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Cytotoxic Alkaloids from *Fusarium incarnatum* Associated with the Mangrove Tree *Aegiceras corniculatum*

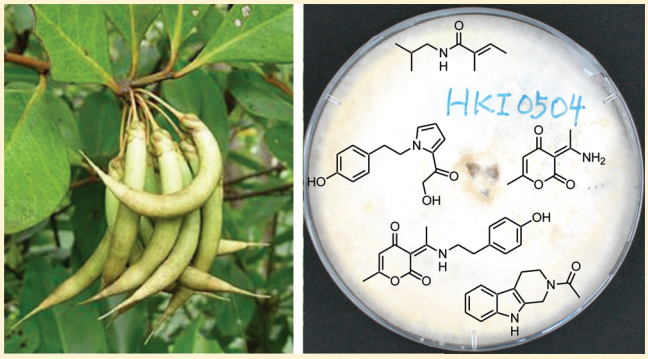
Ling Ding,[†] Hans-Martin Dahse,[†] and Christian Hertweck*[,†,‡]

[†]Leibniz Institute for Natural Product Research and Infection Biology, HKI, Beutenbergstraße 11a, 07745 Jena, Germany

[‡]Friedrich Schiller University, 07737 Jena, Germany

Supporting Information

ABSTRACT: Several unusual alkaloids, *N*-2-methylpropyl-2-methylbutenamide (**1**), 2-acetyl-1,2,3,4-tetrahydro-β-carboline (**2**), fusarine (**3**), fusamine (**4**), and 3-(1-aminoethylidene)-6-methyl-2*H*-pyran-2,4(3*H*)-dione (**5**), were isolated from the culture broth of *Fusarium incarnatum* (HKI0504), an endophytic fungus of the mangrove plant *Aegiceras corniculatum*. Compounds **2**, **4**, and **5** exhibit weak antiproliferative and cytotoxic activities against HUVEC, K-562, and HeLa human cell lines, respectively.



Plants have developed highly diverse mechanisms to cope with their environment. In light of this, it is remarkable that all plants on earth harbor endophytes.[1] It is fascinating to observe that after 400 million years of evolution there are plants that require symbiotic associations for stress tolerance.[2] Coastal sediments are particularly harsh habitats for plants. High temperature, high salinity, high UV radiation, and tides with potential herbivores/pathogens, such as animals, algae, and various microbes, challenge the mangrove plants. To cope with these stress factors, mangroves might employ endophytes to protect the association with bioactive metabolites. Thus, unsurprisingly, endophytes from mangrove plants indeed produce structurally unique compounds.[3−20] *Aegiceras corniculatum* is a widespread mangrove tree distributed along the coastline of Asia and Australia. Traditionally the plant has been used as an antiasthmatic, antidiabetic, and antirheumatic agent and as a fish poison.[21] We wondered whether endophytic microorganisms could produce toxins and thus contribute to the range of defense substances secreted by the plants. Some previous work has demonstrated that its endophytic fungi could produce cytotoxic polyketides[3,4] and tetramic acids,[5] antiviral isoindolones,[6] and indole triterpenoids, blocking large-conductance calcium-activated potassium channels.[7] The fungal isolate *Fusarium incarnatum* (HKI0504), which was retrieved from the fruit of *A. corniculatum*, has drawn our interest because of its high cytotoxicity in the culture broth.[8] In this paper, we report the discovery and characterization of several unusual alkaloids from the culture filtrate of the fungus.

RESULTS AND DISCUSSION

In a bioactivity screening program we noted that the culture filtrate of *F. incarnatum* exhibits high cytotoxicity. HPLC/MS analysis of the crude extract and deconvolution using the HKI compound database indicated that this fungus produces various metabolites in minute amounts. To obtain sufficient material for a full structural elucidation, a 200 L fermentation was carried out. Through bioactivity-guided fractionation using open column chromatography on silica, size exclusion chromatography, and preparative HPLC, we finally obtained pure samples of compounds **1** (2.3 mg), **2** (1.0 mg), **3** (9.0 mg), **4** (0.9 mg), and **5** (1.6 mg) (Figure 1).

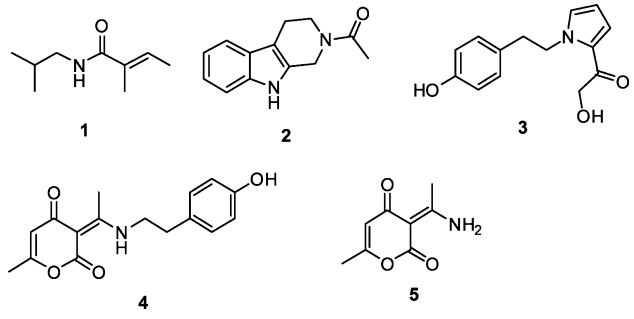


Figure 1. Structures of secondary metabolites produced by *Fusarium incarnatum* (HKI0504).

The molecular formula of **1** was determined by HRESIMS and confirmed by ^{13}CNMR data. Signals of one olefinic proton (δ 6.38), one NH (δ 5.72), and other aliphatic protons were observed in the ^{1}H NMR spectrum. Fragments of *N*-isobutyl and 2-butene were deduced from H,H COSY correlations. The

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carbonyl (δ 169.4, C-1) signal in the ^{13}C NMR spectrum and the HMBC correlations (Figure 2) between H-1′ (δ 3.12) and

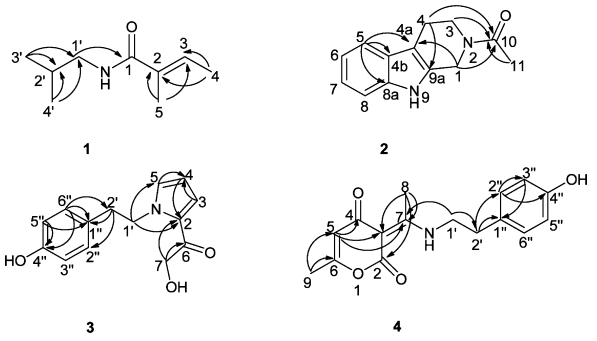

Figure 2. Selected HMBC correlations for compounds **1**, **2**, **3**, and **4**.

H-5 (δ 1.81) and C-1 established the structure of **1** as *N*-isobutyl-2-methylbutenamide. NMR spectroscopic analysis and HRESIMS indicated that a minor component, which was inseparable from **1**, represents *N*-isobutyl-2-methylbutanamide (**1a**, Figure S1 in the Supporting Information). The structurally related compound *N*-(2′*S*)-methylbutanoyl-2-methylbutylamine (**1b**, Figure S1 in the Supporting Information) is known as a female sex pheromone of the longhorn beetle *Migdolus fryanus* Westwood.[22]

According to HRESIMS, compound **2** has a molecular formula of $C_{13}H_{14}N_2O$. The ^{1}H NMR spectrum indicated the presence of a set of anti/syn isomers (ratio 2:1). The 1,2-disubstituted benzene core was deduced from four consecutive aromatic signals (δ 7.39, 7.28, 7.05, 6.98) in the ^{1}H NMR spectrum. Two methylenes (δ 3.84, 2.85) appearing as two triplets, one methylene singlet (δ 4.75), and one methyl singlet (δ 2.23) were also observed. A set of smaller signals corresponding to an isomer of **2** could be detected in the ^{1}H NMR spectrum. An indole moiety was deduced from the ^{13}C NMR signals (δ 138.0, 131.4, 128.2, 122.2, 119.9, 118.5, 111.9, 108.2) and confirmed by HMBC correlations (Figure 2), as H-5 showed correlations to C-4a (δ 108.2), C-4b (δ 128.2), and C-8a (δ 138.0). A signal corresponding to an amide group (δ 172.5, C-10) was detected in the ^{13}C NMR spectrum. HMBC data showed that H-1, H-3, and Me-11 correlate with this carbonyl, and additional correlations from H-1 to C-4a and from H-4 to C-9a supported the proposed carboline partial structure. Compound **2**, identified as 2-acetyl-1,2,3,4-tetrahydro-β-carboline, represents a new member of naturally occurring tetrahydrocarbolines. The minor NMR signals correspond to the syn isomer (Figure 3). The relatively upfield

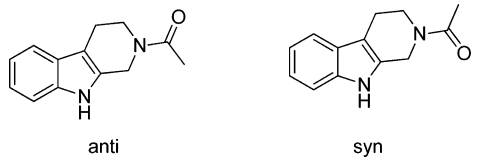

Figure 3. Two rotamers of **2**.

proton signals for H-1 and H-11 were observed, which could be explained by the lower van der Waals effect in the syn isomer.

2-Acetyl-1,2,3,4-tetrahydro-β-carboline and other structurally related tetrahydrocarbolines have been known only as synthetic compounds.[23,24] Carboline derivatives constitute an important group of natural products that possess various biological activities such as antiviral (e.g., euditomins, from tunicates)[25] and cytotoxic and antimicrobial (e.g., manzamines, from sponges) activities.[26] Although most tetrahydrocarbolines were isolated from marine animals, recent findings indicate that microorganisms are capable of producing carbolines such as oxopropaline,[27,28] bauerine,[29] and β-carboline-1-propionic acid.[30] Tryptolines, a subgroup of carbolines postulated as neuromodulators for mammals, have been isolated from tomatoes, kiwis, bananas, pineapples, oranges, and grapefruits.[31] However, this is the first report on a tryptoline from a plant-associated fungus.

High-resolution ESIMS established the molecular formula for compound **3**. A 1,4-disubstituted benzene moiety was inferred from signals of four aromatic protons: δ 6.93 (2H, d, 8.5 Hz) and 6.65 (2H, d, 8.5 Hz) in the ^{1}H NMR spectrum. The pyrrole moiety was deduced from the chemical shifts and coupling constant of the three consecutive protons H-3 (δ 7.04, dd, 4.1, 1.6 Hz), H-4 (δ 6.06, dd, 4.1, 2.5 Hz), and H-5 (δ 6.84, dd, 2.2, 1.9 Hz). Additionally, ^{1}H NMR data suggested the presence of three methylene groups (δ 4.54, t, H-1′; 2.87, t, H-2′; 4.63, s, H-7). The location of an ethylene group between the benzene and pyrrole moieties was determined by an HMBC spectrum, where H-1′ correlated with C-2 and C-5 and H-2′ correlated with C-1″ and C-2″ (C-6″) (Figure 2). The identity of the phenol moiety was readily confirmed by the HMBC data, as H-2″ (H-6″) and H-3″ (H-5″) correlated with C-4″ (δ 157.1), which is attached to a hydroxy group. HMBC correlations from H-7 to C-2 and C-6 supported the substitution of hydroxyacetyl to the pyrrole ring. Finally, **3** was identified as a novel alkaloid, named fusarine. The structure of fusarine is interesting in light of the related pyrrole congeners **6** and **7** identified earlier.[8] Apparently, biogenic amines react with related polycarbonyls to yield various substitution patterns. Only two related natural products are known, pyrrolezanthine (**8**) and ganodine (**9**) (Figure 4), which were isolated from the stem of the pepper

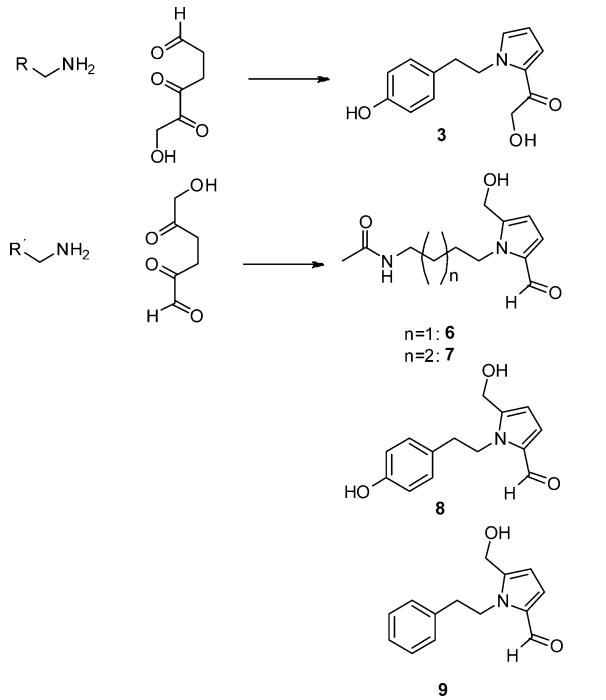

Figure 4. Model for the biosynthesis of **3**, **6** and **7**; structures of related compounds, pyrrolezanthine (**8**) and ganodine (**9**).

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Zanthoxylum simulans[32] and from the mycelium of *Ganoderma capense*,[33] respectively. Isomers of **3** featuring the 2,5-disubstituted pyrrol ring as in **6** and **7** were not detected in *F. incarnatum*.

Interestingly, the tyrosine building block as observed in **3** was also found in the structure of compound **4**. HRESIMS of **4** established its molecular formula as $C_{16}H_{17}NO_4$. The ^{1}H NMR spectrum displayed a rather simple signal pattern, revealing four aromatic protons (δ 7.07, 2H; 6.72, 2H) typical for 1,4-disubstituted benzenes, one olefinic proton (δ 5.69), two mutually coupling methylenes (δ 3.77, 2.89), and two olefinic methyl groups (δ 2.49, 2.10). The ethylene group was attached to the phenol moiety, which was inferred from the HMBC correlations. The ^{13}C NMR and DEPT data revealed several downfield quaternary carbon signals. Apart from the signals from the substituted phenol moiety, the remaining signals (δ 185.6, 177.8, 166.1, 164.3, 108.3, 97.0) suggested a pyronic residue, with diagnostic signals. The partial structure of the pyronic residue was revealed to be 3-(1-aminoethylidene)-6-methyl-2*H*-pyran-2,4(3*H*)-dione, further supported by HMBC correlations (Figure 2). The proposed connection between the pyronic residue and phenol moiety was endorsed by HMBC correlations (H-1′ to C-7). Compound **4**, named fusamine, represents a novel type of natural alkaloid, although related compounds have been reported as synthetic compounds.[34]

Finally, MS and NMR data revealed that compound **5** features a partial structure of **4**, 3-(1-aminoethylidene)-6-methyl-2*H*-pyran-2,4(3*H*)-dione, a compound that has been known only as a synthetic compound.[35]

All isolated compounds were subjected to antiproliferative and cytotoxicity tests. While **1** and **3** were inactive, weak activities were observed for **2**, **4**, and **5**. The detailed results are shown in Table 6.

In summary, we have analyzed the secondary metabolites from a fungal endophyte of the mangrove *A. corniculatum*. The unsual fungal alkaloids comprise an unprecedented tetrahydrocarboline (2-acetyl-1,2,3,4-tetrahydro-β-carboline) and novel tyrosine-derived alkaloids, a pyrrole (fusarine), and a pyrandione-enamine (fusamine). Three of these compounds (**2**, **4**, and **5**) have cytotoxic and antiproliferative activities. Endophytes are known as prolific producers of biologically active compounds,[36−38] and various secondary metabolites from endophytes are candidates for anticancer drugs.[39,40] From an ecological point of view, it is not surprising that in particular mangroves harbor a rich microbial flora, which may protect the plants from pathogens,[17−20] and these organisms continue to be a promising source of natural products.

EXPERIMENTAL SECTION

General Experimental Procedures. UV spectra were obtained using a Varian UV−visible Cary spectrophotometer (Varian, Palo Alto, CA, USA). IR spectra were obtained using an IFS55 spectrometer (Bruker, Karlsruhe, Germany). NMR spectra were recorded on a DPX-600 apparatus (Bruker); ESIMS data were measured using a triple quadrupole mass spectrometer (Quattro; VG Biotech, Cheshire, UK). Open column chromatography was performed on silica gel 60 M (230−400 mesh, Macherey-Nagel, Düren, Germany) and Sephadex LH-20 (Pharmacia Biotech AB, Uppsala, Sweden). TLC analysis was performed on silica gel plates (Sil G/UV254, 0.20 mm, Macherey-Nagel). LC/MS analyses were performed on an Agilent 1100 Series System equipped with a MSD trap and LC/MS column (Zorbax Eclipse XDB-C_8, 150 × 4.6 mm, particle size 5 μm).

Strain Isolation and Fermentation. Strain isolation, taxonomy, and the fermentation details were described previously.[8] The nucleotide sequence spanning the ITS1-5.8S-ITS2-D1/D2 of the nuclear ribosomal DNA cluster was sequenced and deposited under the accession number EU111657 in GenBank at www.ncbi.nlm.nih.gov.[8]

Table 1. NMR Spectroscopic Data (600 MHz, $CDCl_3$) for *N*-2-Methylpropyl-2-methylbutenamide (1)

position	δ_C, type	δ_H (J in Hz)
1	169.4, C	
2	132.0, C	
3	130.2, CH	6.38, dq (6.9, 1.3)
4	13.8, CH_3	1.73, d (6.9)
5	12.3, CH_3	1.81, brs
1′	46.9, CH_2	3.12, dd (6.4, 6.4)
2′	28.5, CH	1.75, m
3′	20.2, CH_3	0.90, d (6.7)
4′	20.2, CH_3	0.90, d (6.7)
NH		5.72, br

Table 2. NMR Spectroscopic Data (600 MHz, CD_3OD) for 2-Acetyl-1,2,3,4-tetrahydro-β-carboline (2, anti and syn isomers)

	anti	anti	syn	syn
position	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
1	41.4, CH_2	4.75, s	41.4, CH_2	4.73, s
2				
3	46.1, CH_2	3.84, t (5.8)	45.4, CH_2	3.93, t (5.8)
4	22.7, CH_2	2.85, t (5.8)	21.9, CH_2	2.76, t (5.8)
4a	108.2, C		108.2, C	
4b	128.2, C		128.1, C	
5	118.5, CH	7.39, d (7.8)	118.6, CH	7.39, d (7.8)
6	119.9, CH	6.98, dd (7.8, 7.0)	119.9, CH	6.98, dd (7.8, 7.0)
7	122.2, CH	7.05, dd (8.0, 7.0)	122.4, CH	7.05, dd (8.0, 7.0)
8	111.9, CH	7.28, d (8.0)	111.9, CH	7.28, d (8.0)
8a	138.0, C		138.1, C	
9				
9a	131.4, C		131.4, C	
10	172.5, C		172.6, C	
11	21.4, CH_3	2.23, s	21.9, CH_3	2.20, s

Table 3. NMR Spectroscopic Data (600 MHz, CD_3OD) for Fusarine (3)

position	δ_C, type	δ_H (J in Hz)
1		
2	127.9, C	
3	120.4, CH	7.04, dd (4.1, 1.6)
4	109.4, CH	6.06 dd (4.1, 2.5)
5	132.6, CH	6.84, dd (2.2, 1.9)
6	189.9, C	
7	65.4, CH_2	4.63, s
1′	52.5, CH_2	4.54, t (7.2)
2′	38.2, CH_2	2.87, t (7.2)
1″	130.5, C	
2″	130.9, CH	6.93, d (8.5)
3″	116.1, CH	6.65, d (8.5)
4″	157.1, C	
5″	116.1, CH	6.65, d (8.5)
6″	130.9, CH	6.93, d (8.5)

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Table 4. NMR Spectroscopic Data (600 MHz, CD_3OD) for Fusamine (4)

position	δ_C, type	δ_H (J in Hz)
1		
2	166.1, C	
3	97.0, C	
4	185.6, C	
5	108.3, CH	5.69, s
6	164.3, C	
7	177.8, C	
8	18.4, CH_3	2.49, s
9	19.6, CH_3	2.10, d (0.9)
1′	47.1, CH_2	3.77, t (6.8)
2′	35.4, CH_2	2.89, t (6.8)
1″	129.6, C	
2″	131.0, CH	7.07, d (8.6)
3″	116.5, CH	6.72, d (8.6)
4″	157.5, C	
5″	116.5, CH	6.72, d (8.6)
6″	131.0, CH	7.07, d (8.6)

Table 5. NMR Spectroscopic Data (600 MHz, $CDCl_3$) for 3-(1-Aminoethylidene)-6-methyl-2*H*-pyran-2,4(3*H*)-dione (5)

position	δ_C, type	δ_H (J in Hz)
1		
2	163.5, C	
3	96.9, C	
4	185.0, C	
5	107.5, CH	5.68, s
6	163.3, C	
7	177.0, C	
8	26.3, CH_3	2.61, s
9	19.9, CH_3	2.11, s

Table 6. Cytotoxic and Antiproliferative Activities of Isolated Compounds (in μM)

	antiproliferative effect		cytotoxicity
compound	HUVEC GI_{50}	K-562 GI_{50}	HeLa CC_{50}
1	>300	>300	>300
2	41.1	33.3	23.8
3	>200	>200	>200
4	37.3	37.6	23.3
5	68.2	9.0	103.6
Imatinib	18.5 (±2.0)	0.17 (±0.01)	65.8 (±2.4)

Extraction and Isolation. The fermentation broth (200 L) was obtained by filtration and loaded onto an Amberchrom 161c resin LC column (200 × 20 cm, 6 L). Elution with a linear gradient of MeOH–H_2O (from 30% to 100% v/v, flow rate 0.5 L/min, in 58 min) afforded seven fractions (F1–F7). By cytotoxicity-guided fractionation F7 was selected and subjected to silica gel chromatography using a gradient of CH_2Cl_2–MeOH (0–50%) as eluent to obtain fractions A–G. B was fractionated by Sephadex LH-20 (MeOH) to afford two major fractions, B1 and B2. Further fractionation of B1 by HPLC (MeOH–H_2O) afforded **3** (9.0 mg) and **4** (0.9 mg), respectively. Compound **5** (1.6 mg) was obtained from B2 by HPLC (MeOH–H_2O). Fraction C was fractionated using a Sephadex LH-20 (MeOH) column and then by HPLC (MeOH–H_2O) to give **1** (2.3 mg). Six fractions (D1–D6) were obtained from fraction D by separation on a Sephadex LH-20 (MeOH) column. Compound **2** (1.0 mg) was obtained by HPLC (MeOH–H_2O) separation of D4.

Antiproliferation and Cytotoxicity Assays. Compounds were assayed against human umbilical vein endothelial cells (HUVEC) and K562 human chronic myeloid leukemia cells (DSM ACC 10) for their antiproliferative effects (GI_{50}) and against HeLa human cervix carcinoma cells (DSM ACC 57) for their cytotoxic (CC_{50}) effects as previously described.[41] Inhibitory concentrations are provided as 50% inhibition of cell growth (GI_{50}; the concentration needed to reduce the growth of treated cells to half that of untreated cells) or 50% cytotoxic concentration (CC_{50}; the concentration that kills 50% of treated cells).

***N*-2-Methylpropyl-2-methylbutenamide (1):** colorless oil; UV (MeOH) λ_{max} (log ε) 208 (3.56) nm; IR (film) ν_{max} 3306, 2959, 2871, 1700, 1660, 1618, 1539, 1457, 1386, 1271, 1156, 1006, 812, 669 cm^{-1}; ESIMS $[M + H]^+$ = 156.2, $[2 M + H]^+$ = 311.4; HRESIMS m/z 156.1381 $[M + H]^+$ (calcd for $C_9H_{18}NO$, 156.1383), NMR data, see Table 1.

2-Acetyl-1,2,3,4-tetrahydro-β-carboline (2): pale yellow solid; ESIMS $[M + H]^+$ = 215.5, $[M + Na]^+$ = 237.7, $[2 M + Na]^+$ = 451.2; HRESIMS m/z 215.1177 $[M + H]^+$ (calcd for $C_{13}H_{15}N_2O$, 215.1179); NMR data, see Table 2.

Fusarine (3): colorless solid; UV (MeOH) λ_{max} (log ε) 287 (2.72), 223 (2.66), 200 (2.81) nm; IR (film) ν_{max} 2923, 1700, 1684, 1653, 1559, 1515, 1472, 1419, 1243, 1172, 1082, 1041, 1002, 953, 833, 767, 669 cm^{-1}; ESIMS $[M + H]^+$ = 246.1, $[M + Na]^+$ = 268.1, $[2 M + Na]^+$ = 513.2; HRESIMS m/z 246.1128 $[M + H]^+$ (calcd for $C_{14}H_{16}NO_3$, 246.1125),; NMR data, see Table 3.

Fusamine (4): pale yellow solid; UV(MeOH) λ_{max} (log ε) 312 (4.28), 228 (4.33), 202 (4.41) nm; IR (film) ν_{max} 3138, 2923, 1704, 1686, 1659, 1559, 1515, 1472, 1425, 1392, 1355, 1329, 1270, 1237, 1171, 1127, 1107, 1074, 1033, 1001, 953, 889, 834, 811, 767, 712, 669, 651, 624 cm^{-1}; ESIMS $[M + H]^+$ 288.9, $[M + Na]^+$ 310.3, $[2 M + Na]^+$ 597.0; HRESIMS m/z 288.1225 $[M + H]^+$ (calcd for $C_{16}H_{18}NO_4$, 246.1230); NMR data, see Table 4.

3-(1-Aminoethylidene)-6-methyl-2*H*-pyran-2,4(3*H*)-dione (5): pale yellow solid; ESIMS $[M - H]^-$ 166.1, $[M + Na]^+$ 190.4, $[M + H]^+$ 168.1; HRESIMS m/z 168.0660 $[M + H]^+$ (calcd for $C_8H_{10}NO_3$, 168.0665); NMR data, see Table 5.

■ ASSOCIATED CONTENT

Supporting Information

NMR spectra of compounds **1**–**5**. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: + 49-3641-5321100. Fax: (+) 49-3641-5320804. E-mail: christian.hertweck@hki-jena.de.

Notes

The authors declare no competing financial interest.

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