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Ustilipids, Acylated β-D-Mannopyranosyl D-Erythritols from *Ustilago maydis* and *Geotrichum candidum*

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Ustilago species produce an extracellular oil that shows activity in various pharmaceutical assays. We isolated several complexes of this heterogeneous glycolipid from cultures of *Ustilago maydis* DSM 11494 and *Geotrichum candidum* ST 002515, and determined the chemical structures of these new compounds, termed ustilipids, on the basis of NMR experiments, mass spectra, and fatty acid analyses. They all possess a 4-*O*-β-D-mannopyranosyl D-erythritol basic framework, the configuration of which was confirmed, after initial solvolysis, by a single-crystal X-ray structure analysis. All the investigated ustilipids and related compounds are similarly constructed: the three hydroxy groups of the erythritol side chain are free in all cases, whereas the hydroxy groups of the mannose residue are for the most part acylated. Medium-chain fatty acids have for the first time been detected as components of glycolipids produced by *Ustilaginales*. While the 2-hydroxy group of the mannose residue is esterified with a C_2-C_8 carboxylate side chain, the 3-hydroxy group is in all cases esterified by a longer, C_{12}-C_{20} fatty acid residue. The oxygen functionalities at the 4 and 6 positions are either acetylated or present as free hydroxy groups.

Ustilipids antagonize dopamine D3 receptors in micromolar quantities; other members of this class of compounds have been found to possess an inhibitory action on the neurotensin receptor. The hemolytic activity of ustilipids is low.

In modern pharmaceutical research, the identification of highly diverse sources of chemical raw materials is of great importance to the drug discovery process. Thus, despite the widespread application of combinatorial chemistry, the importance of natural products research continues to grow, as nature, *e.g.* in the form of plants or microbial cultures, has provided us with an unrivaled abundance of substance types. We have consequently examined fungal extracts in biochemical test models and have discovered natural products that have frequently shown activities in various assays.

As long ago as 1969, A. L. FLUHARTY and J. S. O'BRIEN[1] detected some mannose- and erythritol-containing glycolipids in the mycelium of the corn smut fungus *Ustilago maydis* which accounted for roughly one third of the total lipid content of the organism. They identified a tetraacyl β-D-mannopyranosyl D-erythritol moiety as the basic framework of this class of compounds, though the heterogeneity of the isolated substances made it impossible to establish the structures of individual compounds at the time. Molecular masses of between 1210 and 1236 Da were postulated.

In 1980 W. STEGLICH *et al.*[2] isolated the compounds schizonellin A and B from cultures of *Schizonella melanogramma*, another member of the order Ustilaginales, the structures of which were established by chemical degradation and spectroscopic methods. These esterified mannose derivatives were found to be triacyl β-D-

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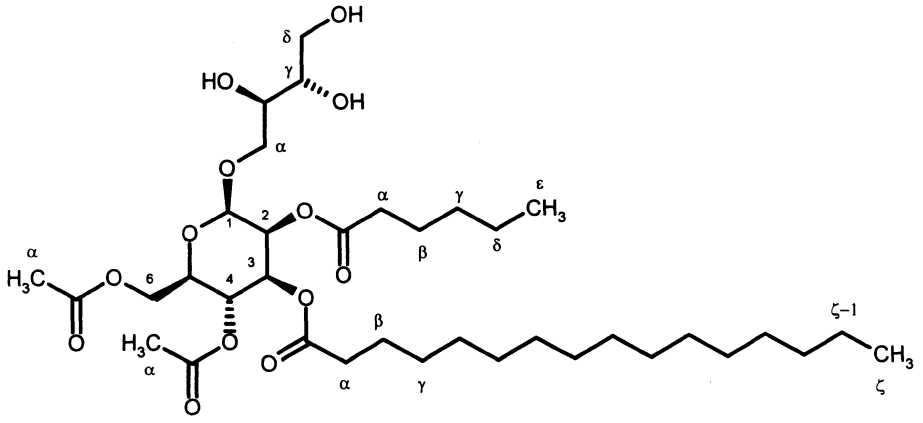
Fig. 1. Structure of ustilipid A (**1**).



mannopyranosyl D-erythritols, which, because of their amphiphilic properties, show strong hemolytic activity. The molecular weights of schizonellin A and B according to the molecular formulae cited are 564 and 606 Da respectively.

In the course of a screening program for the discovery of new active substances, we isolated lipid fractions from the mycelium of *Ustilago maydis* DSM 11494 (ATCC 14826) that attracted our attention on account of their dopamine antagonism[3]. Their biological and physicochemical properties were found to differ from previously published data, so we embarked on an investigation of the composition and structure of this class of compounds.

Geotrichum candidum is an extremely common fungus with a worldwide distribution and is the causative agent of geotrichosis. Pulmonary infection is the most frequently reported form of the disease, but bronchial, oral, vaginal, cutaneous, and alimentary infections have also been reported. This cosmopolitan species is mainly saprophytic on vegetables, fruits, milk, and cheese[4]. While screening for neurolysin receptor antagonists, we encountered constituents of *Geotrichum candidum* that showed activity in the receptor assay. These natural products were found to be identical in part to the glycolipids isolated from *Ustilago maydis*. In this paper we report the structures of new glycolipids from *Ustilago maydis* and *Geotrichum candidum* ST 002515, which we named ustilipids.

Results

The strain *Ustilago maydis* ATCC 14826 was isolated from smutted corn cobs (*Zea mays*) from Canada and is described as the producer of ustilagic acid and an extracellular triglycerol lipase.

The compounds that showed activity in the dopamine assay were extracted from the mycelium of *Ustilago maydis* DSM 11494 and purified by size-exclusion chromatography, followed by chromatography on silica gel with chloroform/methanol mixtures as eluent. Further purification by reversed-phase HPLC afforded material suitable for mass spectrometric characterization. The molecular masses of the isolated lipid fraction and the intensities of the molecular peaks spanned a wide range from approx. 600 Da to approx. 720 Da in the form of a Gaussian curve, mass differences of 14 ($-CH_2-$) pointing to the presence of homologous series, with molecular masses of 634, 648, 662, 676, and 704 being the most common. The ustilipid complexes A, B, C and schizonellin B were isolated from cultures of *Ustilago maydis* DSM 11494 and the neurolysin receptor antagonists ustilipid complexes C, D, E, and F from the cell mass of *Geotrichum candidum* ST 002515.

The various substances that were obtained were of the same type; all were stained by both I_2 vapor and α-naphthol/sulfuric acid and they were clearly glycolipids. To evaluate the fatty acid composition, some of the isolated ustilipids were subjected to methanolysis and then investigated by GC/MS. The results of the fatty acid analyses are shown in Table 1. The observed compositions showed that although the separated complexes were substantially enriched, they were by no means single substances, even though the analyzed substances appeared

Table 1. Gas-chromatographic fatty acid methyl ester analyses of the isolated ustilipids A, B, and C and of a ustilipid mixed fraction with a molecular weight range of 606~704 Da.

Fatty acid methyl ester	Ustilipid A	Ustilipid B	Ustilipid C	Lipid mixture
Caproic acid	12	n.t.	2.5	5.9
Caprylic acid	1.9	1.0	-	0.4
Capric acid	1.1	0.2	0.3	0.3
Lauric acid	0.7	0.9	-	2.4
Tetradecenoic acid	-	-	-	4.9
Myristic acid	3.6	9.5	0.9	7.2
Hexadecenoic acid	-	-	-	6.6
Palmitic acid	23.3	16.0	29.9	10.0
Stearic acid	3.8	0.3	0.2	0.5
Oleic acid	1.3	1.5	4.8	1.3
Linoleic acid	0.4	2.0	0.6	2.4

Only fatty acids with a chain length of $\geq C_6$ and a content of $\geq 0.2\%$ are shown.

Table 2. ^1H chemical shifts of the glycolipids.

	Ustilipid A	Ustilipid B	Ustilipid C	Ustilipid D1	Ustilipid D2	Ustilipid E1[a)]	Ustilipid F1[b)]	Schizonellin
1	4.72	4.73	4.81	4.81	4.77	4.75	4.80	4.72
1α	3.99/3.82	3.99/3.79	4.02/3.78	4.02/3.78	3.96/3.79	3.99/3.80	4.03/3.78	4.00/3.84
1β	3.74	3.73	3.77	3.77	3.72	3.74	3.78	3.75
1γ	3.65	3.63	3.63	3.63	3.63	3.64	3.65	3.68
1δ	3.75	3.71	3.71	3.71	3.71	3.72	3.73	3.76
2	5.50	5.50	5.48	5.48	5.45	5.51	5.49	5.49
2α	2.43	2.41	2.69	2.69	2.64	2.46	2.70	2.15
2β	1.65	1.68	1.21	1.21	1.20	1.17	1.22	-
2γ	1.34	0.98	1.18	1.18	1.18	-	1.19	-
2δ	1.34	-	-	-	-	-	-	-
2ε	0.90	-	-	-	-	-	-	-
3	5.06	5.06	5.09	5.09	4.94	5.07	5.10	4.92
3α	2.21	2.20	2.19	2.28	2.28	2.22	2.21	2.31
3β	1.53	1.52	1.52	1.52	1.57	1.53	1.54	1.59
3γ	1.24	1.24	1.24	1.24	1.28	1.24	1.25	1.28
3δ-3ζ-3	1.28-1.22	1.28-1.21	1.28-1.22	1.28-1.22	1.28-1.22	1.28-1.22	1.27-1.22	1.28-1.21
3ζ-2	1.28	1.28	1.28-1.22	1.28-1.22	1.28-1.22	1.25	1.26	1.28-1.21
3ζ-1	1.28	1.28	1.28	1.28	1.28	1.28	1.29	1.28
3ζ	0.87	0.87	0.87	0.87	0.87	0.87	0.88	0.88
4	5.24	5.24	5.15	5.15	3.78	5.25	5.17	3.80
4α	2.03	2.02	2.02	2.02	-	2.03	2.05	-
5	3.70	3.70	3.56	3.56	3.56	3.71	3.57	3.57
6	4.25/4.19	4.25/4.18	3.70/3.64	3.70/3.64	4.43	4.26/4.19	3.71/3.66	4.44
6α	2.09	2.09	-	-	2.12	2.10	-	2.14

a) Chemical shifts of components E2 an E3 have not be assigned completely.

b) Chemical shifts of components F2 an F3 have not be assigned completely.

Table 3. ^{13}C chemical shifts of the glycolipids.

	Ustilipid A	Ustilipid B	Ustilipid C	Ustilipid D1	Ustilipid D2	Ustilipid E1[a)]	Ustilipid F1[b)]	Schizonellin
1	99.30	99.27	99.00	99.00	99.23	99.24	99.04	99.33
1α	72.29	72.22	71.76	71.76	71.94	72.19	71.77	72.21
1β	71.16	71.13	70.96	70.96	71.12	71.12	71.01	71.24
1γ	71.85	71.89	72.16	72.16	72.01	71.91	72.15	71.93
1δ	63.57	63.51	63.32	63.32	63.49	63.50	63.35	63.63
2	68.60	68.63	68.73	68.73	68.85	68.71	68.70	69.03
2-C'	173.46	173.29	176.92	176.92	176.81	174.20	176.86	170.58
2α	34.10	36.01	33.92	33.92	34.10	27.47	33.99	20.85
2β	24.67	18.48	19.26	19.26	19.16	9.17	19.26	-
2γ	31.11	13.50	18.77	18.77	18.77	-	18.79	-
2δ	22.27	-	-		-	-	-	-
2ε	13.85	-	-		-	-	-	-
3	70.65	70.68	70.86	70.86	73.21	70.68	70.81	73.05
3-C'	172.72	172.71	172.76	172.76	173.59	172.73	172.71	173.53
3α	34.00	33.98	33.99	33.99	34.03	33.97	33.93	34.08
3β	24.69	24.65	24.67	24.67	24.67	24.67	24.67	24.71
3γ	29.07	29.07	29.0-29.7	29.0 -29.7	29.0-29.7	28.98	29.02	29.0-29.7
3δ-3ζ-3	29.2-29.7	29.2-29.7	29.0-29.7	29.0-29.7	29.0-29.7	29.2-29.6	29.0-29.7	29.0-29.7
3ζ-2	31.89	31.89	31.88	31.88	31.88	31.87	31.86	31.91
3ζ-1	22.65	22.65	22.64	22.64	22.64	22.63	22.64	22.67
3ζ	14.08	14.08	14.07	14.07	14.07	14.05	14.07	14.09
4	65.90	65.88	66.27	66.27	65.62	65.88	66.26	66.60
4-C'	169.44	169.45	170.09	170.09	-	169.47	170.08	-
4α	20.63	20.61	20.67	20.67	-	20.62	20.67	-
5	72.50	72.43	74.78	74.78	74.40	72.41	74.81	74.62
6	62.36	62.34	61.36	61.36	63.12	62.32	61.40	63.17
6-C'	170.76	170.74	-	-	171.67	170.76	-	171.67
6α	20.69	20.67	-	-	20.77	20.67	-	20.80

a) Chemical shifts of components E2 an E3 have not be assigned completely.

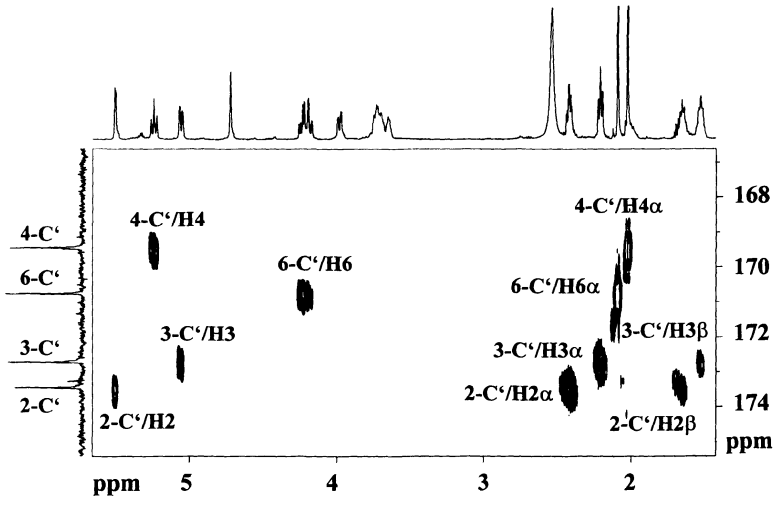
b) Chemical shifts of components F2 an F3 have not be assigned completely.

chromatographically homogeneous. The fatty acid composition of a mixed fraction reflects the diversity of the lipids and largely corresponds to the data reported by A. L. FLUHARTY and J. S. O'BRIEN[1]. Although the presence of acetate in the *Ustilago maydis* glycolipids had previously been reported, the new isolated ustilipids were, to our surprise, found to contain medium-chain fatty acids from C_2 to C_{10}, the abundance of which decreases with chain length from C_2 to C_{10} and then increases again as far as palmitic acid, the most common fatty acid.

The structures of the main components were determined by various two-dimensional NMR techniques including DQF-COSY[5], TOCSY[6], HMQC[7], and HMBC[8] experiments. A complete assignment of the proton and carbon chemical shifts of seven of these compounds and of schizonellin B is given in Tables 2 and 3. Analysis of homo- and heteronuclear spectra revealed the presence of a pyranoside moiety in which the anomeric carbon forms an ether bridge to a 1,2,3,4-tetrahydroxybutane unit. Most of the other mannosyl hydroxyl groups are esterified with different carboxylate residues. The exact substitution pattern can be verified from the HMBC spectra, since each carboxyl carbon shows long-range heteronuclear correlations to aliphatic protons of the carboxylate moiety and to the protons in position 2, 3, 4, or 6 of the sugar ring (see Fig. 2).

As can be seen from Fig. 3, the greatest diversity of substituents is at position 2, with acetate, propionate, butyrate, isobutyrate, and caproate side chains all found at this center. At position 3 all the constituents contain a saturated fatty acid residue with a chain length of 14 or 16 carbon atoms. The exact length of the long fatty acid side

Fig. 2. Carbonyl region of the HMBC spectrum of ustilipid A (**1**).



Each carbonyl carbon shows heteronuclear long-range correlations to aliphatic protons of the carboxylate moiety and to the protons in position 2, 3, 4, or 6 of the sugar ring.

chain was confirmed by MS and fatty acid analysis. The remaining hydroxyl functions at positions 4 and 6 are either free or acetylated. The structure of several minor components could not be established unambiguously by NMR spectroscopy. However, according to the fatty acid analysis and MS studies the carboxylate side chain at position 2 of the mannose residue varies from C_2 to C_8 and at position 3 from C_{12} to C_{20}.

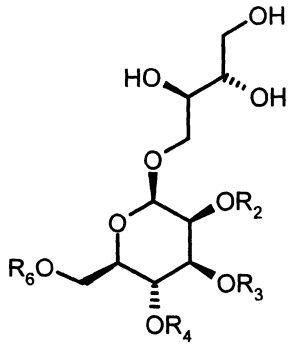
B. BOOTHROYD *et al.*[9], A. L. FLUHARTY and J. S. O'BRIEN[1], and W. STEGLICH's working group[2] had previously described the saccharide core of the 'extracellular oil' obtained from *Ustilago* species as 4-*O*-*β*-D-mannopyranosyl D-erythritol. To confirm that the configuration and identity of the carbohydrate backbone of the ustilipids was that of 4-*O*-*β*-D-mannopyranosyl D-erythritol, the ustilipid mixture was subjected to alkaline methanolysis. Starting from 5 g of material, chromatographic purification yielded 1.28 g of mannopyranosyl erythritol as the sole compound detectable with the carbohydrate reagent *α*-naphthol/H_2SO_4. The starting material prior to hydrolysis was thus clearly a mixture of related compounds all containing mannopyranosyl erythritol as the carbohydrate backbone. The measured specific rotation $[\alpha]_D^{20}$ was $-37°$ (c=1.96, H_2O), which is in good agreement with the literature value of $-38°$. The compounds could thus be assumed to be of identical configuration, *i.e.* that of 1-*O*-*β*-D-manno-pyranosyl L-erythritol.

For definitive confirmation of the configuration, the purified sugar was crystallized from methanol and its X-ray crystal structure determined.

Figure 4 shows the result of the X-ray single crystal structure analysis. The relative and the absolute configuration (in connection with measuring the optical rotation of D-Mannose) of a compound, whose constitution was elucidated earlier[2], could be unambiguously determined. D-Manno-pyranosyl-erithritol has a conformation, which allows a maximum number of intermolecular hydrogen bonds. A partly intramolecular bifurcated hydrogen bond additionally stabilizes this conformation. The carbon chain of the erythritol part is "coplanar" with the pyran skeleton, what reduces the effective volume of the molecule.

On the basis of the single-crystal X-ray structure, the fatty acid analyses (shown here only in part), the molecular mass distribution determined by the HPLC-MS analyses, and in particular the NMR experiments, a general formula can be established for the isolated ustilipids (**2**) in which position 2′ of the mannose residue (R_2 in Fig. 2) bears an acetate to max. caproate side chain and position 3′ a longer acyl residue (R_3) of chain length C_{12} to C_{20}, with substituents R_4 and R_6 usually acetate, less commonly H.

Fig. 3. Substitution patterns of ustilipid components (**2**).



Compound	Empirical formula	M.W.	Pos. 2	Pos. 3	Pos. 4	Pos. 6
Ustilipid A	$C_{36}H_{64}O_{13}$	704	C6 (caproate)	C16	Ac	Ac
Ustilipid B	$C_{34}H_{60}O_{13}$	676	C4 (butyrate)	C16	Ac	Ac
Ustilipid C	$C_{32}H_{58}O_{12}$	634	C4 (isobutyrate)	C16	Ac	H
Ustilipid D1	$C_{32}H_{58}O_{12}$	634	C4 (isobutyrate)	C16	Ac	H
Ustilipid D2	$C_{32}H_{58}O_{12}$	634	C4 (isobutyrate)	C16	H	Ac
Ustilipid E1	$C_{33}H_{58}O_{13}$	662	C3 (propionate)	C16	Ac	Ac
Ustilipid E2	$C_{32}H_{56}O_{13}$	648	Ac	C16	Ac	Ac
Ustilipid E3	$C_{34}H_{60}O_{13}$	676	C4 (isobutyrate)	C16	Ac	Ac
Ustilipid F1	$C_{30}H_{54}O_{12}$	606	C4 (isobutyrat)	C14	Ac	H
Ustilipid F2	$C_{29}H_{52}O_{12}$	592	C3 (propionate)	C14	Ac	H
Ustilipid F3	$C_{28}H_{50}O_{12}$	578	Ac	C14	Ac	H
Schizonellin B	$C_{30}H_{54}O_{12}$	606	Ac	C16	H	Ac

Please note that all ustilipid complexes are still mixtures (see experimental part).

Biological Properties

The ustilipids were initially of interest as antagonists of dopamine D_2 and D_3 receptors. Dopamine affects its target cells in brain and endocrine tissues by interacting with D_1, D_2, and D_3 receptors. These receptor subtypes differ in their pharmacological specificity for neuroleptics, a class of dopamine antagonists used to alleviate the main symptoms of schizophrenia[3]. The D_3 receptor has a predominantly *mesolimbic* distribution, and is found in the shell of the *nucleus accumbens*, in areas where antipsychotics are thought to act[10]. Since drugs able to regulate the activity of dopaminergic neurons by acting selectively on dopamine autoreceptors would be therapeutically useful, there is considerable interest in finding selective D_3 receptor antagonists[11].

Ustilipid complexes A, B, and C antagonize dopamine receptors D_2 and D_3 only weakly and to differing extents. The inhibition constants are given below in Table 4. The procedure described by D. S. KREISS *et al.*[12] was used to carry out the determinations[13]. Due to the fact that all ustlipid complexes are mixtures a more detailed analysis including structure activity relation ships is not possible so far.

Extracts of *Geotrichum candidum* ST 002515 were screened for their suitability as antagonists of the neurolysin receptor and the active substances were isolated. Neurotensin is a 13-amino acid hormonal peptide which is present in the digestive tract as well as in the central nervous system. It has a variety of biological activities as a

Fig. 4. Absolute and relative configuration of 1-*O*-β-D-Manno-pyranosyl-(2*R*,3*S*)-erythritol.

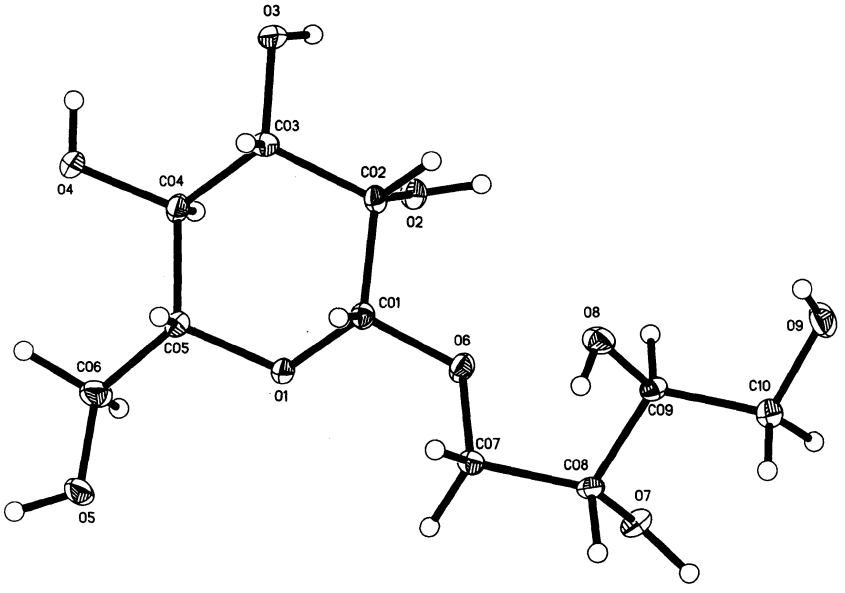


Table 4. Inhibition of dopamine D_2 and D_3 receptors by ustilipids (IC_{50} in μg/ml).

	Dopamine D_2 receptor	Dopamine D_3 receptor
Ustilipid A:	30 μg/ml	5 μg/ml
Ustilipid B:	16 μg/ml	7 μg/ml
Ustilipid C:	9 μg/ml	12 μg/ml

central neurotransmitter or neuromodulator and as a peripheral hormone. NT receptors have been characterized in a variety of tissues and cell lines of peripheral and central organs. From a clinical perspective, studies with neurotensin have pointed to possible involvement in schizophrenia and Parkinson's and Alzheimer's diseases.

Compounds with an affinity for the neurotensin receptor will displace [^{3}H]neurotensin from its binding sites, resulting in a decrease in measured radioactivity.

Ustilipid complexes D, E, and F inhibit the neurolysin receptor with IC_{50} values of 24 μg/ml (ustilipid complex E and F) and 64 μg/ml (ustilipid complex D).

The hemolytic activity of ustilipids is low. As can be seen from Table 5, the new compounds that have been investigated do not cause unacceptable hemolysis at concentrations of over 0.1 mg per ml[13].

Discussion

As long ago as 1956 B. BOOTHROYD *et al.*[9] reported an 'extracellular oil' which was heavier than water and was formed in abundance in cultures of *Ustilago* spec., *i.e.* in quantities of some 15 g/liter. No unambiguous structures have so far been proposed for the isolated substances. DEML *et al.*[2] published the structural formulae of schizonellins A and B, two compounds present in the 'extracellular oil' complex but only secondary components of the Ustilago glycolipid mixtures, with a content of less than 10% by mass.

Even though the isolated ustilipids could not be obtained in a completely pure form, use of modern analysis techniques allied with the available analysis data allowed us to assign structures to the new compounds described here

Table 5. Hemolytic behavior of ustilipids A, B and C in a human erythrocyte model[13] (%).

	Ustilipid A	Ustilipid B	Ustilipid C
Concentration			
250 µg/ml	11.8%	2.2%	4.1%
125 µg/ml	0.1%	0.4%	0.8%
62.5 µg/ml	0.0%	0.0%	0.5%
32 µg/ml	0.0%	0.0%	0.4%
16 µg/ml	0.0%	0.0%	0.4%
8 µg/ml	0.0%	0.0%	0.0%
4 µg/ml	0.0%	0.0%	0.0%
Control	100%	100%	100%

and to postulate a common structural principle: In each of the investigated compounds, position C2 of the mannose residue bears a C_2-C_8 acyl side chain, C3 a C_{12} to C_{20} acyl side chain, and C4 and C6 either acetate or free OH. The acylation pattern is the same in all six new compounds, though a reason for this observed uniformity has yet to be found.

The ustilipids, new glycolipids peracylated on the mannose residue, clearly not only occur in Ustilago species, but are also produced by *Geotrichum candidum* ST 002515 and other fungal species. Because ustilipids frequently exhibit activity in biological test systems, greater attention should be paid to the occurrence of these common glycolipids during screening of pharmacologically active natural products.

Experimental

General

Quantitative ultraviolet absorption spectra were recorded using a Cary 118 B spectrometer (Varian, Darmstadt, Germany); for all other purposes, including the performance of HPLC analyses, Hewlett-Packard series 1100 equipment fitted with diode array detectors was used. Preparative HPLC was performed using Pharmacia equipment (Uppsala, Sweden).

Mass spectrometric studies were performed on Finnigan/MAT LCQ and TSQ 700 instruments. MS and MS/MS spectra were obtained by electrospray ionization (ESI) in positive and negative modes.

Fermentation Conditions

Ustilago maydis DSM 11494:

Ustilipids were produced by fermentation in a stirred 10 liter stainless steel Braun fermentation vessel. 500 ml conical flasks containing 100 ml of seed medium were inoculated with a suspension of *Ustilago maydis* DSM 11494 spores at a concentration of 0.2%. The seed medium consisted of 20 g/liter malt extract, 2 g/liter yeast extract, 10 g/liter glucose, and 0.5 g/liter $(NH_4)_2HPO_4$ in tap water. The seed flasks were incubated for 72 hours at 25°C on a rotary shaking machine at 140 rpm. The fermenter was charged with 8 liters of a medium consisting of 20 g/liter malt extract, 2 g/liter yeast extract, 10 g/liter glucose, and 2 g/liter $(NH_4)_2HPO_4$, pH 6.0 before sterilization. The nutrient solution was sterilized in the fermenter at 121°C for 1 hour. The fermenter was inoculated with the seed flask culture at a concentration of 5%. The temperature during fermentation was maintained at 25°C. The stirring rate was 200 rpm and the air flow rate was 0.5 v/v/minute. Antifoam (Desmophen) was added initially at a concentration of 0.01%. The fermentation was terminated after 48 hours.

Geotrichum candidum ST 002515:

Ustilipids were produced by fermentation in 300 ml shaker flasks containing 100 ml of production medium. The 300 ml flasks were inoculated with a suspension of *Geotrichum candidum* ST 002515 spores at a concentration of 0.2%. The production medium consisted of 20 g/liter malt extract, 2 g/liter yeast extract, 10 g/liter glucose, and 0.5 g/liter $(NH_4)_2HPO_4$ in tap water. The seed flasks were incubated for 144 hours at 25°C on a rotary shaking machine at 140 rpm. The nutrient solution was sterilized in the shaker flasks at 121°C for 1 hour. The temperature

during fermentation was maintained at 25°C.

Isolation of Ustilipids from Cultures of *Ustilago maydis* DSM 11494

30 liters of culture liquor from the *Ustilago maydis* fermentation was centrifuged and the resulting cell mass (approx. 3 liters) was extracted with 9 liters of methanol. The organic extract was concentrated under reduced pressure to give an oily material which was redissolved in dry methanol. 300 ml of this concentrate with an oil content of approx. 10 g was loaded onto a 4 liters column (10 cm× 50 cm) packed with Fractogel TSK HW-40 (F), which was then eluted with methanol at a flow rate of 50 ml/minute, with the eluate collected in 65 ml fractions. The dopamine D3 antagonists were eluted in fractions 20~30, which were pooled and concentrated. The crude product was then dissolved in chloroform and purified further on a silica gel 60 (Merck, Darmstadt, Germany) column (4×23 cm), which was eluted with a gradient from chloroform to 5% methanol in chloroform, with the pump set to a flow rate of 35 ml/minute. Fractions (each 50 ml) 28~32 and 49~52 exhibited the strongest dopamine D3 antagonism and were in each case pooled; fractions 38~48 were also pooled. All the pooled fractions contained only compounds that were stained by both I_2 vapor and α-naphthol/sulfuric acid. The pooled fractions 28~32 and 49~52 were both homogeneous to thin-layer chromatography on silica gel using chloroform/methanol (9 : 1) as the mobile phase (Rf values of 0.41 and 0.2 respectively), but not to reversed-phase HPLC. Separation of pooled fraction 28~32 on LiChrosorb RP-select B (7 μm; 25×250 mm) with a gradient of 50 to 80% acetonitrile in 0.05% trifluoroacetic acid gave 110 mg of ustilipid complex A, 330 mg of complex B. Separation of pooled fraction 49~52 with a gradient of 40 to 90% acetonitrile in 0.05% trifluoroacetic acid gave 10 mg of schizonellin B and 100 mg of ustilipid complex C. As could be shown by MS and NMR, complexes A, B and C are mixtures of the same 3 components [ESI+ MS: m/z=705 (MH^+), corresponding to $C_{36}H_{64}O_{13}$ for component A, ESI^+-MS: m/z=677 (MH^+), corresponding to $C_{34}H_{60}O_{13}$ for component B, and ESI+ MS: m/z=635 (MH^+), corresponding to $C_{32}H_{58}O_{12}$ for component C]. The ratios of components A : B : C were determined by ^{13}C-NMR: in complex A 74% : 19% : 7%, in complex B 40% : 45% : 15%, in complex C 15% : 25% : 60%. Additional ustilipid-containing fractions of variable composition were obtained here too. NMR data are summarized in Tables 3 and 4.

Isolation of Ustilipids from *Geotrichum candidum*

2 liters of culture liquor was freeze-dried and the resulting material exhaustively extracted by stirring with methanol (approx. 2 liters). The extract was evaporated under reduced pressure and the residue (approx. 12 g) dissolved in 5% aqueous acetonitrile. This solution was loaded onto a 1 liter column (6×25 cm) packed with an adsorption resin (MCI gel CHP20P), which was eluted with a gradient of 5 to 100% acetonitrile in water. The biologically active fractions were pooled, concentrated under reduced pressure, and freeze-dried. Further purification was achieved by size-exclusion chromatography on Fractogel TSK HW-40 (Merck, Darmstadt, Germany) using methanol as eluent. The glycolipid-containing fractions were again collected and the solvent was removed. The residue was loaded onto a Nucleosil 100-7 C_{18}HD column (Macherey-Nagel, Düren, Germany; 21×250 mm) and was separated by elution with a gradient of 65 to 100% acetonitrile in 0.05% trifluoroacetic acid. The active fractions were finally purified on a silica gel 60 column (40~63 μm, 3.5×30 cm), eluting with dichloromethane/methanol (9 : 1). This yielded 111.7 mg of ustilipid complex D, 41.3 mg of complex E and 30.7 mg of complex F. Complex D is a mixture of two components (D1 and D2) with the same molecular weight [ESI^+-MS: m/z=635 (MH^+), corresponding to $C_{32}H_{58}O_{12}$] in an approximate ratio of 57 : 43% (determined by ^{13}C-NMR). Complex E is a mixture of three components (E1, E2, and E3) in an approximate ratio of 70% : 20% : 10% [ESI^+-MS: m/z=663 (MH^+), corresponding to $C_{33}H_{58}O_{13}$ for E1, m/z=649 (MH^+), corresponding to $C_{32}H_{56}O_{13}$ for E2 and 677 (MH^+), corresponding to $C_{34}H_{60}O_{13}$ for E3]. Complex F is also a mixture of 3 components (F1, F2 and F3) in an approximate ratio of 56% : 22% : 22% [ESI^+-MS: m/z=607 (MH^+), corresponding to $C_{30}H_{54}O_{12}$ for F1, m/z=593 (MH^+), corresponding to $C_{29}H_{52}O_{12}$ for F2 and 579 (MH^+), corresponding to $C_{28}H_{50}O_{12}$ for F3]. The glycolipid spots on the thin layer chromatograms were detected by spraying with α-naphthol in sulfuric acid followed by heating at 100°C for 5 minutes[14].

Hydrolysis of the Ustilipids/isolation of 1-*O*-β-D-Mannopyranosyl L-Erythritol

Fractions 38~48 from the above silica gel column were stripped of solvent under reduced pressure and the oily residue (5 g) was dissolved in 200 ml methanol. Methanolic sodium methoxide solution was then slowly added until the pH of an aqueous sample reached 8. The course of the methanolysis was monitored by thin-later chromatography. The reaction was terminated after 3.5 hours and the reaction solution was concentrated under reduced pressure. The concentrate was adjusted to pH 3 and partitioned

between water and ethyl acetate. The ethyl acetate phase was evaporated to dryness under high vacuum, affording 1.2 g of a fatty acid mixture. The aqueous phase was freeze-dried to yield a hygroscopic product, which was purified by size-exclusion chromatography on Fractogel TSK HW-40 (Merck, Darmstadt, Germany), with methanol as eluent. Yield: 1.28 g. $[\alpha]_D^{20}=-37°$ ($c=1.96$, H_2O) Literature: $-38°$. Spectroscopic data for 1-*O*-β-D-mannopyranosyl L-erythritol: ^{1}H NMR (500 MHz, D_2O, 300K): $\delta=4.70$ (d, 1H, $J=1.0$, H1′), 4.09 (dd, 1H, $J=10.9$, 2.8, Hα), 4.05 (dd, 1H, $J=1.0$, 3.3, H2′), 3.94 (dd, 1H, $J=12.2$, 2.3, H6′), 3.83 (ddd, 1H, $J=9.5$, 6.7, 2.8, Hβ), 3.78 (dd, 1H, $J=11.6$, 3.0, Hδ), 3.76 (dd, 1H, $J=10.9$, 6.7, Hα), 3.74 (dd, 1H, $J=12.2$, 6.6, H6′), 3.73 (ddd, 1H, $J=9.5$, 6.6, 3.0, Hγ), 3.66 (dd, 1H, $J=3.3$, 9.7, H3′), 3.64 (dd, 1H, $J=11.6$, 6.6, Hδ), 3.58 (dd, 1H, $J=9.7$, 9.7, H4′), 3.39 (ddd, 1H, $J=9.7$, 2.3, 6.6, H5′); ^{13}C NMR (125 MHz, D_2O, 300K): $\delta=103.37$ (C1′), 79.09 (C5′), 75.73 (C3′), 74.68 (Cγ), 73.57 (Cα), 73.47 (Cβ), 73.30 (C2′), 69.73 (C4′), 65.40 (Cδ), 63.92 (C6′).

X-Ray Structure of 1-*O*-β-D-Mannopyranosyl L-Erythritol

Crystals were made by recrystallisation from methanol. A crystal of dimensions $0.5\times0.05\times0.02$ mm^3 was sealed in a Lindemann-glass capillary. 1359 reflections were used to determine the cell parameters on a computer controlled three circle diffractometer, equipped with a CCD area-detector (SIEMENS) and an X-ray generator with a rotating anode (Mo-Kα radiation, 0.5×5 mm^2 focus, 50 kV, 100 mA). The intensities were measured on the same apparatus: ω-scanning with steps of 0.3°; 6536 reflections ($\vartheta_{min}=1.89$, ($\vartheta_{max}=25.62$; $-5<h<5$, $-12<k<14$, $-24<l<24$), 2145 of which were unique ($R_{int}=0.053$, $R_\sigma=0.0369$) and they were all used for the structure analysis. Direct methods for solving the phase problem[15], refinement of the structure parameters by Least-Squares methods {minimization of $(F_o^2-F_c^2)^2$ [16]; weighting scheme: $w=1/[\sigma^2(F_o^2)+(0.0110*P)^2+1.2614*P]$, $P=(Max(F_o^2,0)+2*F_c^2)/3$, where σ is according to the counting statistics, 253 parameters}, the coordinates of the H atoms were got from a difference synthesis, $S=1.222$, $R=0.0829$ ($R=0.0509$ for $|F_o|>4\sigma$, 1686 reflections), $R_w=0.1024$, minimum and maximum peak in the difference map: -0.278 and 0.273 electrons/A^3. All calculations were done by a DEC 3000/900 AXP with the SHELXS-90[15], the SHELXTL-PLUS[17] and SHELXL-93[16] programs. Further tables of the results are available from one of the authors (E. F. P.).

The average estimated standard deviation (e.s.d.) of a C–C bond is 0.005A, that one of an O.C bond 0.005 A. The e.s.d. of bond angles is 0.3 and that of torsion angles 0.4°.

NMR Spectroscopy

All NMR spectra were recorded on Bruker DRX 600 and DRX 500 spectrometers operating at 600 and 500 MHz respectively. All spectra were acquired at 27°C using a solution of 10~20 mg of glycolipid in 0.5 ml $CDCl_3$. The data were processed on an indigo2 station (Silicon Graphics) using Bruker XWINNMR software.

Homonuclear COSY[5] and TOCSY[6] experiments were performed with a spectral width of 6 ppm. In all the experiments, spectra were recorded with 512 increments in t_1 and 4096 complex data points in t_2 (8 scans). For the TOCSY experiment, a mixing time of 70 ms was used (spin-lock field 10 kHz, mixing sequence MLEV17[6]). For HMQC spectra[7], 512 increments (16 scans) with 2048 complex data points in t_2 were collected using a sweep width of 6 ppm in the proton and 100 ppm in the carbon dimension. The HMBC spectra[8] were acquired with a sweep width of 6 ppm in the proton and 200 ppm in the carbon dimension. A total of 48 transients were averaged for each of 512 increments in t_1, and 2048 complex points in t_2 were recorded. A delay of 70 ms was employed for the development of long range correlations.

Neurotensin Receptor Binding Assay

The neurotensin receptor ligand-binding test was performed using a scintillation proximity assay[18,19] (SPA: Amersham Biosciences UK), using PVT-WGA beads (wheat germ agglutinin, Amersham) and 96-well Isoplates (Wallac). Into each well of the plate was placed 50 μl of test sample, 50 μl of 0.32 mg/ml human neurotensin receptor protein (provided by J. E. ROEHR, Aventis Pharma USA, final concentration: 16 μg) expressed in human embryonic kidney cells, 50 μl of 20 mg/ml PVT-WGA beads (final concentration: 1 mg), and 50 μl of 4 nM [^{3}H]neurotensin (Amersham, final concentration: 1 nM). The plates were sealed and incubated for 2 hours at room temperature on a shaker (1100 rpm).

Prior to counting (MicroBeta Trilux, Wallac) the beads were allowed to settle for at least 20 minutes. 1 μM (L-α,γ-diaminobutyryl)-neurotensin (Amersham) was used to determine nonspecific binding.

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