Eutypoids B−E Produced by a *Penicillium* sp. Strain from the North Sea

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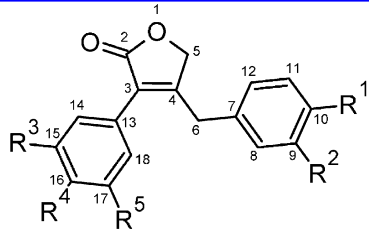
Crude extracts of the *Penicillium* sp. strain KF620 isolated from the North Sea showed antimicrobial activities against *Xanthomonas campestris* and *Candida glabrata*. Purification of the extracts led to the isolation of the new aromatic butenolides eutypoids B (**1**), C (**2**), D (**3**), and E (**4**). Their structures were elucidated by NMR spectroscopy and supported by HRESIMS and UV data. The antibacterial activity of the crude extracts was due to the presence of the known diketopiperazine fellutanine (cyclo(Trp-Trp)). The eutypoids were neither cytotoxic nor antibacterial, but inhibited the activity of glycogen synthase kinase-3β.

Various strains of the genus *Penicillium* are known for their ability to produce bioactive compounds.[1,2] During our investigations of marine fungi isolated from the North Sea, *Penicillium* sp. KF620 was selected for detailed study. This strain showed profound antibacterial activity in combination with a diverse metabolite spectrum. The metabolites were isolated and identified as the new aromatic butenolides eutypoids B−E (**1**−**4**) as well as the known diketopiperazine fellutanine (cyclo(Trp-Trp)).[3] The only previously reported phenyl- and benzyl-substituted butenolide natural products without a substitution at C-5 and C-6 are eutypoid A[4] and gymnoascolide A.[5]



	R^1	R^2	R^3	R^4	R^5
Gymnoascolide A	-H	-H	-H	-H	-H
Eutypoid A	-H	-H	-H	-OH	-H
Eutypoid B (**1**)	-OH	-H	-H	-OH	-H
Eutypoid C (**2**)	-OH	-OH	-H	-OH	-H
Eutypoid D (**3**)	-OH	-H	-OH	-H	-OH
Eutypoid E (**4**)	-OH	-OH	-OH	-H	-OH

***Penicillium* sp.** KF620 was grown in static culture and extracted with EtOAc to give a crude extract. The extract was fractionated by column chromatography on Sephadex LH-20 (MeOH) and finally by preparative HPLC to yield compounds **1**−**4** as colorless solids.

High-resolution ESIMS mass measurements of **1** (measured 305.0792, calculated 305.0784 $[M + Na]^+$) gave the molecular formula $C_{17}H_{14}O_4$, which required 11 degrees of unsaturation and was fully consistent with the spectroscopic data of eutypoid B. The structure of **1** was established on the basis of one- and two-dimensional NMR spectra (^1H, ^{13}C (^1H decoupled and DEPT), $^1H-^1H$ COSY, $^1H-^{13}C$ HSQC, and $^1H-^{13}C$ HMBC). The ^{13}C NMR spectrum included four particularly intense signals accounting for two magnetically equivalent carbons each, C-8 plus C-12 (δ_C 130.6 ppm), C-9 plus C-11 (δ_C 116.8 ppm), C-14 plus C-18 (δ_C 131.5 ppm), and C-15 plus C-17 (δ_C 116.4 ppm), indicating the presence of two para-substituted aromatic rings. Two quaternary carbons with the shifts δ_C 128.6 ppm (C-7) and 122.3 ppm (C-13), which connected the aromatic rings to the rest of the molecule, and two oxygen-bearing aromatic carbons, C-10 (δ_C 157.7 ppm) and C-16 (δ_C 159.1 ppm), were also detected. The ^{13}C NMR spectrum revealed the presence of an ester/lactone carbonyl group C-2 (δ_C 176.3), two methylene carbons C-5 (δ_C 72.8 ppm) and C-6 (δ_C 33.8 ppm), and two sp^2-hybridized carbons C-3 (δ_C 127.2 ppm) and C-4 (δ_C 162.4 ppm), consistent with a tetrasubstituted double bond conjugated to the carbonyl function. The structural elements already deduced from the ^{13}C NMR spectrum could be confirmed by the analysis of the ^1H and the two-dimensional NMR spectra. Thus, the equivalent aromatic methine protons H-8 plus H-12 (δ_H 6.99) and H-9 plus H-11 (δ_H 6.73) gave a doublet each due to coupling with each other and showed $^1H-^{13}C$ HMBC correlations to the quaternary carbons C-7 and C-10. The same pattern could be observed for the second aromatic ring consisting of C-14 plus C-18 (δ_H 7.35), C-15 plus C-17 (δ_H 6.87), and the quaternary carbons C-13 and C-16. The para substitution of each aromatic ring with a hydroxy group was obvious from the shifts of C-10 and C-16. The second aromatic ring was directly bonded to the double bond $\Delta^{3,4}$, while the first one was connected via C-6, which could be proven by $^1H-^{13}C$ HMBC correlations from H_2-6 (δ_H 3.87 ppm) to C-8 plus C-12. Additionally, the signal of H_2-6 coupled to C-13 from the second aromatic ring and all carbons belonging to the α,β-unsaturated γ-butyrolactone. The methylene group belonging to the γ-butyrolactone ring, CH_2-5 (δ_C 72.8, δ_H 4.71), had to be located adjacent to an oxygen atom, as proven by its chemical shifts. As the double bond, the ester carbonyl, and the two aromatic rings accounted for only 10 degrees of unsaturation, the presence of a lactone ring, which was already indicated by the chemical shifts, was confirmed. Thus, the structure of eutypoid B (**1**) was established.

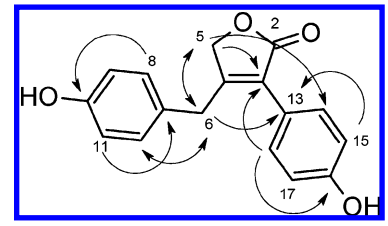


Figure 1. Selected $^1H-^{13}C$ HMBC correlations relevant for the structure elucidation of **1**.

1 and the three derivatives eutypoids C (**2**), D (**3**), and E (**4**) could be shown to share the same α,β-unsaturated γ-butyrolactone

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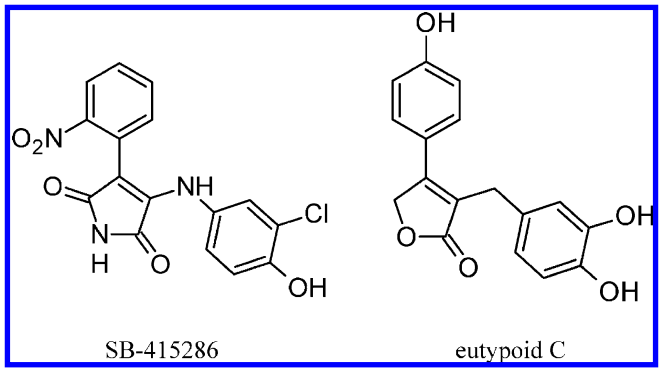

Figure 2. Structures of SB-415286 and **2**.

substructure substituted with different aromatic moieties. Their structures, just as that of **1**, were confirmed by the analysis of different one- and two-dimensional NMR spectra. The substitution patterns of the aromatic rings could be deduced from the ^{1}H NMR spectra. The high-resolution mass measurement of **2** (measured 321.0733, calculated 321.0733 $[M + Na]^+$) and the resulting molecular formula $C_{17}H_{14}O_5$ indicated the substitution of the two aromatic rings with three hydroxy groups. As in **1**, H-14 plus H-18 (δ_H 7.35 ppm) and H-15 plus H-17 (δ_H 6.87 ppm) were shown to be equivalent aromatic methines, which coupled with a coupling constant of $J = 8.7$, evidence of a para-substituted phenyl ring. The other aromatic ring was substituted with two hydroxy groups adjacent to each other, as shown by the signal splitting patterns of the aromatic protons. H-12 showed ^3J coupling ($J = 8.0$) to H-11 and ^4J coupling ($J = 2.1$) to H-8, which proved the second aromatic ring to be 1,2,4-substituted.

3 had the same molecular formula as **2** and also contained one para-substituted aromatic ring. The signals of the remaining aromatic protons of **3** were partly overlapping, but it was obvious that all coupling constants were below 3 Hz, which proved that none of them were neighboring protons. Therefore, the second aromatic ring could only be 1,3,5-substituted.

4 showed a mass increase of 16 compared to **2** and **3**, accounting for one additional hydroxy group. From the signal splitting patterns one 1,2,4- and one 1,3,5-substituted aromatic ring could be deduced. The structures of all three derivatives were confirmed by analysis of the two-dimensional NMR spectra.

The simplest structure of a butenolide would be 2-furanone, which is a common substructure of natural products from various sources including fungi and bacteria.[6] Phenyl- and benzyl-substituted butenolides have previously been described as fungal metabolites. Related structures are gymnoascolides A−C produced by *Gymnoascus reessii*,[5] eutypoid A isolated from *Eutypa* sp.,[4] microperfuranone reported from *Anixiella micropertusa*,[7] and 9-hydroxymicroperfuranone described as a metabolite of *Emericella quadrilineata*.[8] The highly hydroxylated aromatic rings in combination with no substitution at C-5 and C-6 sets the new compounds apart from the known metabolites.

1−**4** were evaluated in several *in vitro* enzyme assays including glycogen synthase kinase-3β (GSK-3β) as a target. The serine/threonine protein kinase GSK-3β is involved in numerous signaling pathways and has emerged as a key target for the treatment of Alzheimer's disease and type 2 diabetes.[9]

Eutypoids B−E show moderate inhibitory activities against the enzyme GSK-3β with IC_{50} values in the low micromolar range. Interestingly, the synthetic inhibitor of GSK-3β SB-415286 (3-(3-chloro-4-hydroxyphenylamino)-4-(2-nitrophenyl)-1*H*-pyrrole-2,5-dione), discovered in a chemical-synthesis screening approach, shows some structural relationships with the eutypoids (Figure 2).[10] Thus, high-throughput screening of synthetic compound libraries and traditional natural product research can result in the discovery of structurally similar compounds as inhibitors of the same target. However, the eutypoids are approximately 30 times less active than SB-415286 (Table 3).

Eutypoids B−E were inactive in cytotoxicity and antimicrobial bioassays. No biological activity has been reported for eutypoid A, gymnoascolides B/C, and microperfuranone and its hydroxide, either, although gymnoascolide A is active against *Septoria nodorum*.[5] Eutypoids B−E were inactive against *Septoria tritici*.

Experimental Section

General Experimental Procedures. UV spectra were obtained on a NanoVue photometer (GE Healthcare). NMR spectra were recorded on a Bruker DRX500 spectrometer (500 and 125 MHz for ^{1}H and ^{13}C NMR, respectively), using the signals of the residual solvent protons and the solvent carbons as internal references (δ_H 3.31 and δ_C 49.0 ppm for methanol-d_4). High-resolution mass spectra were acquired on a benchtop time-of-flight spectrometer (MicrOTOF, Bruker Daltonics) with positive electrospray ionization. Analytical reversed-phase HPLC-UV/MS experiments were performed using a C_{18} column (Phenomenex Onyx Monolithic C18, 100 × 3.00 mm) applying an H_2O (A)/MeCN (B) gradient with 0.1% HCOOH added to both solvents (gradient: 0 min 5% B, 4 min 60% B, 6 min 100% B; flow 2 mL/min) on a VWR Hitachi Elite LaChrom system coupled to an ESI-ion trap detector (Esquire 4000, Bruker Daltonics).

Preparative HPLC was carried out using a Merck Hitachi system consisting of an L-7150 pump, an L-2200 autosampler, an L-2450 diode array detector, and a Phenomenex Gemini C18 110A AXIA, 100 × 21.20 mm, or a Phenomenex Luna silica 5 μm, 250 × 10.00 mm column.

Microorganisms and Fermentation. Strain KF620 was isolated from a water sample collected during a research cruise on the North Sea. By morphological characteristics the fungus could be identified as a member of the genus *Penicillium.* Sequence analysis of the 18S rRNA gene and the internal spacer region (ITS-1) revealed 99% similarity to *P. verrucosum*, *P. viridicatum*, *P. allii*, *P. albocoremium*, *P. neoechinulatum*, *P. hordei*, and *P. tricolor*, but did not allow identification on the species level.

For the production of secondary metabolites the fungus was cultivated in 9 L of liquid saline (3% Tropic Marin sea salt, Dr. Biener GmbH, Wartenberg, Germany) Wickerham medium[11] (12 × 750 mL) at 28 °C for 21 days in static cultures.

Isolation of Eutypoids B−E. A 9 L portion of a *Penicillium* sp. KF620 culture was separated into culture filtrate and mycelium. The culture filtrate was extracted with EtOAc. The organic extract was concentrated *in vacuo* to dryness to give a crude extract of 0.64 g. The extract was fractionated by column chromatography on Sephadex LH-20 (3 × 80 cm, MeOH). Compounds **1**−**4** eluted in one fraction and fellutanine in an other. All fractions were purified by preparative HPLC (Phenomenex Gemini C18 110A AXIA column; linear gradient: 0 min 20% MeCN, 15 min 50% MeCN; flow 15 mL/min; detection at 280 nm) to yield 35 mg of compound **1** (t_R5.8 min), 110 mg of compound **4** (t_R 7.6 min), a mixture of compound **2** and **3** (t_R 9.3 min), and 29 mg of fellutanine (t_R 9.7 min). The mixture of compounds **2** and **3** was further purified by preparative HPLC (Phenomenex Luna silica 5 μm, linear gradient: 0 min 85% 2-propanol, 10 min 95% 2-propanol; flow 3 mL/min; detection at 280 nm) to yield 20 mg of compound **2** (t_R 4.1 min) and 36 mg of compound **3** (t_R 5.1 min).

Table 1. NMR Spectroscopic Data (500 MHz, methanol-d_4) of Eutypoid B (**1**)

position	δ_C, mult.	δ_H, (J in Hz)	COSY	HMBC
2	176.3, C			
3	127.2, C			
4	162.4, C			
5	72.8, CH_2	4.71, s	6	2, 3, 4, 6, 13, 14 + 18
6	33.8, CH_2	3.87, s	5, 8 + 12	2, 3, 4, 5, 7, 8 + 12, 13
7	128.6, C			
8 + 12	130.6, CH	6.99, d (8.6)	6, 9 + 11	6, 8 + 12, 9 + 11, 10
9 + 11	116.8, CH	6.73, d (8.6)	8 + 12	7, 10, 9 + 11
10	157.7, C			
13	122.3, C			
14 + 18	131.5, CH	7.35, d (8.8)	15 + 17	3, 14, 15 + 17, 16
15 + 17	116.4, CH	6.87, d (8.8)	14 + 18	13, 15 + 17, 16
16	159.1, C			

Table 2. NMR Spectroscopic Data (500 MHz, methanol-d_4) of the Eutypoids

	eutypoid B (**1**)		eutypoid C (**2**)		eutypoid D (**3**)		eutypoid E (**4**)	
position	δ_C, mult.	δ_H, (J in Hz)	δ_C, mult.	δ_H, (J in Hz)	δ_C, mult.	δ_H, (J in Hz)	δ_C, mult.	δ_H, (J in Hz)
2	176.3, C		176.3, C		176.3, C		176.3, C	
3	127.2, C		127.2, C		127.2, C		127.2, C	
4	162.4, C		162.4, C		162.4, C		162.4, C	
5	72.8, CH_2	4.71, s	72.8, CH_2	4.71, s	72.7, CH_2	4.68, s	72.7, CH_2	4.68, s
6	33.8, CH_2	3.87, s	34.0, CH_2	3.79, s	33.8, CH_2	3.86, s	34.0, CH_2	3.80, s
7	128.6, C		129.4, C		129.3, C		129.4, C	
8	130.6, CH	6.99, d (8.6)	116.6, CH	6.59, d (2.1)	130.7, CH	6.99, d (8.5)	116.8, CH	6.59, d (2.1)
9	116.8, CH	6.73, d (8.6)	146.4, C		116.7, CH	6.73, d (8.5)	146.7, C	
10	157.7, C		145.5, C		157.6, C		145.5, C	
11	116.8, CH	6.73, d (8.6)	116.7, CH	6.71, d (8.0)	116.7, CH	6.73, d (8.5)	116.8, CH	6.71, d (8.0)
12	130.6, CH	6.99, d (8.6)	120.9, CH	6.49, dd (8.0, 2.1)	130.7, CH	6.99, d (8.5)[a]	120.9, CH	6.51, dd (8.0, 2.1)
13	122.3, C		122.0, C		127.2, C		127.2, C	
14	131.5, CH	7.35, d (8.8)	131.4, CH	7.35, d (8.7)	117.2, CH	7.00, m[a]	117.2, CH	7.03, dd (1.0, 1.5)
15	116.4, CH	6.87, d (8.8)	116.4, CH	6.87, d (8.7)	146.4, C		146.4, C	
16	159.1, C		159.0, C		122.0, CH	6.85, m[a]	122.0, CH	6.85, m[a]
17	116.4, CH	6.87, d (8.8)	116.4, CH	6.87, d (8.7)	147.1, C		147.1, C	
18	131.5, CH	7.35, d (8.8)	131.4, CH	7.35, d (8.7)	116.8, CH	6.85, m[a]	116.3, CH	6.85, m[a]

[a] Signals are overlapping.

Table 3. GSK-3β Inhibitory Activity of the Eutypoids and SB-415286

	GSK-3β inhibition IC_{50} [μM]
SB-415286	0.09 (±0.01)
eutypoid B	0.78 (±0.02)
eutypoid C	0.67 (±0.04)
eutypoid D	4.08 (±1.4)
eutypoid E	2.26 (±0.13)

Eutypoid B (1): colorless, amorphous solid; UV (MeOH) λ_{max} (log ε) 277 (4.05), 224 (4.23) nm; for 1D and 2D NMR data see Table 1; HRESIMS m/z 305.0792 (calcd for $C_{17}H_{14}NaO_4$, 305.0784).

Eutypoid C (2): colorless, amorphous solid; UV (MeOH) λ_{max} (log ε) 282 (3.91), 222 (4.20) nm; for 1D and 2D NMR data see Table 2−3 and SI; HRESIMS m/z 321.0733 (calcd for $C_{17}H_{14}NaO_5$, 321.0733).

Eutypoid D (3): colorless, amorphous solid; UV (MeOH) λ_{max} (log ε) 282 (3.91), 222 (4.20) nm; for 1D and 2D NMR data see Table 2 and SI; HRESIMS m/z 321.0733 (calcd for $C_{17}H_{14}NaO_5$, 321.0733).

Eutypoid E (4): colorless, amorphous solid; UV (MeOH) λ_{max} (log ε) 288 (3.94), 222 (4.25) nm; for 1D and 2D NMR data see Table 2 and SI; HRESIMS m/z 337.0680 (calcd for $C_{17}H_{14}NaO_6$, 337.0683).

Antibacterial and Antifungal Assays. Antimicrobial assays were performed using *Bacillus subtilis* (DSM 347), *Erwinia amylovora* (DSM 50901), *Escherichia coli* K12 (DSM 498), *Pseudomonas fluorescence* (NCIMB 10586), *Propionibacterium acnes* (DSM 1897), *Pseudomonas aeruginosa* (DSM 50071), *Pseudomonas syringae* pv *aptata* (DSM 50252), *Ralstonia solanacearum* (DSM 9544), *Staphylococcus epidermidis* (DSM 20044), *Staphylococcus lentus* (DSM 6672), *Xanthomonas campestris* (DSM 2405), the yeast *Candida glabrata* (DSM 6425), and the fungus *Septoria tritici*. The antimicrobial assays were performed as recently described by Schneeman et al. (2010).[12]

Cytotoxic Assays. The sensitivity of the cell lines NIH-3T3, HepG2, and HT-29 to the isolated compounds was evaluated by monitoring of the metabolic activity using the CellTiter-Blue cell viability assay (Promega, Mannheim, Germany). The cultivation of the cell lines and the bioassays were performed as described by Schneeman et al. (2010).[12]

GSK-3β Inhibition Assay. To measure the inhibitory effect of the isolated compounds, they were tested in an *in vitro* activity assay adapted from a luminescent assay described by Baki et al. (2007).[13]

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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