin A is a well-known and obvious endogenous pain-relieving peptide, neurotensin has been reported to have analgesic properties when applied centrally in animal models [2]. Therefore, neurolysin inhibitors are likely to enhance the analgesic properties of neurotensin and/or dynorphin A by inhibiting cleavage and inactivation of these peptides. Accordingly, selective inhibitors of neurolysin are likely to emphasize the analgesic effects of the aforementioned peptides, which accumulate if their inactivation is prevented. Hence, neurolysin inhibitors appear useful alternatives to complement or substitute therapy with morphine and other opiates in the treatment of severe pain. However, only few specific inhibitors of this target have hitherto been found. Those mostly constituted peptides [3～5], and otherwise there are few examples for non-peptidic inhibitors of this enzyme, such as particular hydroxamates [6]. Therefore, a high throughput screening (HTS) for novel selective non-peptidic neurolysin inhibitors

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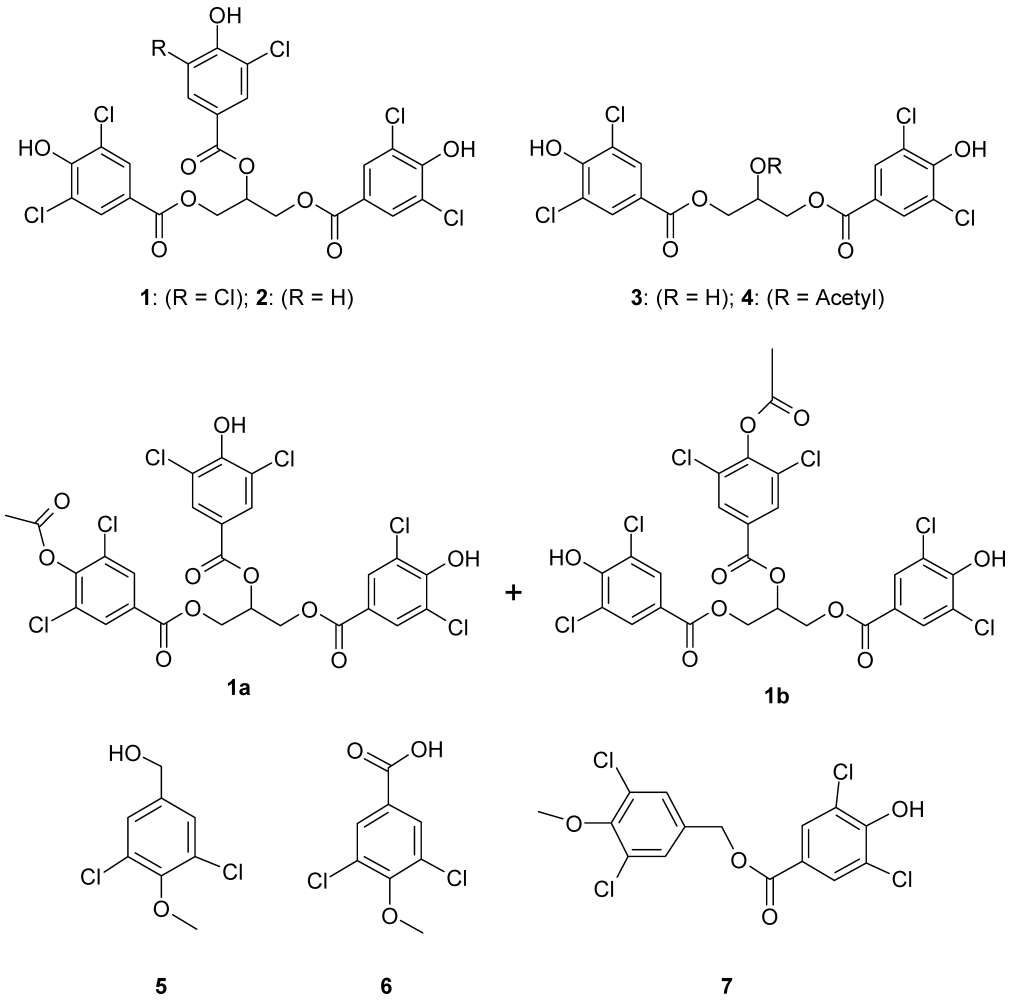


**Fig. 1** Chemical structures of metabolites isolated from *Agaricus macrosporus*.

**1**: Agaricoglyceride A; **2**: Agaricoglyceride B; **1a**/**b**: Monoacetyl-agaricoglycerides A (isolated as inseparable mixture); **3**: Agaricoglyceride C; **4**: Agaricoglyceride D; **5**: DCMB; **6**: 3,5-Dichloro-4-anisic acid; **7**: Agaricic ester.

from microbial crude extracts was carried out, followed by identification of bioactive principles from the most promising hits by bioassay-guided fractionation.

The current paper deals with the discovery of a new class of biologically active natural products from cultures of edible mushrooms and other basidiomycetes. Their production, isolation, and biological properties are described here, while their physicochemical parameters, structure elucidation, derivatization and total synthesis have been compiled in a patent application [7]. Further details on their structure elucidation will be reported concurrently.

## Results and Discussion

### Isolation and Biological Activities of Agaricoglycerides (1~4) and Their Co-metabolites (5~7)

Agaricoglyceride A (**1**) was identified as the main active principle of the crude extract of *A. macrosporus* and later produced in gram scale as described in the Experimental for intensified biological evaluation, including a derivatization and *in vivo* studies. Chromatographic separation by preparative HPLC (Fig. 2) yielded several further congeners (structures see Fig. 1), of which only the mixture of monoacetyl agaricoglycerides (**1a**/**b**) was more active than the parent compound (Table 1). Since the monoacetyl derivatives of compound **1** were detected in methanolic crude extracts prepared from growing cultures, they constitute original natural products rather than

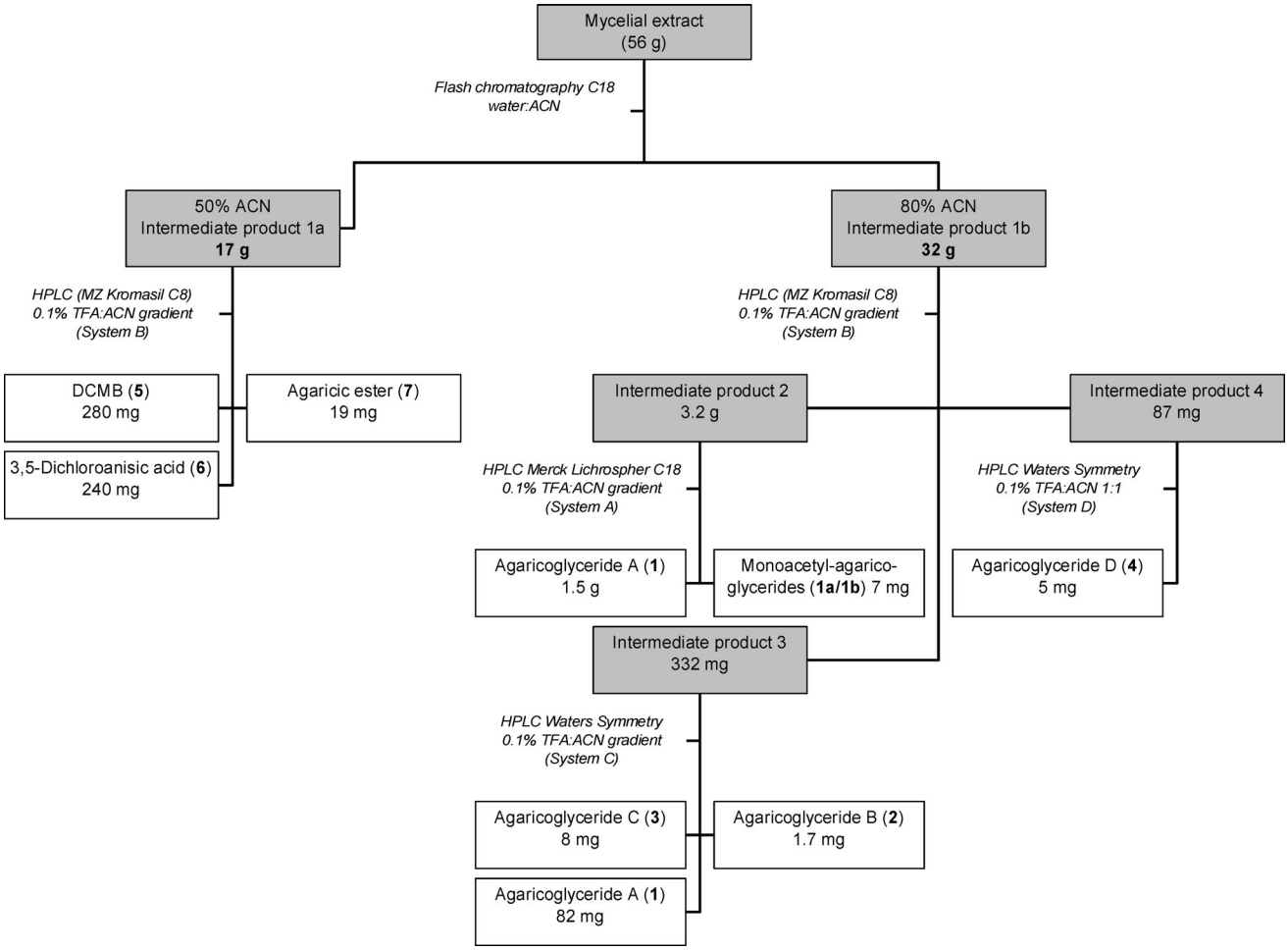


**Fig. 2** Scheme illustrating the isolation of secondary metabolites from the mycelial crude extract of *Agaricus macrosporus* (200 liters scale; Q6/2 medium)

isolation artifacts. Their biological activities were confirmed for the mixture **1a**/**b** , which were later on also prepared using the synthetic scheme depicted in Fig. 3. Synthetic agaricoglyceride A (**1**) was also obtained *via* this route and showed identical biological activities to that of the natural product [7].

Agaricoglyceride B (**2**) still showed activities in the micromolar range. However, despite differing in only one chlorine substitution from the main component, its activity was significantly lower than that of agaricoglyceride A (**1**). Agaricoglycerides C and D (**3**, **4**), as well as the known co-metabolites (**5** and **6**) and the novel agaricic ester (**7**) were found devoid of activity against neurolysin up to 10 $\mu$M.

**Table 1** Biological activities of compounds **1**~**7** against neurolysin and ACE

| Compound | $IC_{50}$ [$\mu$M] against neurolysin | $IC_{50}$ [$\mu$M] against ACE |
|---|---|---|
| **1** | 0.2 | >10 |
| **1a**,**b** | 0.05 | >10 |
| **2** | 1 | >10 |
| **3** | >10 | >10 |
| **4** | >10 | >10 |
| **5** | >10 | >10 |
| **6** | >10 | >10 |
| **7** | >10 | >10 |

### *In vivo* Activity

As demonstrated in Fig. 4, agaricoglyceride A monoacetate (**1b**) showed significant *in vivo* activity in the cold plate model at 10 mg/kg. Agaricoglyceride A (**1**) was also active in about the same concentration range, and morphine caused a similar effect, albeit already at a dosage of 3 mg/kg. The results suggest a great analgesic potential for

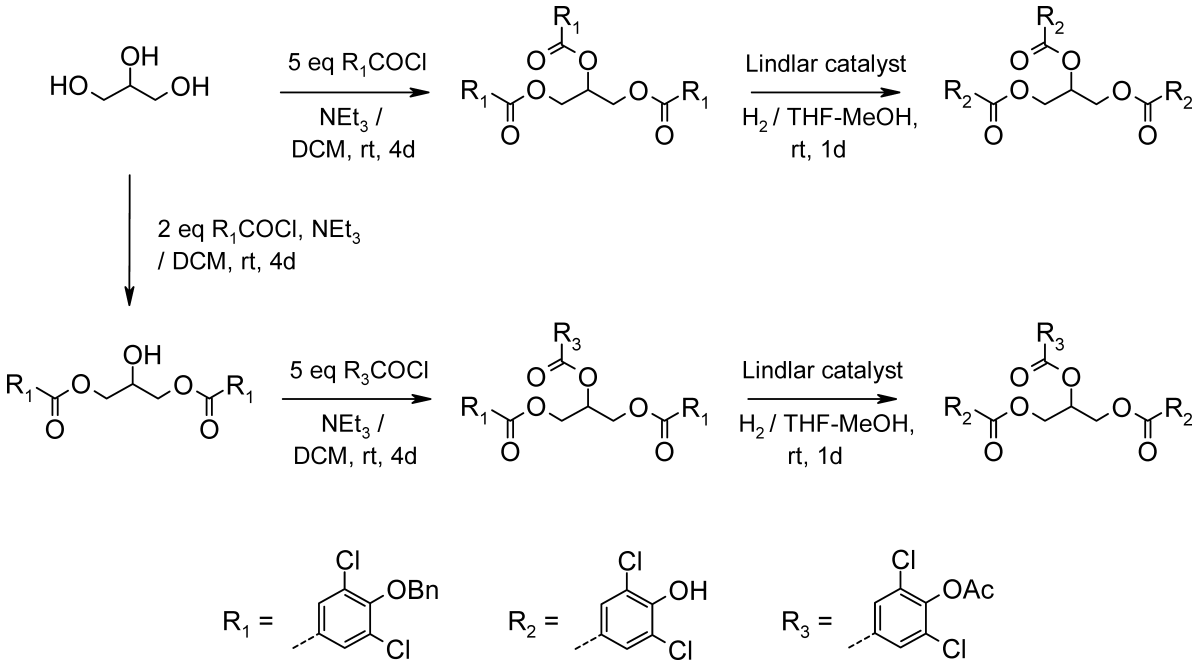


**Fig. 3** General scheme for synthetic preparation of agaricoglycerides. For detailed descriptions, see Ref. 7.

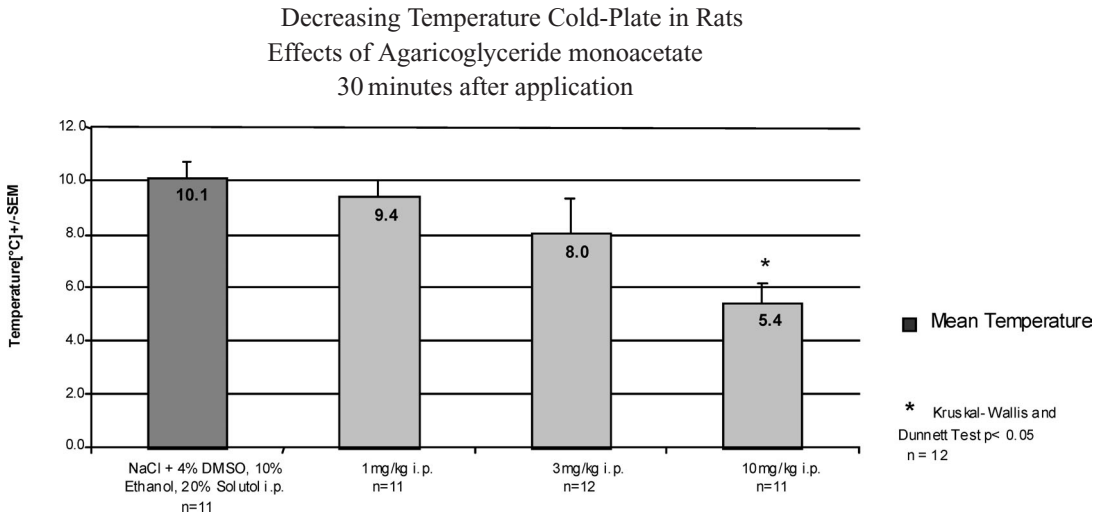


**Fig. 4** *In vivo* analgesic activities of agaricoglyceride A monoacetate (**1a/b**).

agaricoglycerides, albeit their biological activities remain to be optimized and these *in vivo* effects confirmed in other animal models. Interestingly, the agaricoglycerides were devoid of activity in a large number of biochemical and cellular assays, emphasizing their selectivity towards neurolysin. Enzyme classes that were not inhibited by agaricoglycerides included, *e.g.*, trypsin, chymotrypsin, phosphodiesterases 1~6, caspases 1~7, cathepsins B~D, several protein phosphatases, serine/threonin and thyrosine kinases, and matrix metalloproteases. No modulation of adrenergic, glutamate, gamma aminobutyric acid and other receptors was noted up to concentrations of 10 $\mu$M. Furthermore, the pure agaricoglycerides neither showed cytotoxic nor modulatory effects in various reporter cell assays that were conducted concurrently up to concentrations of 10 $\mu$M.

## HPLC Analyses during Fermentation and Optimization of Culture Media

The agaricoglycerides were detected in various

**Table 2** Producers of agaricoglycerides, origin, taxonomy, and (in brackets) maximal production rates for agaricoglyceride A in Q6/2 medium at 300 hours of fermentation. Aside from strains DSM 14593 and DSM 14594, which were subjected to preparative work, all other producers were identified by analytical HPLC of their crude extracts only

| Species | Strain | Origin/collector/isolator [production rates] |
|---|---|---|
| *Agaricus arvensis* Schaeff.:Fr. | MUCL 35030 | leg. C. Decock, 1992, from Warcoing (Belgium)**<br>[2.4 mg/liter] |
| *A. bisporus* (Lange) Imbach* | DSM 14900 | leg. H. Müller, September 1991, from spores of cultivated mushrooms, Wuppertal (Germany)<br>[4.9 mg/liter] |
| *A. bitorquis* (Quél.)Sacc. | DSM 14895 | leg. M. Stadler, STMA 98023, 9. May 1998, from Hamburg (Germany), near paved path of lawn in Volkspark), culture from context of pileus<br>[7.7 mg/liter] |
| | DSM 14894 | leg. M. Stadler, STMA 97106, 9. September 1997, from Wuppertal-Elberfeld,Germany, humus in flowerbed near roadside), culture from context of pileus<br>[2.4 mg/liter] |
| | DSM 14594 | leg. M. Stadler, STMA 97106, 9. September 1997, from Wuppertal-Elberfeld, Germany, humus in flowerbed near roadside), culture from basidiospores<br>[1.9 mg/liter] |
| | MUCL 28516 | leg. G. L. Hennebert, no date, from Kapellen (Belgium)*<br>[2.5 mg/liter] |
| *A. campestris* var. *campestris* L.:Fr. | MUCL 29004 | leg. G. L. Hennebert, 1996, from Louvain-la-Neuve (Belgium), on lawn*<br>[<1 mg/liter] |
| *A. macrosporus* (Moeller & J. Schaeffer) Pilát | MUCL 28242 | leg. G. L. Hennebert, 1993, Bois de Boulaide (Luxembourg)*<br>[6.6 mg/liter] |
| | CBS 585.76 | leg. W. Gams, September 1976, from Empolder near Embrugge (Netherlands)*<br>[5 mg/liter] |
| | DSM 14593 | leg. M. Stadler, STMA 97106, 7. August 1999, from Wuppertal-Elberfeld, Germany, lawn of Bayer Pharma Research Center, culture from context of pileus<br>[14.5 mg/liter] |
| | DSM 14594 | leg. M. Stadler, STMA 97106, 7. August 1999, from Wuppertal-Elberfeld, Germany, lawn of Bayer Pharma Research Center, culture from basidiospores<br>[13 mg/liter] |
| *A. xanthoderma* Genev.* | DSM 14901 | leg. H. Müller, September 1991, from Wuppertal, Germany, culture from basidiospores<br>[<1 mg/liter] |
| *Hypholoma marginatum* (Pers.:Fr.) J. Schröt. | DSM 14898 | leg. M. Stadler, STMA 00034, 11. September 1997, from Schopp, Rheinland-Pfalz, Germany, Finsterbrunnertal, on wood of *Pseudotsuga*, culture from context of pileus<br>[3 mg/liter] |
| *Hypholoma* sp.* | DSM 14902 | leg. R. Seeger, 25.9.1992, as *Hypholoma subviride* (Berk. & M.A. Curtis) Dennis, from the vicinity of Coburg, Germany<br>[<1 mg/liter] |

**Table 2** (Continued)

| Species | Strain | Origin/collector/isolator [production rates] |
|---|---|---|
| *Psathyrella prona* (Fr.) Gillet | DSM 14903 | leg. H. Dörfelt, 3. September 1999, from Neuengönna (Thüringen, Germany) on meadow, culture from basidiospores<br>[<1 mg/liter] |
| *Stropharia rugosoannulata* Farlow ex Murrill | DSM 11373 | no data on collector available**<br>[1.5 mg/liter] |
| *Stropharia squamosa* (Pers.:Fr.) Quél. | DSM 14899 | leg. M. Stadler, STMA 97126, 22.September 1997, near Johanniskreuz/Pfalz, Germany, Burgalbspring<br>[2.3 mg/liter] |

* No herbarium specimen extant to confirm taxonomy; but morphology and secondary metabolism of the culture in agreement with other strains of the respective taxa. ** Further information as to the correspondence of these strains may be retrieved from the catalogues or by contacting the staff of the public collections where these strains are deposited.

**Table 3** Retention times (Rt) of compounds **1**∼**7** upon analytical HPLC

| Compound | Rt (HPLC-UV/Vis) [min.] | Rt (HPLC-MS) [min.] |
|---|---|---|
| **1** (Agaricoglyceride A) | 9.45∼9.53 | 4.65∼4.67 |
| **1a,b** (Monoacetyl agaricoglyceride mixture) | 9.49∼9.57 | 4.72∼4.74 |
| **2** (Agaricoglyceride B) | 9.05∼9.17 | 4.57∼4.58 |
| **3** (Agaricoglyceride C) | 8.7∼8.85 | 4.29∼4.32 |
| **4** (Agaricoglyceride D) | 7.92∼8.01 | 3.88∼3.93 |
| **5** (DCMB) | 7.05∼7.12 | 3.61∼3.65 |
| **6** (Dichloro anisic acid) | 7.8∼7.85 | 3.72∼3.74 |
| **7** (Agaricic ester) | 8.1∼8.15 | 3.95∼3.97 |

Parameters described in Ref. 26 (HPLC-MS) and Ref, 30 (HPLC-UV/Vis), respectively.

basidiomycetes (Table 2) by analytical HPLC profiling as described in the Experimental. The HPLC characteristics of all compounds detected are compiled in Table 3. In case of *A. macrosporus* strains that were chosen for intensified evaluation, similar yields were isolated from shake flasks and stirring fermentors, and the main component, agaricoglyceride A (**1**) was always encountered in substantial quantities in various culture media with different constituents (Table 4). Estimation of agaricoglycerides by HPLC analysis of crude extracts revealed that agaricoglyceride A (**1**) was present after 192 hours in all strains and culture media employed. In MGP and Q6/2, an increase of production was observed at prolonged fermentation times, and these media were chosen for 30 and 200 liter scales. While an analogous fermentation was also successful in MGP medium, the large scale production of agaricoglycerides is here only exemplified for a 200 liter culture in Q6/2 medium. The other agaricoglycerides (**1a**/**b** and **2**∼**4**) were generally only present in traces. HPLC profiling of several related strains (data not shown), regardless whether they belonged to the genera *Agaricus*, *Hypholoma*, *Stropharia* or *Psathyrella*, frequently revealed only DCMB (**5**) and the conceivable acidic precursor of agaricoglycerides, 2,4-dichloro-anisic acid (**6**), but these strains apparently lacked the ability to form glycerol conjugates of the latter compound under the chosen conditions.

### Increase of Production of Agaricoglycerides in *A. macrosporus* by Precursor Feeding

In attempts to increase production of agaricoglycerides, the culture medium of strain DSM 14593 was supplemented with putative precursors as described in the Experimental. As compared to the control (12∼13 mg/liter of **1** in

**Table 4** Estimated yields (HPLC/UV) of agaricoglyceride A (**1**) [mg/liter] in the mycelial extracts of strain DSM 14593 prepared from shake cultures in various culture media and at various stages of fermentation

| Medium | Fermentation time [h] | Estimated prduction rates [mg agaricoglyceride A/liter culture broth] |
|---|---|---|
| MGP medium | 192 | 6.5 |
| | 300* | 13.0 |
| | 360 | 10.4 |
| Q6/2 medium | 96 | 4.0 |
| | 192 | 9.4 |
| | 300* | 14.5 |
| | 360 | 12.9 |
| YMG medium | 192 | 7.6 |
| | 300 | 8.5 |
| Mycorrhiza medium* | 192 | 5.4 |
| ZM/2 medium* | 192 | 5.7 |

* Maximum yields observed upon comparison of daily samples taken in the time range of 96～360 hours.

conventional MGP), agaricoglyceride A (**1**) increased to 20.4～22.6 mg/liter upon supplementation by the synthetic acid (**6**), and to 18.2～19.0 mg/liter in the presence of 4-hydroxybenzoic acid. Since a rather effective total synthesis of agaricoglycerides, and a synthetic approach to related compounds was concurrently elaborated, which appeared more practical for variation of the agaricoglyceride skeleton in attempts to increase bioactivity and establish structure-activity relationships [7], such supplementation experiments were not performed at a larger scale.

**Occurrence of Agaricoglycerides and Chemically Related Compounds in Nature**

The outcome of this study suggests that the production of agaricoglycerides and related metabolites in culture is widespread in the genus *Agaricus* (Agaricaceae). Moreover, several species belonging to other genera and families, such as *Psathyrella* (Coprinaceae), *Hypholoma* and *Stropharia* (Strophariaceae) are able to synthesize these aromatic triglycerides. Actually, chlorinated aromatics are well-known from basidiomycetes, including the genera *Hypholoma* and *Stropharia* [8～10] from which agaricoglycerides are reported here. *Agaricus* spp. themselves have been shown to yield in culture several unique biological active metabolites such as blazeisporols [11,12]. Even strobilurins were once obtained from an African *Agaricus* species [13]. Moreover, the cultivated mushroom, *A. bisporus*, was shown to produce the antibiotic, drosophilin A (*i.e.*, *p*-methoxy tetrachlorophenol) [14]. In contrast, fruitbodies of *Agaricus* spp. are known to yield various aromatic heterocycles, including, *e.g.*, the toxins of the poisonous *A. xanthoderma*, which have, however, so far not been found in cultures of these fungi [15]. A concurrent study on all materials designated STMA in Table 2, employing analytical HPLC of methanolic fruitbody extracts revealed that agaricoglycerides were not present in any of the fruitbodies of those fungi that were shown to produce them in culture.

Whereas simple chlorinated aromatics appear to be widespread especially in cultures of those basidiomycetes inhabiting humus or wood [16,17], there are relatively few examples of metabolites consisting of several aromatic chlorinated carboxylic acids. Only recently, the antiviral cytonic acids were found in cultures of the endophytic fungus, *Cytonaema* sp., which constitute esters of tridepsides [18]. The dichloro-anisic acid (**6**) is also a formal constituent of kaitocephalin, a glutamate receptor inhibitor from cultures of *Eupenicillium shearii* [19]. Furthermore, this building block also occurs in azaphilones of the falconensine type, which are metabolites of *Emericella falconensis* [20], as well as in the depsidones of some lichens [21]. Thielavins from ascomyceteous fungi of the genera *Thielavia* and *Chaetomium* constitute polyesters of three aromatic building blocks and were reported to act as glucose 6-phosphatase inhibitors [22]. They have also been reported previously to exhibit various other bioactivities (see summary in the latter reference). As glycerol esters of aliphatic fatty acids are essential constituents of the membranes of most prokaryotic and eukaryotic organisms, it is surprising that no aromatic di- and triesters of glycerol have so far been reported from natural sources. Remote structural similarities to the agaricoglycerides are only to be found in few natural products such as the kelletinins. These compounds were isolated from, *e.g.*, mollusks of the genus *Kelletia* [23]. They are aromatic polyesters of benzoic acid and butane tetrol (erythrol) and were reported to possess antiviral and antimicrobial activities. In addition, they show cytostatic effects, which are probably due to inhibition of eukaryotic RNA-polymerase alpha [24].

## Experimental

### General

If not indicated otherwise, all chemicals were provided by Sigma Aldrich (Deisenhofen, Germany), while media ingredients, solvents for chromatography and spectroscopy were obtained from Merck Eurolabs (Darmstadt, Germany).

Culture Media

For isolation of basidiomycete cultures, YMG[25], or Mycorrhiza medium (malt extract 0.8%, glucose 0.7%, asparagine 0.05%, casein hydrolysate 0.1%, $KH_2PO_4$ 0.05%, $MgSO_4$ 0.05%, yeast extract 0.1%, ad 1 liter deionized water, pH 5.05, for solid media 1.5% Difco Bacto Agar were added before autoclaving) were employed. To avoid bacterial contamination upon preparation of primary cultures, the media were supplemented by antibiotics, adding sterile aqueous solutions of streptomycin sulfate and penicillin G (Sigma Aldrich, Deisenhofen, Germany), immediately after sterilization and adjustment of temperature to 55°C in a water bath, to final concentrations of each 125 mg/liter for both antibiotics. For screening and fermentation, ZM/2 medium [26], MGP medium [27], and Q6/2 medium (glucose 2.5 %, glycerol 1 %, cotton seed meal 0.5 %, $CaCO_3$ 0.05 %, ad 1 liter tap water, no pH adjustment) were also employed besides liquid YMG and Mycorrhiza media. All media used for propagation of the fungi on either agar plates or shake cultures were sterilized at 121°C for 20 minutes, while media in fermentors were autoclaved *in situ* with steam for 60 minutes.

### Origin, Isolation and Taxonomy of Producer Strains

All strains studied were derived from basidiocarps of agaricoid basidiomycetes and are listed in Table 2. Those from materials designated STMA were identified by microscopic examination of the teleomorphs according to Refs. [28, 29]. These cultures were obtained from either spore prints or mycelial plugs taken from the context of the fruitbody tissue under sterile conditions upon plating the fungal materials on YMG medium or Mycorrhiza medium containing antibiotics (see above). Microscopic examination of the cultures showed the same microscopic and macroscopic features as those of related strains from public collections (which even showed similar secondary metabolite profiles by HPLC). Voucher specimens, which showed the characteristics of the genera and species, are kept in the herbarium of M. S. (Wuppertal, Germany). Some additional strains from the Bayer culture collection, as well as other ones from public collections were studied for comparison. All cultures are deposited with either CBS (Utrecht, Netherlands), DSMZ (Braunschweig, Germany), or MUCL (Louvain-la-Neuve, Belgium) as stated in Table 2. They are also maintained at the BHC Research Center, Wuppertal, Germany, on YMG agar slants and in 10% glycerol under liquid $N_2$.

### Fermentation

For screening and small scale production of desired compounds, all strains were propagated in 1 liter Erlenmeyer flasks containing 300 ml of the above culture media. The flasks were inoculated using ten agar disks (*ca.* 1 cm diameter) from well-grown mycelial cultures (9～12 days old) on YMG agar. Submerged fermentations were carried out on a rotary shaker at 23°C and 140 rpm. After 96 hours, daily samples were taken, extracted and analyzed by HPLC as described below. Large scale fermentations were carried out in stirring fermentors, using either MGP or Q6/2 media. 600 ml of well-grown YMG shake cultures prepared as stated above were used as inoculum for 30 liter fermentors, while the culture of a 30 liter fermentor grown for ten days served as inoculum for the 200 liter fermentor. To avoid excessive foaming, media in fermentors were supplied with 1 mg/liter culture medium of silicon antifoam agent SAG 5693 (Union Carbide, USA). Production cultures in 30 liter scale were grown for 331 hours (MGP) and 233 hours (Q6/2), respectively, at 24°C with agitation (150 rpm) and aeration (3 liters sterile air/minute) in a 40 liter Biostat E fermentor (Braun Bioengineering, Melsungen, Germany). Production cultures in 200 liter scale were performed in a stirring fermentor (Bayer Health Care), using Q6/2 medium only. The culture was grown for 115 hours at 24°C under aeration (3 liters air/minute) and agitation (100 rpm).

All strains listed in Table 2 were studied for production of the desired compound in flask scale (see a comparison of production rates in this Table), and some of them also in 30 liters scale, but the best production rates were still observed with *A. macrosporus* strain DSM 14593. Therefore, optimization of production was mainly carried out using this organism.

### Analytical HPLC Methods

The equipment used for analytical and preparative HPLC has been described in detail [30]. Characteristic retention times (Rt) in these two different gradient systems, along with UV/visual and mass spectra in comparison with external and internal standards served for detection of the metabolites in crude extracts and intermediate products obtained during downstream processing and chromatography. For estimation of active principles by

HPLC during fermentation, 10 ml of culture broth were withdrawn under sterile conditions. Mycelia were separated by filtration and extracted with 10 ml acetone for 30 minutes in an ultrasonic bath. The acetone was then evaporated *in vacuo* (40 °C, 250 mbar), the aqueous residue diluted with water to 10 ml and extracted with 10 ml of ethyl acetate (EtOAc). An aliquot of 5 ml of the organic phase was withdrawn, dried over $Na_2SO_4$ and evaporated *in vacuo*. Subsequently, the residue was redissolved in 1 ml MeOH and analyzed by HPLC. The Rt values are summarized in Table 3 and the production rates of compound **1** estimated during fermentation in Table 4, using external and internal standards. HPLC-MS data suggested that no other peak was located beneath the agaricoglyceride A peak, and that the employed methodology was suited well to detect the compound quantitatively.

**Precursor Feeding to Enhance Production of Agaricoglycerides**

Shake cultures of *A. macrosporus* DSM 14593 were propagated in MGP medium containing 1 ml of a solution of 50 mg of the dichloro-anisic acid (**6**) or 4-hydroxybenzoic acid, respectively, in glycerol : MeOH 1 : 1, which was added under sterile conditions immediately before inoculation. Fermentations were carried out for up to 360 hours, using the same inoculum in a control fermentation without supplements. Extracts were prepared and analyzed by HPLC to estimate agaricoglyceride A (**1**) as described above. Five flasks of each medium were used for comparison.

**Production and Isolation of Secondary Metabolites**

Small Scale Isolation

Ten YMG shake flasks of *A. macrosporus* DSM 14593 were grown for 192 hours. Mycelia were separated from the culture fluid by filtration and extracted twice with each 1 liter acetone for 30 minutes in an ultrasonic bath. The extract was filtered, and the acetone was removed *in vacuo* (*ca.* 40°C, 250 mbar) to yield an aqueous residue. This residue was diluted with tap water to 300 ml and subsequently extracted three times with each 300 ml EtOAc. The combined organic phases were dried over $Na_2SO_4$ and evaporated *in vacuo* to yield an oily residue (160 mg). This crude extract was separated by preparative HPLC using system A: flow 9 ml/minute; mobile phase 0,1% TFA (trifluoroacetic acid):ACN (acetonitrile); stationary phase: Merck LichroSorb RP-18 (7 $\mu$m, 250×25 mm); gradient: starting with t=0′ (20% ACN); linear to t=70′ (100% ACN); isocratic to t=88′ (100% ACN). Compound **1** (16 mg) was eluted at Rt 60～64 minutes, detected as single peak at 210 nm. As side metabolite, DCMB (**5**; 5 mg) was obtained at Rt 41～46 minutes.

Large Scale Production/Isolation of Compound 1 and Isolation of Congeners

The combined mycelia from a fermentation of *A. macrosporus* DSM 14593 in 30 liters MGP medium were separated from the culture broth by centrifugation (15 minutes at 3000×*g*) and filtration and extracted four times with each 5 liters acetone under stirring. The resulting fluid was combined and filtered, then the acetone was evaporated *in vacuo* at 250 mbar. The resulting aqueous residue was diluted to 1 liter with $H_2O$ and subsequently extracted three times with each 1 liter EtOAc. The combined organic phases were dried over $Na_2SO_4$ and evaporated *in vacuo* to yield an oily residue (4.5 g). This product was divided into portions containing approximately 2.2 g and subjected to preparative HPLC using system B: flow 9 ml/minute; mobile phase 0.1 % TFA: ACN; stationary phase: MZ Analysentechnik (Mainz, Germany) Kromasil C8 (7 $\mu$m, 250×40 mm); gradient: starting with t=0′ (20% ACN); linear to t=20′ (45% ACN); isocratic to t=55′ (45% ACN); linear to t=90′ (100% ACN); thereafter isocratic at 100% ACN. Agaricoglyceride A (**1**) eluted at Rt=101～108 minutes. Approximately 300 mg of the pure compound (**1**) were thus obtained from mycelia of a 30 liter culture. DCMB (**5**, 56 mg) was obtained at Rt 83～90 minutes and dichloro anisic acid (**6**, 5 mg) at Rt 94～96 minutes. An analogous fermentation/downstream processing procedure yielded 420 mg of agaricoglyceride A from 30 liter of Q6/2, medium. Besides this higher yield, fermentation time was reduced as compared to MGP medium, and Q6/2 was therefore chosen for further scale-up.

The combined mycelia of a fermentation of *A. macrosporus* strain DSM 14593 in 200 liters Q6/2 medium were separated from the culture broth by centrifugation (15 minutes at 3000×*g*) and filtration and extracted four times with each 7 liters acetone under stirring. The resulting fluid was combined and filtered, then the acetone was evaporated *in vacuo* at 250 mbar. The resulting aqueous residue was diluted to 1 liter with water and subsequently extracted three times with each one liter EtOAc. The combined organic phases were dried over $Na_2SO_4$ and evaporated *in vacuo* to yield an oily residue (56 g). This material was dissolved in acetone and absorbed to 50 g of $C_{18}$ functionalized silicagel (Sigma Aldrich 37.763-5). The acetone was evaporated *in vacuo*, and the dried extract/silicagel mix was applied onto a glass column (30×4.8 cm diameter) filled with a bed of 30 g of the same silicagel that had previously been equilibrated to 50 %

ACN. A three-step gradient (each 200 ml of 50%, 80% and 100 % aqueous ACN) was employed to achieve a crude fractionation. The 100% ACN fraction (19 g) lacked agaricoglycerides and biological activities and was discarded. Intermediate product 1a (7 g) eluted at 50% ACN and yielded DCMB (**5**, 280 mg ), 3,5-dichloro anisic acid (**6**, 240 mg) and agaricic ester (**7**, 16 mg; Rt 98～99 minutes) upon repetitive HPLC on Kromasil C8 using gradient system B (see above). Intermediate product *1b* (32 g, eluted after intermediate product 1a at 80% ACN) was also further processed using the preparative HPLC system B as described above. For this purpose, intermediate product 1b was portioned into aliquots of approximately 3.2 g. Repetitive HPLC on Kromasil C8 thus yielded 3.2 g of an intermediate product 2, which was further processed by HPLC on Merck LiChrosorb C18 using system A. An overall amount of 1.5 g of pure agaricoglyceride A (**1**) was finally obtained. An inseparable mixture of monoacetyl agaricoglycerides (**1a**/**b**) eluted under these conditions at Rt 112～114 minutes. The identity of these compounds was later proven by synthesis of the single components and comparison of their spectral data as described in Ref. 7. An intermediate product 3 (332 mg) eluted upon HPLC separation using system B at Rt 94～98 minutes. It was further processed using system C: flow: 9 ml/minute; stationary phase: Waters SymmetryPrep C18 (7 $\mu$m, 300× 19 mm); mobile phase 0.1%TFA : ACN; gradient: t=0～10 minutes; isocratic at 50% ACN; thereafter linear to t=25′(80% ACN); thereafter linear to t=44′(100% ACN); thereafter isocratic at 100% ACN. This procedure afforded agaricoglycerides C (**3**, 8 mg, Rt 25～26 minutes) and B (**2**, 1.7 mg, Rt 29～30 minutes) as well as another 82 mg of agaricoglyceride A (**1**, Rt 30～32 minutes). An intermediate product 4 (87 mg) was also obtained upon purification of intermediate product 1b using HPLC gradient B at Rt=87～92 minutes and further processed using HPLC gradient system D (like system C but isocratic conditions at 50% ACN : 0.1% TFA), affording 5 mg of agaricoglyceride D (**4**) at Rt=12～14 minutes. These procedures are also summarized in the scheme in Fig. 2.

**Neurolysin Inhibition Assay *In Vitro***

For determination of *in vitro* activities against neurolysin (Endopeptidase EC 3.4.24.16), test compounds (extracts from fermentations, intermediate fractions obtained during chromatography and pure compounds) were dissolved in DMSO. Serial dilutions were made in dilution buffer (50 mM Tris/HCl, pH 7.5, 0.0025% Brij-35), resulting in final concentrations of 10 to 0.078 $\mu$M. If necessary, further dilutions were prepared. The assay was performed in OptiPlate 96 well microtiter plates (Packard). Two microliters of the diluted test samples were incubated in each well, and 50 $\mu$l of a neurolysin solution in dilution buffer (final concentration: 1.5 nM; resulting in no more than 70% turnover of the substrate after the subsequent incubation) were added. The peptidic substrate, DABCYL-Glu-Arg-Nle-Phe-Leu-Ser-Phe-Pro-EDANS; was diluted in 50 mM Tris/HCl buffer (pH 7.5) to final concentration of 5 $\mu$M in each well. The enzyme reaction was initiated by addition of 50$\mu$l diluted substrate to the enzyme/test compound mix. The samples were then incubated for 90 minutes at RT. Thereafter, the $IC_{50}$ values were determined by fluorometric methodology, using a FluorStar Fluorometer (BMG LabTechnologies; $\lambda_{ex}$=320 nm, $\lambda_{ex}$= 520 nm) and by plotting the results (substrate concentration *vs.* percentage of inhibition). Selectivity was established by using an analogous readout, employing various other proteases that were concurrently tested at the High Throughput Screening unit of Bayer HealthCare, but no significant activity was observed in any of the samples tested. For further data as to the selectivity of agaricoglycerides see above in Results and Discussion.

**Animal Model**

The methodology for determination of *in vivo* activities was described in detail in the corresponding patent [7]. Briefly, male Wistar rats (strain HsdCpb: WU) were housed in groups of six under standardized conditions. Conventional rat chow (Type R/M-H, Sniff Spezialdiaten GmbH, Soest, Germany) and tap water were given *ad libitum*. Rats were accustomed to the laboratory conditions for at least three days prior to experimental manipulations. The animals were randomly assigned to control or drug treated groups with $n$=11～12 per group. Experimental protocols and conditions were in conformity with the local regulations on animal welfare. Test compounds were pre-solubilized in mixture of DMSO, ethanol and Solutol HS 15 (BASF ChemTrade GmbH, Bargbernheim, Germany). This mixture was subsequently filled up with physiological saline to final concentrations of 4% DMSO, 10% ethanol and 20% Solutol. Intraperitoneal doses of 1, 3 and 10 mg/kg body weight were applied in a volume of 2 ml/kg. The decreasing temperature cold-plate was a self-constructed device. It consisted of twelve plastic chambers (24 cm high) with an aluminium floor (21×24 cm) equipped from beneath with semiconducting Peltier elements. Cooling or heating of the floors was performed under computer control. Each of the twelve chambers could be controlled individually in order to conduct an experiment with twelve animals simultaneously.

Animals were treated with i.p. injection of test compounds or vehicle and placed individually in the cold-

plate chambers. Initially, the floors of the chambers had a pleasant temperature of 30°C. Thirty minutes after application the floors were cooled down with a linear ramp of 3°C/minutes and a cut-off temperature of 2.5°C. The cold pain threshold was reached when the animals either took their forepaws repeatedly to the mouth or began restlessly to trip with the forepaws. When this behavior became evident the experiment was stopped and the threshold temperature was taken as a measure for the pain experienced. A decrease of the threshold temperature was considered to be a drug induced analgesia. Data were processed using the statistical software package SYSTAT Version 10. Experimental data have been analyzed with the Kruskal-Wallis test. Differences in the subsequent post-hoc Dunnett test were considered to be significant if $p<0.05$. Morphine was used as a standard.

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