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Altersetin, a New Antibiotic from Cultures of Endophytic *Alternaria* spp.

Taxonomy, Fermentation, Isolation, Structure Elucidation and Biological Activities

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A novel antibacterial antibiotic, for which the name altersetin is proposed, was isolated from the culture broth of two endophytic *Alternaria* species. The relative and absolute configuration were assigned by NOESY or CD data, respectively. Altersetin is chemically related to equisetin and showed potent MIC against several pathogenic Gram-positive bacteria, whereas Gram-negative bacteria and pathogenic yeast were not or much less susceptible. Moderate *in vivo* efficiacy was observed for altersetin in a murine sepsis model.

Endophytic fungi of higher plants represent a substantial diversity of nutritional modes from biotrophic parasites to interim or facultative saprophytes. Accumulating evidence suggests that these fungi harbor a great reservoir of genetic diversity, implying practical importance with regard to the discovery of novel bioactive secondary metabolites[1~4]. Recently, we found a new antibacterial antibiotic in cultures of two fungal endophytes. In the following we wish to describe the characteristics and fermentation of the producer strains, as well as the isolation, structure elucidation and biological characterization of the active principle.

Results and Discussion

Selection and Characterization of Producer Strains

The endophytic fungi P 0506 and P 0535 were isolated from a leaf of *Vinca minor* and from a fruit of *Eonymus europaeus*, respectively, as described in the Experimental section. Their host plants had been collected near Lindlar, Germany, in September of 1997. In a screening for antimicrobial effects using the classical agar diffusion assay against *Bacillus subtilis* and *Yarrowia lipolytica*, their extracts from submerged cultures showed rather strong antibacterial activity, while the yeast test strain was insensitive.

For morphological characterization the fungal cultures were grown on Difco Potato Dextrose Agar (PDA) and on Difco Oatmeal Agar (OA) at 23°C under 12 hours of fluorescent light. No pigments were released into the culture media. The colonies showed a downy to wooly consistence. Both cultures started to spread whitish mycelia, attaining a grayish green colour after 4~5 days and becoming blackish with age, due to the production of conidia. On PDA, both strains formed effuse, grayish colonies with felty aerial mycelia, attaining a diameter of 5.5 to 6 cm after two weeks of incubation. On OA, strain P 0506 attained a diameter of 4 cm after two weeks of incubation, whereas the culture of strain P 0535 covered a 9 cm agar plate within ten days. In both strains, septate brown hyphae, along with characteristic muriform to ovate, brown poroconidia with several transversal and fewer longitudinal septa were formed from simple or geniculate conidiophores (the latter due to sympodial elongation), revealing the typical characteristics of the form genus *Alternaria* Nees ex Fr. 1821. In both strains, only solitary conidia were produced, *i.e.*, neither conidia production in

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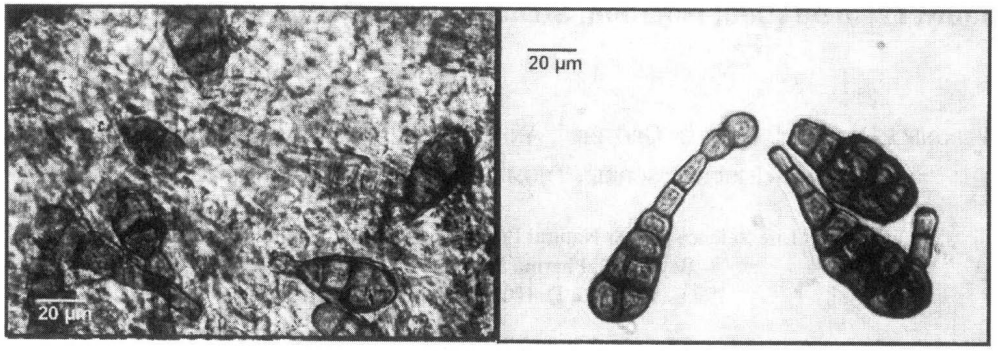
Fig. 1. Conidia of *Alternaria* spp. strains P 0506 (left) and P 0535 (right) from cultures on Difco Oatmeal agar.



Phase contrast microphotographs (1000×, under oil immersion).

acropetal chains nor secondary conidiophore formation was observed. From these results, it was concluded that both species belong to the Section *Noncatenae* ss. Neergard *fide* Rotem[5,6].

Both strains, however, significantly differed from one another in their conidial morphology. In strain P 0535, the mature rostrate conidia were 55~95 µm long×9~16 µm diam., the beak measuring 12~40 µm, which matched best the characteristics of *Alternaria carthami* in the key by ROTEM[6]. The other strain, P 0506 formed conidia without beaks, which were mostly produced singly or in short chains. Moreover, its conidia were smaller than those of strain P 0535 and measured only 30×55×15~22 µm. Out of the species described by ROTEM[6], these conidial characteristics matched best with *A. helianthi*.

Recent studies, employing, *e.g.* three dimensional structures of conidia and conidiophores, RAPD-PCR and secondary metabolite profiles for discrimination of these fungi[7~9] indicate that the taxonomy of *Alternaria* and allies will soon be the subject of considerable changes. As neither of the aforementioned methods was employed as yet to characterize the altersetin-producing strains in comparison with type materials, their current taxonomy is regarded as preliminary. Morphological characteristics of both strains are depicted in Fig. 1.

Detection, Isolation and Time Course of Production of Altersetin (**1**) and Tenuazonic Acid (**2**)

In shake flasks and stirring fermentors, similar secondary metabolite profiles were observed with both strains. Two main metabolites of the crude extracts of both investigated fungi showed antibacterial activities as revealed from testing of aliquots of fractions derived from preparative HPLC. One of those was identified as tenuazonic acid (**2**), a fairly well-known metabolite from *Alternaria* spp.[10,11]. In the course of the fermentation of both endophytic fungi, tenuazonic acid (**2**) was always detected earlier than altersetin (**1**). Regarding the structural similarities of the tetramic acid core of altersetin (**1**) to **2**, the former compound might either be formed from the latter, or both metabolites might be synthesized from a yet unknown precursor.

As exemplified further below, several metabolites of the decalin tetramic acid type antibiotics have previously been reported from cultures of mitosporic fungi. The present report apparently gives first evidence for the co-occurrence of a simple tetramic acid derivative and a substituted derivative of the equisetin type in cultures of the same microorganisms.

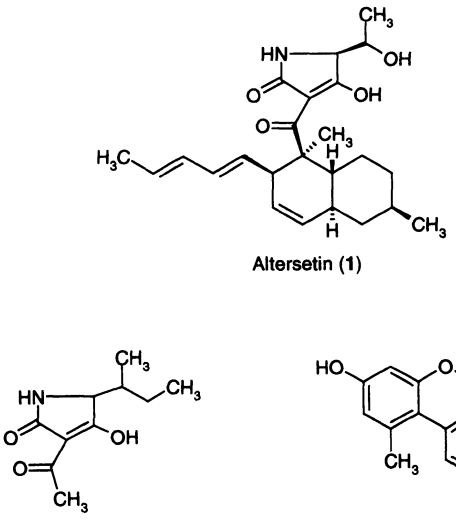
The production of altersetin (**1**) appears to be dependent on the composition of the culture medium. In the low nitrogen medium ZM/2 medium, no altersetin production occurred but instead two further typical *Alternaria* metabolites[10], namely alternariol (**3**) and alternariol monomethylether (**4**), were produced, along with tenuazonic acid (**2**). Their structures are depicted in Fig. 2.

Fermentation of *Alternaria* sp. P 0506 in 10-Liter Scale

Strain P 0506 was preferred to strain P 0535 because of higher altersetin production rates observed during fermentations in shake flasks as estimated by HPLC analyses and biological activity of crude extracts. In YMG

medium the production rate of altersetin (**1**) by strain P 0506 varied from 10 mg/liter culture broth in shake cultures to 1.5 mg/liter culture broth in a stirring fermentor with stirring speed of 200 rpm. This is probably due to sensibility of the producer strains to shear forces at higher stirring rates. Reduction of stirring resulted in increased production of altersetin (**1**). For 10-liter scale fermentation an aeration of 3 liters/minute and a stirring rate of 100 rpm was finally found to be optimal, leading to average production rates of 16.5 mg/liter. During the fermentation whose time course is illustrated in Fig. 3, even yields of 25 mg/liter of altersetin (**1**) were obtained. The compound was isolated from the crude mycelial extract as described in the Experimental section.

Fig. 2. Metabolites from *Alternaria* spp. P 0506 and P 0535.



Structure Elucidation of Altersetin (**1**)

The molecular formula of altersetin (**1**) was determined to be $C_{24}H_{33}NO_4$ by HR-ESI-MS. The proton NMR spectra of **1** in $CDCl_3$ or CD_3OD are complicated by occurrence of several subsets or broadening of NMR-signals. From our experience, the ^1H, ^{13}C and two-dimensional NMR spectra of altersetin (**1**) in [D_6]DMSO show one set of sharp signals (see Fig. 4 and Table 1). Referring to this data set, all 24 carbon and 33 proton atoms are detected, which can be assigned to four methyl groups, three methylene groups, six aliphatic as well as six olefinic methine groups and five quaternary carbon atoms. Three of the detected protons can be exchanged by deuterium.

Structure elucidation based upon the two-dimensional NMR spectra (see Fig. 5 for important HMBC correlations) resulted in the identification of a decalin moiety with an *exocylic* all-*trans*-diene residue ($^3J_{19\text{-H},20\text{-H}}=15.4$ Hz, $^3J_{17\text{-H},18\text{-H}}=14.1$ Hz) and two methyl substituents. From the

Fig. 3. Time course of fermentation of strain P 0506 (10 liters YMG culture).

Altersetin production was observed after *ca.* 190 hours.

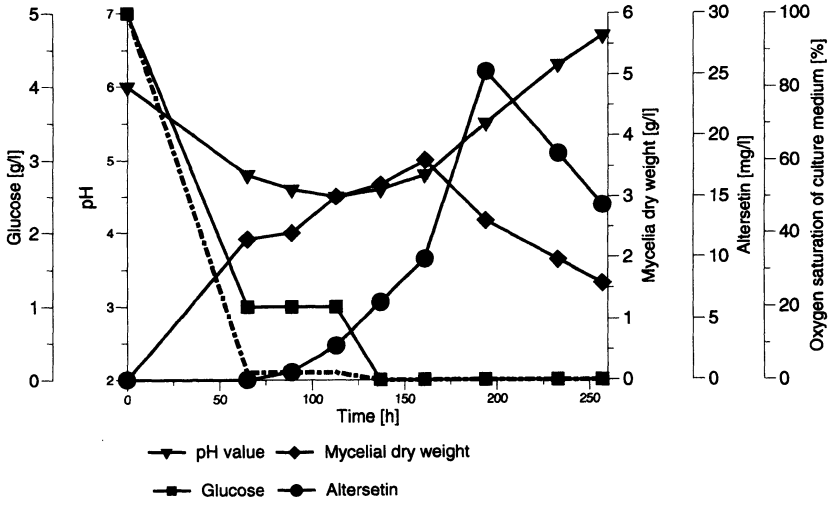


Fig. 4a. ^{1}H NMR spectrum of altersetin (**1**) (400 MHz, [D_6]DMSO).

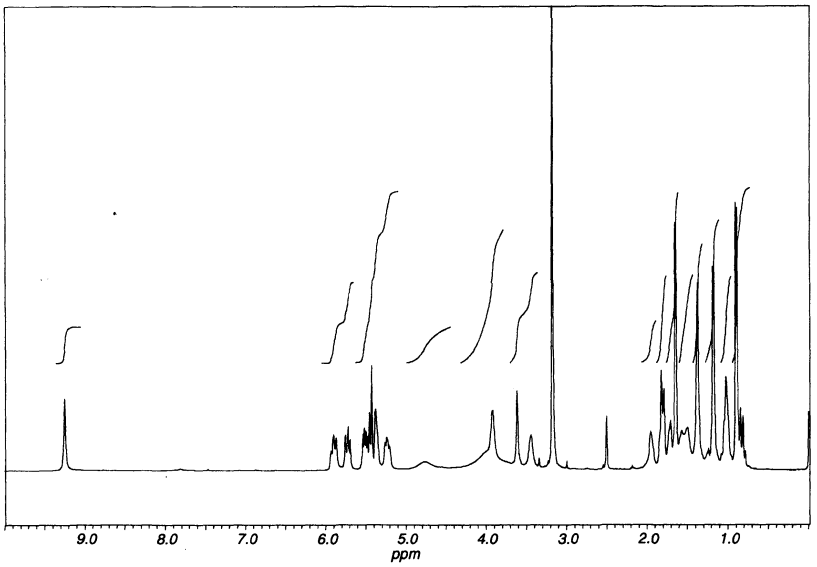
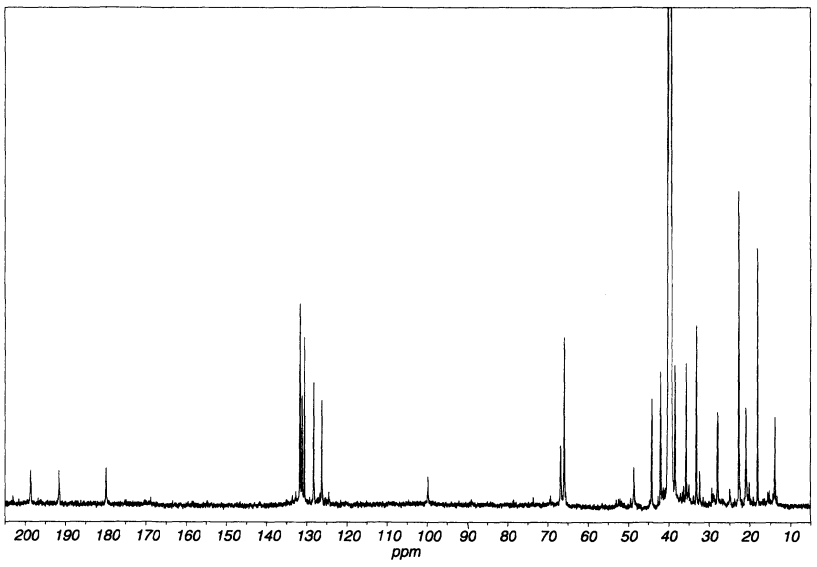


Fig. 4b. ^{13}C NMR spectrum of altersetin (**1**) (100 MHz, [D_6]DMSO).



$^{1}H,^{1}H$-COSY coupling information of the remaining NMR signals a CH_3–CH–CH–NH unit was deduced, which is part of a tetramic acid moiety as supported by comparison to corresponding data from tetramic acids in the literature: Proton NMR spectra of tetramic acids frequently indicate several tautomeric forms due to enolization[11].

Relative Configuration of Altersetin (**1**)

The relative stereochemistry of the decalin core of altersetin (**1**) was determined by a NOESY experiment (see Fig. 5 for NOESY correlations). No NOESY correlation can be detected between 8-H and 13-H in agreement with a *trans* ring fusion of the two six-membered rings. The

Table 1. ^{13}C and ^{1}H NMR data of altersetin (**1**) in [D_6]DMSO (100 and 400 MHz, resp.)

Pos.	δ(^{13}C) [ppm]	δ(^{1}H) [ppm]
NH		9.20 (s, br., 1H)
2	179.8	
3	99.7	
4	191.5	
5	66.8	3.61 (s, br., 1H)
6	198.6	
7	48.6	
8	ca. 39[+]	1.56 (m, 1H)
9α	27.9	1.90 (m, 1H)
9β		1.03 (m, 1H)[a]
10α	35.6	1.00 (m, 1H)[a]
10β		1.70 (m, 1H)
11	33.1	1.50 (m, 1H)
12α	42.0	0.80 (m, 1H)
12β		1.80 (m, 1H)[b]
13	38.4	1.80 (m, 1H)[b]
14	130.6	5.45 (dm, 1H)
15	126.2	5.35 (m, 1H)
16	44.2	3.45 (m, 1H)
17	131.1	5.24 (dd, 1H)
18	131.6	5.70 (dd, 1H)
19	131.6	5.90 (dd, 1H)
20	128.3	5.50 (dq, 1H)
21	18.1	1.64 (d, 3H)
22	13.8	1.35 (s, 3H)
23	22.7	0.85 (d, 3H)
24	65.9	3.91 (m, 1H)
25	21.0	1.17 (d, 3H)

[+] Under solvent signal.
[a],[b] Overlapping of signals in ^{1}H NMR.
J [Hz]: 11~23=6.3; 14~15=10.0; 16~17=9.0; 17~18=14.1; 18~19=10.9; 19~20=15.4; 20~21=6.7; 24~25=6.3

NOESY correlation peak between 10β-H and the bridgehead proton 8-H as well as between 10β-H and the methyl group 23-CH_3 indicates the *syn* relationship of these protons. The second bridgehead proton, 13-H, which is located on the other side of the molecule plane, shows a correlation signal to the methyl group 22-CH_3. Due to the correlation between the methyl group 22-CH_3 and the 16α-H the tetramic acid moiety and the pentadienyl side chain are located on the same side of the plane of the decalin system. No correlation signals can be detected between 8-H and 16-H and 22-CH_3 respectively, as they are located on different sides of the molecule. The overlapping ^{1}H NMR signals of 12β-H and 13-H at δ_H=1.80 and of 9β-H and 10α-H at δ_H=1.03 and δ_H=1.0 respectively, made the interpretation of some of the correlation signals difficult. According to these data the NOESY derived stereochemistry of altersetin (**1**) is depicted in Figs. 2 and 5. The stereochemistry of the tetramic acid moiety was not established by NOESY due to free rotation of the CC-single bond in the side chain of the heterocycle. A comparison of the NMR data of altersetin (see Table 1) with the NMR data of equisetin (**5**), a known tetramic acid with analogous relative configuration, is in agreement with these results[12~14].

Absolute Configuration of Altersetin (1)

The relative stereochemistry of altersetin (**1**) corresponds to that published for equisetin (**5**)[12] and phomasetin (**6**)[15], whose structures are depicted in Fig. 6. Even supported by total synthesis, the absolute configuration of equisetin (**5**) was previously established[16]. Phomasetin (**6**) is a structurally related homologue of equisetin (**5**) with opposite stereochemistry[15]. The CD spectra of all these compounds as well as of the synthetic C-5 equilibrated *ent*-equisetin (**7**) are given in literature (see Table 2). From the close correspondence of the CD data of altersetin (**1**) with the

Fig. 5. Selected HMBC and NOESY correlation signals of altersetin (**1**) (400 MHz, [D_6]DMSO]).

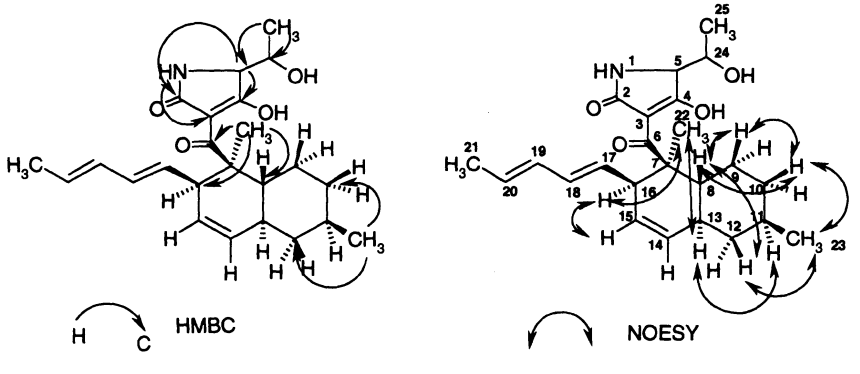


Table 2. CD data of various tetramic acids in MeOH.

λ_{max} ($\Delta\varepsilon$ [cm^2 * $mMol^{-1}$])				
Equisetin (**5**)*	Phomasetin (**6**)*	*Ent*-equisetin (**7**)*	Altersetin (**1**)	Hexahydro-altersetin (**8**)
227 (-5.5) 235 (-7.5)	225 (+3.2) 232 (+4.4)	235 (+6.0)	212 (-2.9) 232(-18.1)	215 (+2.8) 230 (-1.9) 239 (-2.4)
260 (-3.0)	260 (+1.0)	250 (+4.0)	253 (-5.0)	250 (-1.2)
290 (-8.9)	290 (+5.2)	291 (+12.5)	282 (-15.3)	284 (-7.8)
330 (0)	330 (0)	330 (0)	320 (+0.9) 337 (0)	315 (+1.9) 347 (0)

* From SINGH *et al.*[15].

data given for equisetin (**5**)[15], the identical absolute configuration for the decalin moiety is be assigned to altersetin (**1**).

The synthesis of the hydrogenation products **8** and **9** (see Fig. 6 and Experimental) enabled us to obtain CD data of compounds with the same tetramic acid moiety interfering with a less intensive chromophore in the decalin moiety or with no interference at all (Fig. 7). Lacking a chromophore in the decalin moiety, hexahydroaltersetin (**8**) shows a negative maximum at λ=284 nm in correspondence with the CD data in literature for the synthetic tetramic acid **11** (Fig. 7) from a L-amino acid and in contrast to decahydrofuligorubin (**12**) with opposite stereochemistry[17].

The absolute stereochemistry of altersetin (**1**) is assigned to be 5*S*, 7*S*, 8*R*, 13*S*, 16*R*.

Biological Activities

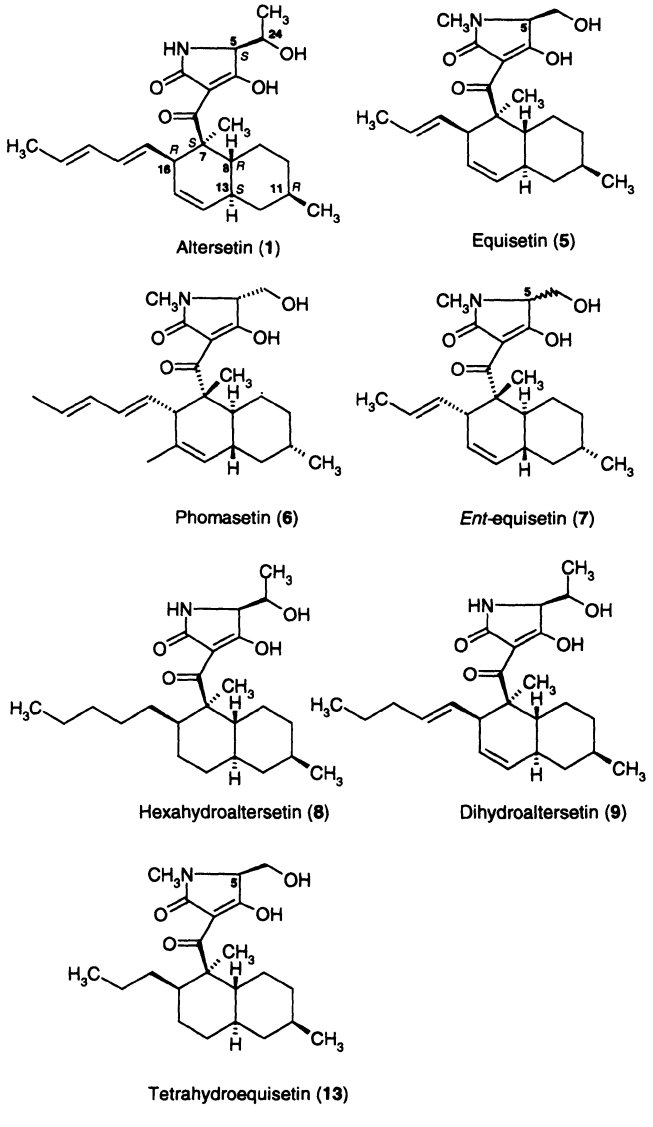
As shown in Table 3, altersetin (**1**) showed strong activities in the serial agar dilution assay against various human pathogenic bacteria. Gram-positive cocci were rather sensitive. The biological activities of altersetin (**1**) resembled those of equisetin (**5**); slightly weaker activities were observed with the hydrogenated activities of both compounds (Table 4).

Altersetin (**1**) showed also moderate *in vivo* activities in the murine sepsis model when administered i.p. in concentrations of 10 or 25 mg per kg. Four out of five animals in each test group had survived after six days, while none of the untreated control animals survived the second day after administration of *S. aureus in vivo*. However, at dosages higher than 25 mg per kg, adverse toxic effects were observed, and below 10 mg per kg, altersetin (**1**) showed no efficiacy.

Equisetin (**5**), the first discovered and best-investigated fungal metabolite of this type, had initially been described as an antibacterial agent and was patented as an antibiotic with selectivity against Staphylococci and Mycobacteria[18]. Later it was postulated that the activity of equisetin be due to inhibition of a specific ATPase[19] or ion carriers in the inner membranes of bacteria and mitochondria[20]. More recently, the HIV integrase inhibitory activity of equisetin and some novel homologues was patented[21]. Further homologous antimicrobial tetramic acids from plant-associated mitosporic fungi include vermisporin[22] and cryptocin[23].

Both the high structural similarity and the matching antibacterial spectrum of altersetin (**1**) and equisetin (**5**) point towards a similar mode of action of both compounds, although this remains to be confirmed, and the exact antibacterial target site of altersetin is also not clear yet.

Fig. 6. Chemical structures of altersetin (**1**), equisetin (**5**) and related compounds.



Experimental

General

Chemicals

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

Culture Media

YMG medium (D-glucose 0.4%, yeast extract 0.4%, malt extract 1.0% and 1 liter tap water, pH adjustment to 6.3 prior to sterilization) was generally used for isolation of endophytic fungi, maintenance and fermentation. For preparation of solid media, 1.5% of agar-agar (Difco) were added to YMG. To avoid bacterial contamination in the isolation medium, YMG agar was supplemented with aqueous solutions of Penicillin G (50 mg/liter) and Streptomycin sulfate (75 mg/liter), which were added under sterile conditions after sterilization of the solid medium. In all cases, the pH was adjusted to 6.3 prior to sterilization at 121°C for 30 minutes. For 10 liter scale fermentations, liquid YMG medium was supplemented with 1 ml of Antifoam SAG 5693 per liter. The sterilization was carried out *in situ* for 60 minutes at 121°C.

Medium $ZM^1/_2$ consisted of melasse 1%, oatmeal 1%, sucrose 8 g, mannitol 8 g, D-glucose 0.3%, $CaCO_3$ 0.3%, edamine 0.1%, $(NH_4)_2SO_4$ 0.1% and 1 liter tap water; pH was adjusted to 7.2 prior to sterilization at 121°C for 30 minutes.

Origin, Isolation and Taxonomy of Producer Strains

Among several other endophytic fungi, strains P 0506 and P 0535 were obtained according to a previosuly described method[24]. Leaves, fruits and other organs of apparently healthy plants were rinsed with deionized water and left for 1 minute in 95% EtOH, followed by incubation for 3 minutes in 3% sodium hypochlorite and again in 95% EtOH for another 30 seconds. Thereafter, the material was cut into pieces of *ca.* 1 cm^2 under sterile conditions and incubated on YMG plates containing antibiotics (see above). The plates were incubated at room temperature for several weeks and controlled daily under a dissecting microscope for mycelial outgrowth. If mycelia were observed, these were transferred immediately onto YMG plates without antibiotics. Pure cultures were obtained after several media transfers. The pure cultures are maintained at the Bayer Pharma Research Center in Greiner 123 278 kryo tubes (Solingen, Germany) containing 1 ml of spore suspensions in 10% glycerol in liquid N_2.

Fusarium equiseti strain CBS 406.86, known from literature to produce equisetin (**5**), was kindly provided by CBS (Baarn, Netherlands) and used for production of reference compounds in YMG shake flasks as described above for *Alternaria* spp.

Fermentation

Shake Flask Fermentations

Submerged cultures were propagated in 1 liter Erlenmeyer flasks without lateral indentation containing 300 ml of culture medium (YMG or $ZM^1/_2$, which were

Fig. 7. CD data ($\Delta\varepsilon$ [cm^2*$mmol^{-1}$]) of compounds **1** and **8** in MeOH and reference CD data of tetramic acids.[17]

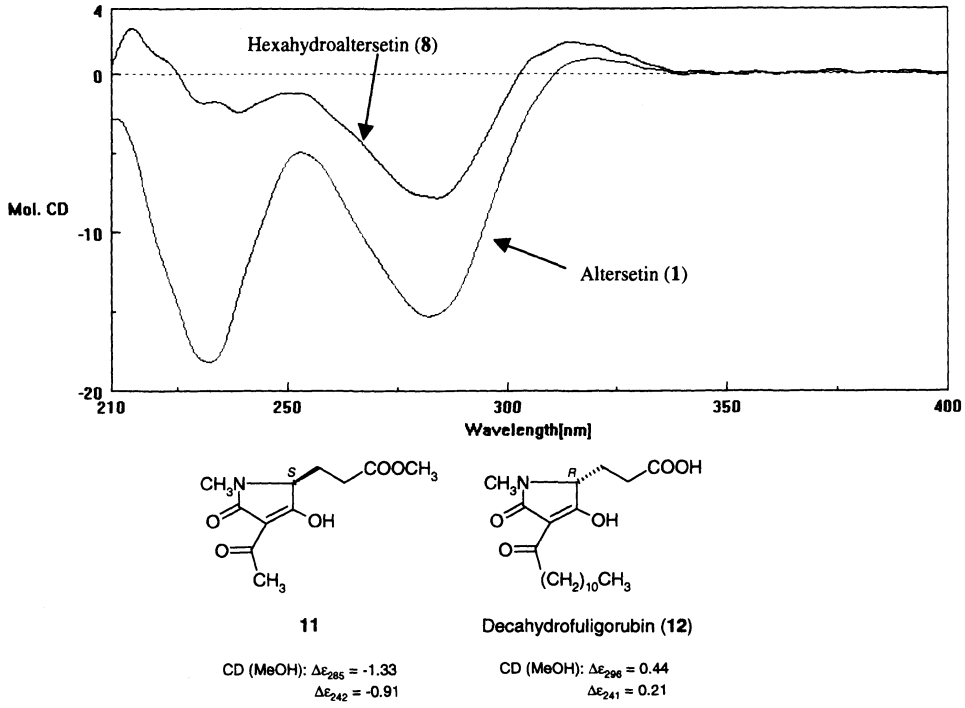


Table 3. MIC of altersetin (**1**) and ampicillin (control) in serial dilution assay against several species of bacteria and *C. albicans* after 20 hours.

Test organism	MIC [μg/ml]	
	Ampicillin (control)	Altersetin (**1**)
Staphylococcus aureus strain 133	< 0.12	0.12-0.25
Staph. aureus 9 TV	0.25-0.5	0.25
Staph. aureus 25508 (44)	0.25-0.5	0.25
Staph. aureus 25701	2	0.12-0.25
Staph. aureus 25470	n.t.	0.25
Streptococcus pyogenes WA	1	1
Str. pyogenes 4851	1	1
Str. pyogenes 4333	< 0.12	0.25
Str. pneumoniae Sp 665	0.25-0.5	0.5
Str. pneumoniae 13	< 0.12	1
Enterococcus faecium	1	1-2
Ent. faecalis	1	1
Branhamella catarrhalis	0.12-0.25	0.5
Proteus vulgaris	8	> 64
Proteus mirabilis	1	> 64
Klebsiella pneumoniae	16	> 64
Escherichia coli	2	> 64
Candida albicans	>64	>64

n.t.: not tested

Concentrations tested: 64 μg/ml and dilutions thereof.

Table 4. MIC of altersetin (**1**), equisetin (**5**) and their hydrogenated derivatives.

Test organism	MIC [μg/ml]#			
	Altersetin (**1**)	Hexahydro-altersetin (**8**)	Equisetin (**5**)	Tetrahydro-equisetin (**13**)
Staph. aureus 133	0.5	2	1	2
Staph. epidermidis 193	1	2	1	2
Staph. aureus 44	0.5	2	1	2
Str. pyogenes Wacker	2	4	(2) 4	8
Ent. faecium L 4001	4	4	4	8
Ent. faecalis 27251	2	2	(2) 4	4

MIC after 20 h

inoculated with 2 ml of a glycerol culture. The fermentations were performed at an incubation temperature of 23°C on a rotary shaker (140 rpm) for 144 hours.

Ten-Liter Scale Production Culture

A Biostat E fermentor (Braun Melsungen, Germany) containing 10-liter sterile YMG medium was inoculated with 300 ml of a YMG seed culture which had been propagated for 96 hours. The stirring speed initially varied from 50, 80, 100, and 150 to 200 rpm; later 100 rpm was found optimal for production. The culture media were supplied with 3 liters of sterile air/minute at 23°C. Daily samples (100 ml) were taken, and the mycelium was separated from the culture broth by centrifugation. The mycelia were extracted with 100 ml of MeOH in an ultrasonic bath for 30 minutes. The extracts were evaporated *in vacuo* and redissolved in 2 ml of MeOH, and 10 μl aliquots of these samples were subjected to HPLC-UV/visual analyses for estimation of altersetin (**1**) and tested in the agar diffusion assay against *E. coli* and *B. subtilis*. After 216 hours the culture was harvested, and the mycelia were separated from the culture supernatant by centrifugation (15 minutes at 1.000×*g*) and thereafter extracted as described below. The supernatant was discarded for lack of altersetin (**1**).

Isolation of Altersetin (**1**)

The combined wet mycelia of 50 YM shake flasks grown on a rotary shaker as described above were extracted two times with each three liters of acetone. The organic solvent was evaporated *in vacuo* to yield an aqueous residue, which was diluted with water to 1 liter and thereafter extracted three times with each 1 liter of EtOAc. The organic layers were pooled and evaporated *in vacuo* to yield an oily crude extract. This crude extract (4.4 grams) was redissolved in 10 ml of MeOH and bound to approximately 25 grams of C18 functionalized silica-gel (Sigma Aldrich 37.763-5). The residue of the extract/silica-gel mixture was applied onto a Flash chromatography column consisting of the same C18 material (column size 30×4.8 cm) equilibrated with 20% ACN. A gradient in three steps (500 ml of 50%, 80% and 100% ACN respectively) was employed for crude fractionation of the extract. While tenuazonic acid (**2**) (*ca.* 300 mg) was contained in the 50% ACN fraction in *ca.* 55% purity and the 80% ACN fraction was devoid of bioactive compounds, altersetin (**1**) only eluted at 100% ACN (fraction containing *ca.* 750 mg).

Final purification of altersetin (**1**) was achieved by preparative HPLC at room temperature. A Gilson Abimed (Ratingen, Germany) system (Software: Gilson Unipoint; Hardware: 306 binary pump system, 205 fraction collector, 119 UV/Vis detector, 806 manometric module, and 811C dynamic mixer) was employed. The following parameters were selected: Flow: 7 ml/minute; mobile phase 0.1% TFA:ACN; column: Merck LichroSorb RP-18, 7 μm, 250×25 mm; gradient: Starting with t=0′ (20% ACN); linear to t=60′ (100% ACN); isocratic to t=88′ (100% ACN). The 100% ACN eluate from the previous separation was portioned, and aliquots of *ca.* 250 mg thereof were applied in each independent HPLC experiment. Altersetin (**1**) eluted from the column at Rt of 72~73 minutes. From 50 shake flasks (equivalent to 15 liters of culture medium), final yields of 142 mg of pure altersetin (**1**) were obtained. Similar yields (approximately 10 mg/liter) were obtained upon scale-up from the mycelial extract of a 10-liter culture performed under the above described conditions, applying an analogous downstream processing procedure.

Spectral Analysis

NMR spectra were recorded at 300 K with a Bruker DRX 400 spectrometer (400.13 MHz) with the solvent peak as internal reference ([D_6]DMSO: δ_H 2.49, δ_C 39.7). The NOESY experiment was recorded with the Bruker pulsprogram 'noesytp' (delay d8: 0.6 s).

HPLC-UV/visual was carried out on a HP1090 (Hewlett Packard, Waldbronn, Germany) unit with automated sample injector and diode array detector employing the following instrumental conditions (system 1): Column: Eurospher-100 C_{18}, 5 μm; 2×125 mm (Knauer); eluant A: water+0.05% TFA, eluant B: acetonitrile+0.05% TFA; gradient: 0 minute 10% B; 1 minute: 10% B; 15 minutes: 100% B; 17 minutes: 100% B; 18 minutes: 0% B; flow 0.4 ml/minute; temperature 40°C. UV spectra were recorded in the range of 200~400 nm. Preparative HPLC was performed with a Gilson 305/306 unit. A Gilson 215 liquid handler was used for automated injection and fraction collection. For detection a Gilson 170 diode array detector was employed. The Rt of the isolated compounds are further experimental parameters described below. During fermentation, altersetin (**1**) was estimated using external and internal standards.

HPLC-MS analyses were performed using the following conditions (System 2): TSP liquid chromatograph directly coupled with a MAT 900S mass spectrometer (Finnigan, Bremen, Germany) in the positive electrospray ionisation (ESI pos.) mode with the following instrumental conditions: Column: Symmetry-C_{18}, 5 μm; 2.1×150 mm (Waters); eluant A: 0.01 M HCl, eluant B: acetonitrile; gradient: 0 minute 10% B; 9 minutes: 90% B; 18 minutes: 90% B; flow 0.6 ml/minute. temperature: 50°C. MS parameters: scan speed: 1.5 seconds/decade against scan; scan range 150~1200; resolution: 2000; capillary voltage: 4.750 V; identity of nebulizer gas: N_2 99.999%; nebulizer gas pressure: 5 bar; heated capillary temperature: 220°C. EI-MS and HR-MS were performed on a Finnigan MAT 95. CD spectra were recorded on a Jasco J 500 spectrometer at the University of Göttingen, Germany, Institute of Organic Chemistry by Prof. Dr. A. ZEECK and coworkers.

Altersetin (**1**)

Colorless solid; HPLC: Rt=13.0 minutes (system 1); UV λ_{max} MeOH nm (lg ε) 234 (4.37), 286 (3.84); CD λ_{max} MeOH nm: see Table 2; ^{1}H-NMR (400 MHz, [D_6]DMSO): see Fig. 4 and Table 1; ^{13}C-NMR (100 MHz, [D_6]DMSO): see Fig. 4 and Table 1; HPLC-MS: Rt=9.9 minutes (system 2), ESI pos. *m/z* (%) 441 (17) $[M+CH_3CN+H]^+$, 400 (100) $[M+H]^+$, 382 (7) $[M-H_2O+H]$; HR-MS for $C_{24}H_{34}NO_4$: calcd. 400.2488, found 400.2495.

Hydrogenation of Altersetin (**1**)

To a solution of 20 mg (0.05 mmol) altersetin (**1**) in MeOH, palladium (10% Pd/C) was added in catalytic amount. The mixture was hydrogenated for 1 hour at room temperature under atmospheric pressure. The catalyst was removed by filtration. The residue of the filtrate was purified by preparative reverse phase HPLC using the following conditions: Column: Nucleosil C_{18}, 7 μm; 8×125 mm (MZ Analysentechnik); eluant A: water+0.5% TFA, eluant B: acetonitrile+0.5% TFA; gradient: 0 minute 10% B; 25 minutes: 100% B, flow 5.25 ml/minute. Rt was 22~23 minutes (**8**) and 21~22 minutes. (**9**). A tetrahydroaltersetin (**10**) was detected as a side product in small amounts by means of HPLC-UV/Visual and HPLC-MS, but was not isolated.

Hexahydroaltersetin (**8**)

Yield: 2 mg (0.005 mmol, 10%); HPLC: Rt=14.9 minutes (system 1); UV λ_{max} MeOH nm (lg ε) 248 (3.82), 285 (4.02); CD λ_{max} MeOH nm: see Table 2; ^{1}H-NMR (400 MHz, [D_6]DMSO): assigned signals δ 9.22 (s, 1H, 1-NH), 3.89 (m, 1H, 24-H), (1H, 5-H, obscured by water signal), 3.16 (s, OH), 2.07 (s, OH), 1.64~1.57 (m, 3H, 9_a-H, 10_a-H, 12_a-H), 1.45 (m, 1H, 13-H), 1.39 (s, 3H, 22-CH_3), 1.34 (m, 1H, 11-H), 1.18~1.12 (m, 20-H), 1.15 (d, $J_{24,25}$=6.6 Hz, 25-CH_3), 0.97~0.88 (m, 2H, 9_b-H, 10_b-H), 0.82 (d, $J_{11,23}$=6.4 Hz, 3H, 23-CH_3), 0.77 (t, $J_{20,21}$=7.0 Hz, 21-CH_3), 0.74 (m, 1H, 12_b-H); HPLC-MS: Rt=11.5 minutes (system 2), ESI pos. *m/z* (%) 447 (9) $[M+CH_3CN+H]^+$, 406 (100) $[M+H]^+$; EI-MS *m/z* (%) 405 (5) $[M^+]$, 362 (12), 251 (100), 175 (18), 160 (20), 149 (39), 121 (32), 105 (22), 91 (26), 83 (19), 69 (26), 57 (44), 43 (43), 41 (42); HR-MS for $C_{24}H_{39}NO_4$: calcd. 405.2879, found 405.2830.

Dihydroaltersetin (**9**)

Yield: 2 mg (0.005 mmol, 10%); HPLC: Rt=14.1 minutes (system 1); UV λ_{max} MeOH nm (lg ε) 250 nm (3.60), 286 (3.77); CD λ_{max} MeOH nm ($[\theta]_M$) 223 (−4255), 231 (−7447), 234 (−7234), 239 (−8298), 248 (−7021), 282 (−22538), 322 (+3134), 346 (0); ^{1}H-NMR (400 MHz, [D_6]DMSO): assigned signals δ 9.24 (s, 1H, 1-NH), 5.40~5.37 (m, 2H, 17-H and 18-H), 5.14~5.15 (m, 2H, 14-H and 15-H), 3.93 (m, 1H, 24-H), 3.58 (s, 1H, 5-H), (1H, 16-H, obscured by water signal), 1.95 (m, 1H, 9_a-H), 1.85~1.70 (m, 3H, 13-H, 12_a-H, 10-H), 1.59 (m, 1H, 8-H), 1.50 (m, 1H, 11-H), 1.37 (s, 3H, 22-CH_3), 1.17 (d, $J_{24,25}$=6.6 Hz, 25-CH_3), 1.20~1.16 (m, 20-H), 1.05~1.00 (m, 2H, 10-H and 9_b-H), 0.83~0.80 (m, 1H, 12_b-H), 0.90 (d, $J_{11,23}$=6.6 Hz, 3H, 23-CH_3), 0.74 (t, $J_{20,21}$=7.3 Hz, 21-CH_3); HPLC-MS: Rt=10.4 minutes (system 2), ESI pos. *m/z* (%) 443 (2)

$[M+CH_3CN+H]^+$, 402 (100) $[M+H]^+$, 384 (11) $[M-H_2O+H]^+$; EI-MS *m/z* (%) 401 (1) $[M^+]$, 362 (10), 251 (100), 175 (14), 160 (20), 149 (23); HR-MS for $C_{24}H_{35}NO_4$: calcd. 401.2566, found 401.2557.

Tetrahydroaltersetin (**10**)

Not isolated; HPLC-MS: Rt=10.9 minutes (system 2), ESI pos. *m/z* (%) 404 (100) $[M+H]^+$, 386 (2) $[M-H_2O+H]^+$.

Hydrogenation of Equisetin (**5**)

Equisetin (**5**) was isolated from fermentations of *Fusarium equiseti* strain CBS 406.86 in YMG medium, using the same protocols for fermentation and isolation as in case of altersetin (see above). The compound eluted from the prep. C18 column at Rt=64~65 minutes. After prep. HPLC, 14 mg of the pure compound were obtained from the mycelia of five shake flasks containing an overall culture volume of 1500 ml.

HPLC: Rt=13.0 minutes (system 1); HPLC-UV/visual: λ_{max}=233 nm, 293 nm; HPLC-MS: Rt=9.6 minutes (system 2), ESI pos. *m/z* (%) 374 (100) $[M+H]^+$, 356 (5) $[M-H_2O+H]^+$.

To a solution of 10 mg (0.027 mmol) equisetin (**5**) in MeOH, palladium (10% Pd/C) was added in catalytic amount. The mixture was hydrogenated for 1 hour at room temperature under atmospheric pressure. The catalyst was removed by filtration. The residue of the filtrate was purified by preparative reverse phase HPLC using the following conditions: Nucleosil C_{18}, 7 μm; 8×125 mm (MZ Analysentechnik); eluant A: water+0.05% TFA, eluant B: acetonitrile+0.05% TFA; gradient: 0 minute 10% B; 25 minutes: 100% B, flow 5.25 ml/minute. Rt of the product (**13**)=21 minutes.

Tetrahydroequisetin (**13**)

Yield: 2.2 mg (0.0058 mmol, 22%); HPLC: Rt=13.9 minutes (system 1); HPLC-UV/visual: λ_{max} 230 nm, 293 nm.

Tenuazonic Acid (**2**), Alternariol (**3**) and Alternariol Monomethylether (**4**)

The identification of these compounds was accomplished by comparison with their matching HPLC-UV/visual and HPLC-MS data (Rt and spectra) with those of authentic standards provided by Sigma-Aldrich.

Biological Assays

Agar Diffusion Assay

The agar diffusion assay was carried out for bioassay-guided isolation of altersetin (**1**) according to the classical method described by ZÄHNER[25], using *B. subtilis* strain ATCC 6363 and *Yarrowia lipolytica* (Strain HT 20, Bayer) as test organisms. Aliquots of MeOH solutions equivalent to 10~100 μg of crude extracts and fractions obtained during fermentation and chromatography, respectively, were pipetted on paper disks (diameter 6 mm) and air-dried. The paper disks were incubated on agar plates containing the respective organisms in initial titers of 5×10^{-4} cells, and the diameters of inhibition zones were determined after 24 hours of incubation. For example, 100 μg of pure altersetin (**1**) gave an inhibition zone of 43 mm against *B. subtilis* but was devoid of activity against *Y. lipolytica*.

MIC values were determined using a serial dilution test on Isosensitest-agar (Oxoid). The inoculum was approximately $10\times e^4$ bacteria per inoculation point (Denley inoculator). MIC were determined after incubation of the test strains for 16~20 hours at 37°C.

Characteristics of Pathogenic Test Strains

All test strains used in the current study were clinical isolates from human infections. The strains are maintained at the Bayer Pharma Research Center, Wuppertal, Germany, at −80°C after purification and identification according to standard procedures.

Murine Sepsis Model (*In Vivo*)–Systemic Infection with *S. aureus* 133

Bacteria from an overnight culture of *S. aureus* strain 133 were grown to the logarithmic growth phase in BHI broth. The culture was precipitated and washed twice with phosphate buffered saline. Bacteria were suspended in phosphate buffered saline containing 10% mucin to a cell number of $4\times e^6$ cells/ml. Female CFW1-mice (20 g) were infected with 0.25 ml of the bacterial suspension. The tests were dosed i.p. at 30 minutes post infection. The survival in each treatment group was monitored for 6 days.

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Dedication

This paper is dedicated to the memory of our dear colleague, Prof. Dr. KLAUS SCHALLER (Bayer AG, Anti-infective Research), deceased in 2000.

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