BIOLOGICALLY ACTIVE METABOLITES FROM FUNGI.

3. SPOROTHRIOLIDE, DISCOSIOLIDE, AND 4-*epi*-ETHISOLIDE— NEW FUROFURANDIONES FROM *Sporothrix* sp., *Discosia* sp., AND *Pezicula livida*

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In the course of our screening program for biologically active compounds from fungi we have investigated three strains of genera from which the secondary metabolites had not yet been studied: *Sporothrix* sp. (strain No. 700), *Discosia* sp. (strain No. 1290), and *Pezicula livida* (strain No. 1156). The strains were selected for their antifungal, antibacterial, and (in part) herbicidal activities (inhibition of alfalfa and seedlings of garden cress). This paper describes the isolation, physico-chemical properties, structure determination and biological activity of three novel furofurandiones named sporothriolide (**1a**), discosiolide (**3a**) and 4-*epi*-ethisolide (**3c**) isolated from the above mentioned fungi.

Fungal Strains

Discosia sp. Lib. (strain 1290) was isolated from a soil sample from Teneriffe. The sample originated from foggy wooded area (elevation 1,100 m). The genus *Discosia* belongs to the Coelomycetes (Fungi Imperfectae), which are characterized by their ability to form pycnidia. They grow parasitically or saprophytically on leaves or fruiting bodies of various plants and are considered to be plurivorous.

Pezicula livida (Berk. and Br.) Rehm (strain 1156) was isolated as an endophyte following surface sterilization[1] from leaves of *Betula pendula*. *Pezicula* belongs to the Helotiales (Ascomycetes). Under laboratory conditions, the fungus does not develop the teleomorphic form, but sporulates in acervuli of the anamorphic (*Cryptosporiopsis*) form.

Sporothrix sp. Hektoen and Perkins (strain 700) was isolated in West Borneo from a soil sample of a slag heap left by gold miners. *Sporothrix* is the anamorphic form of the genus *Ophiostoma* (Sphaeriales, Ascomycetes). The *Ophiostoma* species are found on decaying wood and associated with bark-beetles.

Conditions of Culture

Strains 700 (*Sporothrix* sp.) and 1156 (*Pezicula livida*) were grown for 14 days at 25°C in shaken culture (2,000-ml Erlenmeyer flasks) each containing 800 ml biomalt (50 g/liter) medium (Vitaborn, Hameln) at 125 rpm. Strain 1290 (*Discosia* sp.) was cultivated on a semi-solid (0.3% agar) MPY-medium (malt extract (20.0 g) - yeast extract (2.5 g) - peptone (2.5 g) in 1,000 ml H_2O) for 33 days at room temperature. These lengths of culture were chosen because they yielded a maximum on fungicidal and herbicidal activity as measured in our tests.

Extraction and Purification

The submerged cultures of *Sporothrix* sp. (20 liters) and *Pezicula livida* (10 liters) were filtered and the filtrate extracted three times with ethyl acetate (each strain with a total of 3 liters). The biological activity was located in the organic extract. The mycelium showed no activity.

Discosia sp. was grown on semi-soft agar (33 1.8-liter Fernbach flasks each containing 250 ml of medium). The semi-soft agar was homogenated with a Waring blender, diluted with 5 liters of water and then extracted five times with ethyl acetate. The organic extracts were dried (Na_2SO_2), filtered, and evaporated *in vacuo*. The biologically active compounds in the crude extracts were located by means of biodiagrams (silica gel TLC plates sprayed with *Cladosporium*, see ref.[2]). The active compounds could also be detected on TLC at 254 nm UV-light. Table 1 summerizes the amount of crude extract and the location of active components on TLC.

Table 1. Crude extract and location of active components.

Fungus	Extract (g)	Rf of active compound
Sporothrix sp.	1.6	0.7[a]
Discosia sp.	3.4	0.65[b]
Pezicula livida	0.2	0.25 and 0.45[b]

[a] Dichloromethane - methanol (99.5 : 0.5).
[b] Dichloromethane.

Each of the crude extracts was redissolved in dichloromethane, filtered and applied to columns of silica gel that had been pre-washed with dichloromethane. The elution was carried out with dichloromethane using increasing amounts of methanol (99.5:0.5~98:2). The respective biologically active fractions (*Cladosporium*-test) were then further purified by layer chromatography on silica gel plates (1 mm, repeated development with dichloromethane) yielding from strain No. 700, 430 mg of **1a**, mp 101 °C (dichloromethane-petroleum ether); from strain No. 1290, 420 mg of **3a**, mp 66°C (dichloromethane-*n*-hexane); from strain No. 1156, 37 mg of (−)-mellein (**5**) (3,4-dihydroxy-8-hydroxy-3-methyl-1*H*-2-benzopyran-1-one) and 20 mg (oil) of compound **3c** (Rf 0.25).

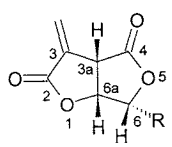
Physico-chemical Properties and Structure Determination

The compound from *Sporothrix* sp. was obtained as white needles which melted at 100.5~101°C. It was readily soluble in most solvents of medium polarity such as chloroform, dichloromethane or tetrahydrofuran and was less soluble in hydrocarbons such as pentane or hexane. The compoumd was optically active ($[\alpha]_D^{25} = -146.5$ ($c=0.66$ in chloroform)). The molecular formular of the antibiotic was established as $C_{13}H_{18}O_4$ by the mass spectrum (M^+ m/z 238) and microanalysis. The ^1H NMR spectrum showed typical signals for one methyl and five methylene groups of an unbranched hexyl side chain. An exocyclic methylene group could be deduced from signals at $\delta=6.15$ and 6.47 ppm ($J=2.0$ Hz) and the signal at 127.33 ppm in the ^{13}C NMR spectrum. In addition, signals for three coupling methine protons can be seen at $\delta=4.02$ (dt), 4.65 (dt) and 5.15 (dd) ppm. Further signals for quaternary carbon atoms at 167.45 and 172.09 are typical for an ester or lactone group that was confirmed by a characteristic double resonance at 1780~1750 cm^{-1} in the IR spectrum.

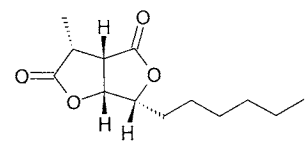
The sum of these data is indicative for structure **1a** that is mentioned as a racemic synthetic compound in a patent of ALDRIGE *et al.*[3], but no data for comparison are given in the patent. To elucidate the absolute configuration comparison was made with the antifungal natural product (−)-canadensolide (**1b**)[4]. The relative configuration of (−)-canadensolide was later corrected by KATO *et al.*[5,6] and the absolute configuration assigned by total synthesis by the group of FRASER-REID as (3a*S*,6a*R*,6*R*)-6-butyltetrahydro-3-methylenefuro-[3,4-*b*]furan-2,4-dione (**1b**)[7,8]. The ^1H NMR data for the new compound closely resemble the values given for the analogue (−)-canadensolide (**1b**). The *cis*-orientation of 6- and 6a-H is proven by a coupling of 4.6 and 4.5 Hz for **1a** and **1b**, respectively, whereas the corresponding *trans*-configuration of 6- and 6a-H results in a coupling of 1.5 Hz[5]. Thus, on the basis of the similar negative optical rotation for **1a** and (−)-canadensolide (**1b**) ($[\alpha]_D^{20} = -141°$), the structure of the new furofuranone is assigned as **1a** and the compound named sporothriolide according to its origin. Catalytic hydrogenation with palladium on charcoal as the catalyst afforded dihydrosporothriolide (**2**). The β-stereochemistry of the methyl group at C-3 as shown in **2** is assigned on the basis of *exo*-addition of hydrogen and the high coupling constant of 3a-H and 3-H = 10.1 Hz for a *cis*-relationship of these protons.

The major compound from *Discosia* sp. forms white needles with a melting point of 66°C and solubility comparable to that of **1a**; the optical rotation was $[\alpha]_D^{25} = -36.1$ ($c=0.43$ in chloroform). The molecular formula of $C_{17}H_{26}O_4$ was established from the mass spectrum (M^+ m/z 294) and microanalysis. A triplet in the ^1H NMR spectrum at $\delta=0.85$ ppm and the multiplet at 1.25~1.82 for 18 protons (combined with the corresponding signals in the ^{13}C NMR spectrum) designate an unbranched C_{10}-side chain.

As for **1a**, signals at $\delta=167.5$ and 169.82 indicate a lactone structure, confirmed by a resonance at 1771 cm^{-1} in the IR spectrum. The exocyclic methylene group is deduced from signals in the ^1H NMR-spectrum at $\delta=5.88$ and 6.47 ppm that show a *geminal* coupling of 2.4 Hz. Furthermore, signals for three coupling methine protons can be seen at $\delta=3.58$ (ddt), 4.43 (dt) and 5.00 (d) ppm. From



Sporothriolide (**1a**) R = *n*-C_6H_{13}
(−)-Canadensolide (**1b**) R = *n*-C_4H_9



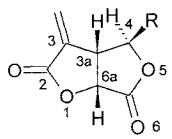
Dihydrosporothriolide (**2**)

these data the skeleton of a 3-methylene-furo[3,4-*b*]-2,6-dione can be deduced. A compound with that arrangement and a *n*-$C_{10}H_{21}$ side chain has not as yet been mentioned in the literature as a natural or synthetic product. However, several related natural products are known as (−)-avenaciolide (**3b**)[9], (−)-isoavenaciolide (**4a**)[10] and ethisolide (**4b**)[10]. ALDRIDGE *et al.*[10] recognised that **3c** and **4b** are epimeric at C_4. The *cis*-configurated protons at C_{3a} and C_4 in **4a** and **4b** show a coupling constant of 9 Hz, whereas the corresponding *trans*-orientation in **3b** had a coupling of 3.8 Hz, which was also measured in the new metabolite discosiolide (**3a**). The absolute configuration of **3b** originally assumed by HUGHES *et al.*[9] has been reversed by OHRUI and EMOTO[11] and ANDERSON and FRASER-REID[12,13] following synthetic work starting from D-glucose. The optical rotation of (−)-avenaciolide of $[\alpha]_D^{25} = -41$ ($c = 0.274$ in chloroform) is in good agreement with that found for the new natural product that could thus be assigned structure **3a**.

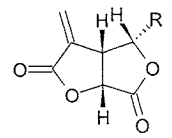
The first compound from *Pezicula livida* (strain 1156) was identified as (−)-mellein Rf 0.45, mp 50~51°C, $[\alpha]_D^{25} = -102$ ($c = 0.97$ in chloroform)[14]. The second compound with Rf = 0.25 was isolated as an oil in only small amount (20 mg). The spectral data (^1H, ^{13}C NMR, IR, UV) showed great similarity to those found for discosiolide (**3a**) with the exception of the signals for the ethyl side chain which occurred instead of those for the *n*-decyl side chain. The data indicate a molecular formula of $C_9H_{10}O_4$ ($M^+ + H^+$ *m/z* 183) and the relative configuration could be established as shown in **3c** by the coupling constant of $J_{3a,4} = 4.0$ Hz. The new metabolite is thus epimeric to ethisolide (**4b**) isolated from *Penicillium decumbens*[10]. The racemic 4-*epi*-ethisolide has been prepared synthetically by TAKAI *et al.*[15].

Biological Activity

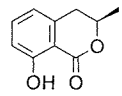
The abscess curing effect of the synthetic racemic sporothriolide *rac*-**1a** is described in a patent[3]. The fungicidal activity of (−)-avenaciolide (**3b**)[16] and (−)-canadensolide (**1b**), particularly against *Botrytis allii*[4], was also previously known. Table 2 summarizes the antibacterial, fungicidal and algicidal and Table 3 the herbicidal activity of the new natural products **1a**, **3a** and **3c** and its hydrogenation product **2** in initial agar diffusion tests. The test organisms were three fungi, a Gram-positive and a



Discosiolide (**3a**) R = $C_{10}H_{21}$
(−)-Avenaciolide (**3b**) R = C_8H_{17}
4-*epi*-Ethiosolide (**3c**) R = C_2H_5



(−)-Isoavenaciolide (**4a**) R = C_8H_{17}
Ethiosolide (**4b**) R = C_2H_5



(−)-Mellein (**5**)

Table 2. Fungicidal, antibacterial and algicidal activity of **1a**, **2**, **3a** and **3c** in agar diffusion tests.

	Concentration (mg/ml)	Radius of inhibition (mm)					
		B.m.	*E.c.*	*Ust.*	*M.m.*	*Eur.*	*Chl.*
Sporothriolide (**1a**)	10	2	2	6	3	11	2
hydrog. product (**2**)	10	0	0	9	2	0	—
Discosiolide (**3a**)	10	2	1	5	1	9	1
Epiethisolide (**3c**)	20	5	3	15	17	16	3

Organisms: *B.m.*, *Bacillus megaterium*; *E.c.*, *Escherichia coli*; *Ust.*, *Ustilago violacea*; *M.m.*, *Mycotypha microspora*; *Eur.*, *Eurotium repens*; *Chl.*, *Chlorella fusca*. The substances to be tested (50 µl of the methanol or acetone solution) were pipetted onto antibiotic filter discs (9 mm in diameter). These were placed onto appropriate agar media for the fungal, bacterial and algal tests and sprayed with a suspension of the test organisms. The radius of the zone of inhibition was measured on the 1~4 day, depending on the growth of the test organism.

Table 3. Herbicidal activity of **1a**, **2**, **3a**, and **3c** in seedling germination tests.

	% Inhibition of growth	
	Lepidium sativum	*Medicago sativa*
Sporothriolide (**1a**)	95	95
Dihydrosporothriolide (**2**)	0	100
Discosiolide (**3a**)	0	0
Epiethiosolide (**3c**)	100	100

Seeds of *Lepidium sativium* and *Medicago sativa* were placed onto filter paper in petri dishes (4.5 cm in diameter) to each of which 2.25 ml of sterile water and 0.25 ml of one of the above substances in acetone of methanol solution was added. The final concentrations were **1a**: 4 mg/ml, **2**: 1 mg/ml, **3a**: 5.1 mg/ml and **3c**: 20 mg/ml. The length of the seedlings was measured and compared to the controls after 2 and 5 days and evaluated as % inhibition of growth.

Gram-negative bacteria, the alga *Chlorella fusca* and the seedlings *Lepidium sativum* and *Medicago sativa*.

All of the tested substances are biologically active against the fungal and algal test organisms. All but the hydrogenation product are antibacterially active. (Compound was not tested with *Chlorella fusca*.) **1a**, **2** and **3c** are active as herbicides, inhibiting growth of the 2 seedlings tested. It is noteworthy that the antifungal activity of sporothriolide (**1a**) is not lost in the hydrogenation product **2**. Additionally, **2** still possesses remarkable herbicidal activity against seedlings of *Medicago sativa*. This activity is thus not correlated with the chemically reactive α-methylene butyro-lactone. In contrast to the furo[3,4-*b*]furan-2,4-diones **1a** and **2**, the furo[3,4-*b*]furan-2,6-dione derivative **3a** with a long C_{10} chain does not exhibit herbicidal activity. If the side chain is shortened to C_2 as in **3c**, herbicidal activity can again be observed.

Sporothriolide (**1a**) showed most promising effects against *Botrytis cinerea*. For testing the effectiveness of sporothriolide in controlling pepper seedlings of the variety "Neusiedler Ideal Elite" infected with *B. cinerea*, the plants—after having developed 4~5 leaves—were sprayed with a watery suspension, the dry matter of which contained 80% active substance and 20% emulsifier. When the sprayed surface had dried, the plants were sprayed with a 1.7×10^6 spore suspension of *B. cinerea* in 2% biomalt and then cultured in a 22~24°C growth chamber with high humidity. After 5 days the disease had developed to such an extent on the untreated leaves that the necroses convered 70% of the leaf surfaces (evaluation of 2 on a scale of 1~8). In contrast, there was no disease development on the leaves that had been treated with 500 ppm sporothriolide (evaluation of 8). Discosiolide was not active in this test. The other compounds, **2** and **3c**, were not tested.

Physical and Spectroscopic Data

Melting points are determined with a Büchi apparatus (Dr. TOTTOLI) and are not corrected. Optical rotations are measured with a Perkin-Elmer 241 polarimeter. Infrared (IR) spectra are obtained with a Nicolet 320 FT-IR spectrometer. Nuclear magnetic resonance (^1H and ^{13}C NMR) spectra are recorded with a Bruker AM 400 (400 and 100 MHz, respectively). Chemical shifts are recorded in ppm (δ) relative to tetramethylsilane as internal standard (in $CDCl_3$). Ultraviolet/visible spectra are recorded on a Beckman UV 5230 spectral photometer. Mass spectra are obtained with a Finnigan MAT 8430 mass spectrometer (70 eV). Elemental analyses are performed by the microanalytical laboratory at the Pharmaceutical Institute, TU Braunschweig.

Sporothriolide; (3a*S*,6a*R*,6*R*)-6-*n*-Hexyl-3,3a,-6,6a-tetrahydro-3-methylenfuro[3,4-*b*]furan-2,4-dione (**1a**)

mp 101°C; (ref[3]: (*rac*)-**1a** (66~68°C)); $[\alpha]_D^{25} = -146$ ($c = 0.66$ in chloroform). IR (KBr): $\nu(cm^{-1}) =$ 2970 (C–H), 2920, 2850, 1780, 1758 (γ-lactone), 1660, 1460, 1400, 1360, 1340, 1300, 1270, 1200, 1170, 1070, 1020, 960, 940, 900, 780. UV (methanol): λ_{max} ($\log \varepsilon$) = 211 (3.862). ^1H NMR ($CDCl_3$): δ (ppm) = 0.89 (t, $J = 6.8$ Hz, 3H, CH_3CH_2), 1.31~1.87 (10H, $5\,CH_2$), 4.02 (dt, $J_{3a,3'} = 2.0$ Hz, $J_{3a,6a} = 6.7$ Hz, 1H, 3a-H), 4.65 (dt, $J_{6,6a} = 4.6$ Hz, $J_{6,6'} = 6.5$ Hz, 1H, 6-H), 5.15 (dd, $J_{6a,6} = 4.6$ Hz, $J_{6a,3a} = 6.8$ Hz, 1H, 6a-H), 6.15 (d, $J_{3',3a} = 2.0$ Hz, 1H, 3'-H), 6.47 (d, $J_{3'3a} = 2.0$ Hz, 1H, 3'-H). ^{13}C NMR ($CDCl_3$): δ (ppm) = 14.05 (p, CH_3), 22.53 (s), 25.38 (s), 28.79 (s), 28.87 (s), 31.56 (s), 46.22 (t, C-3a), 77.19 (t, C-6a), 82.82 (t, C-6), 127.33 (s, C-3'), 129.88 (q, C-3), 167.45 (q, C-4), 172.09 (q, C-2). MS (EI; 100°C): m/z (%) = 240 (10) ($M^+ + 2$), 239 (8) ($M^+ + 1$), 238 (2) (M^+), 220 (46) ($M^+ - H_2O$), 192 (70), 165 (40), 151 (60), 124 (62), 109 (96), 96 (100), 68 (68), 41 (44). MS (CI/NH_3 positive; 120°C): m/z (%) = 256 (24) ($M + NH_4^+$), 239 (30) ($M + H^+$), 194 (30), 109 (72), 96 (96), 68 (54), 55 (52), 41 (100).

Anal Calcd for $C_{13}H_{18}O_4$ (238.2): C 65.61, H 7.62
Found: C 65.76, H 7.73

Hydrogenation of sporothriolide (**1a**) to dihydrosporothriolide (**2**) (3a*S*,6a*R*,6*R*)-6-Hexyltetrahydro-3-methylfuro[3,4-*b*]furan-2,4-dione

A solution of 32 mg of sporothriolide (**1a**) in 10 ml of methanol is shaken under an atmosphere of hydrogen (1 atm) at ambient temperature in presence of 10 mg of 10% Pd/charcoal until no more hydrogen is consumed (2 hours). The solution is filtered, the solvent evaporated at reduced pressure and the residue purified by TLC chromatography on silica gel (dichloromethane - 0.5% methanol, Rf = 0.6) to afford 13.5 mg (48%) of **2**; mp 57°C (Ref[3] mp for racemic **2**: 55~57°C).

^{1}H NMR ($CDCl_3$): δ (ppm) = 0.89 (t, J = 6.8 Hz, 3H, CH_3CH_2), 1.47 (d, $J_{3',3}$ = 7.5 Hz, 3H, 3'-H), 1.30~1.92 (10H), 3.08 (dq, $J_{3,3'}$ = 7.5 Hz, $J_{3,3a}$ = 10.1 Hz, 1H, 3-H), 3.45 (dd, $J_{3a,3}$ = 10.1 Hz, $J_{3a,6a}$ = 5.9, 1H, 3a-H), 4.52 (dt, $J_{6,6a}$ = 4.0 Hz, $J_{6,6'}$ = 6.2 Hz, 1H, 6-H), 5.02 (dd, $J_{6a,6}$ = 4.0 Hz, $J_{6a,3a}$ = 5.9 Hz, 1H, 6a-H). MS (50°C): m/z (%) = 240 (4) (M^+), 194 (28), 130 (22), 98 (100), 69 (30).

Discosiolide; (3a*R*,4*R*,6a*R*)-4-Decyl-3a,6a-dihydro-3-methylenfuro-[3,4-*b*]furan-2,6(3*H*,4*H*)-dione) (**3a**)

mp 66°C; $[\alpha]_D^{25} = -37$ (c = 0.43 in chloroform). IR (KBr): ν (cm^{-1}) = 2918 (C–H), 2898, 1771 (γ-lactone), 1463, 1295, 1270, 1235, 1113, 1040, 996. UV (methanol): λ_{max} (log ε) = 210 (3.850). ^{1}H NMR ($CDCl_3$): δ (ppm) = 0.88 (t, J = 6.8 Hz, 3H, CH_3), 1.26~1.55 (16H, 8 CH_2), 1.82 (m, 2H, CH_2), 3.58 (ddt, $J_{3a,4}$ = 3.8 Hz, $J_{3a,6a}$ = 8.4 Hz, $J_{3a,4'}$ = 2.0 Hz, 1H, 3a-H), 4.43 (dt, $J_{4,3a}$ = 4.0 Hz, $J_{4,4'}$ = 4.0 Hz, 1H, 4-H), 5.0 (d, $J_{6a,3a}$ = 8.6 Hz, 1H, 6a-H), 5.88 (d, J_{gem} = 2.2 Hz, 1H, 3'-H), 6.47 (d, J_{gem} = 2.2 Hz, 1H, 3'-H). ^{13}C NMR ($CDCl_3$): δ (ppm) = 14.11 (p, CH_3), 22.67 (s), 24.84 (s), 29.12 (s), 29.28 (s), 29.37 (s), 29.46 (s), 29.53 (s), 31.87 (s), 36.06 (s), 44.16 (t, C-4), 74.32 (t, C-3a), 85.22 (t, C-6a), 126.30 (s, C-3'), 134.62 (q, C-3), 167.54 (q), 169.82 (q). MS (20°C): m/z (%) = 294 (4) (M^+), 276 (6) ($M^+ - H_2O$), 249 (6), 219 (30), 169 (20), 109 (44), 96 (100).

Anal Calcd for $C_{17}H_{26}O_4$ (294.39): C 69.36, H 8.90
Found: C 69.41, H 8.97

4-*epi*-Ethisolide (**3c**) [(3a*R*,4*R*,6a*R*)-4-Ethyl-3a,6a-dihydro-3-methylenfuro-[3,4-*b*]furan-2,6-(3*H*,4*H*)-dione] (oil)

(Ref[15] for racemic material: mp: 64~65°C). IR (KBr): ν (cm^{-1}) = 2950 (CH_3), 1780 (γ-lactone), 1610, 1450, 1290, 1100, 1050. UV (methanol): λ_{max} (log ε) = 209 (3.776). ^{1}H NMR ($CDCl_3$): δ (ppm) = 1.09 (t, J = 7.4 Hz, 3H, CH_3CH_2), 1.85 (m, 2H, CH_2CH_3), 3.59 (ddt, $J_{3a,6a}$ = 8.3 Hz, $J_{3a,4}$ = 4.1 Hz, J = 2.4 Hz, 1H, 3a-H), 4.41 (dt, $J_{4,3a}$ = 3.9, $J_{4,4'}$ = 6.4 Hz, 1H, 4-H), 5.05 (d, $J_{6a,3a}$ = 8.6 Hz, 1H, 6a-H), 5.90 (d, J_{gem} = 2.2 Hz, 1H, 3'-H), 6.48 (d, J_{gem} = 2.2 Hz, 1H, 3'-H). ^{13}C NMR ($CDCl_3$): δ (ppm) = 9.09 (p, CH_3), 29.02 (s, CH_2CH_3), 43.69 (t, C-4), 74.29 (t, C-3a), 86.33 (t, C-6a), 126.40 (s, C-3'), 134.62 (q, C-3), 167.0 (q), 169.84 (q). MS (50°C): m/z (%) = 183 (2) ($M+H^+$), 109 (92), 96 (100) ($C_5H_4O_2^+$), 81 (40), 68 (22), 57 (12) ($C_2H_5O^+$).

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