Guignardic Acid, a Novel Type of Secondary Metabolite Produced by the Endophytic Fungus *Guignardia* sp.: Isolation, Structure Elucidation, and Asymmetric Synthesis

by **Katia F. Rodrigues-Heerklotz**[a]), **Konstantin Drandarov**[b]), **Jörg Heerklotz**[b]), **Manfred Hesse***[b]), and **Christa Werner***[b])

[a]) Departamento de Micologia, Instituto Oswaldo Cruz, *FIOCRUZ*, CP 926, BR-21045-900, Rio de Janeiro, RJ, Brazil

[b]) Organisch-chemisches Institut der Universität Zürich, Winterthurerstrasse 190, CH-8057 Zürich

A UV-guided fractionation of the AcOEt extract of the fermentation broth of *Guignardia* sp., an endophytic fungus from the leaves of the tropical tree *Spondias mombin*, resulted in the identification of the new metabolite (−)-(2*S*,5*Z*)-2-(1-methylethyl)-4-oxo-5-(phenylmethylene)-1,3-dioxolane-2-carboxylic acid (**1**), isolated as NH_4^+ salt **1a**. The metabolite **1** was designated (−)-(*S*)-guignardic acid. This first member of a new class of natural compounds contains a dioxolanone moiety formed by fusion of 2-oxo-3-phenylpropanoic acid (phenylpyruvic acid) and 3-methyl-2-oxobutanoic acid (dimethylpyruvic acid), products of the oxidative deamination of phenylalanine and valine, respectively. The structure of **1a** was deduced from spectral data (UV, IR, MS, ^{1}H- and ^{13}C-NMR) and confirmed by asymmetric synthesis.

Introduction. – The interest in endophytic fungi as a source of novel bioactive compounds is increasing mainly because of the difficulty to find new interesting lead compounds from extensively investigated organisms. In Brazil, *Spondias mombin* L. (Anacardiaceae) has been used in traditional medicine because of its antimicrobial properties [1].

Species of *Guignardia* (Ascomycetes) and/or its anamorph genus *Phyllosticta* have been frequently isolated as endophytes [2–4]. *Phyllosticta*, a genus of asexual fungi, is a source of various secondary metabolites. The most abundant, the phytotoxin phyllosinol, containing an α,β-unsaturated ketone moiety, shows antibacterial activity [5][6]. Recently the sesquiterpene lactone heptelidic acid and two analogues, which are toxic against spruce budworm larvae, have been reported [7].

During an initial screening of secondary metabolites produced by strains of different fungal species isolated as endophytes from *S. mombin* that could contain useful antimicrobial properties [8], we selected a *Guignardia* strain based on some degree of antibacterial activities demonstrated by its crude extract. In the present communication, we describe the isolation, structure elucidation, and asymmetric synthesis of the new metabolite (−)-(*S*)-guignardic acid (**1**) as ammonium salts **1a** or **1b**.

Results and Discussion. – (−)-(*S*)-Guignardic acid (**1**), isolated as its NH_4^+ salt **1a**, was obtained as amorphous solid and identified as (−)-(2*S*,5*Z*)-2-(1-methylethyl)-4-oxo-5-(phenylmethylidene)-1,3-dioxolane-2-carboxylic acid, as deduced from UV, IR, MS, ^{1}H-, ^{13}C-NMR spectral data.

We failed to obtain useful data of **1** by the usual positive mode in EI, CI, or ESI mass spectrometric methods, but the ESI-MS data of ammonium guignardate **1a** in the negative mode furnished a $[M-H]^-$ peak at

m/*z* 261 (molecular mass 262 for $C_{14}H_{14}O_5$). The tandem mass spectrum (MS/MS) from the parent ion at *m*/*z* 261 showed two daughter ions: one at *m*/*z* 217, which obviously arises by loss of CO_2, and one at *m*/*z* 189 (the base fragment ion), which can be written as $[M - H - CO_2 - CO]^-$.

The UV spectrum of the salt **1a**, showing a strong absorption band at 301 nm, is similarly shaped as those of (*E*)-cinnamic acid derivatives and suggests a similarly arranged extended chromophore system. The presence of the strong bands at 1646 and 1783 cm^{-1} in the IR spectrum of **1a**, corresponding to conjugated C=C and C=O bonds, supports such a correlation. Moreover, the strong shorter wavelength shift (at 1783 cm^{-1}) of the second band indicates that the corresponding C=O group is part of a five-membered lactone ring. In addition, the IR spectrum of **1a** contains a broad band at 3404 cm^{-1} (OH) and a strong band at 1666 cm^{-1} (C=O), which evidently belong to a COOH group.

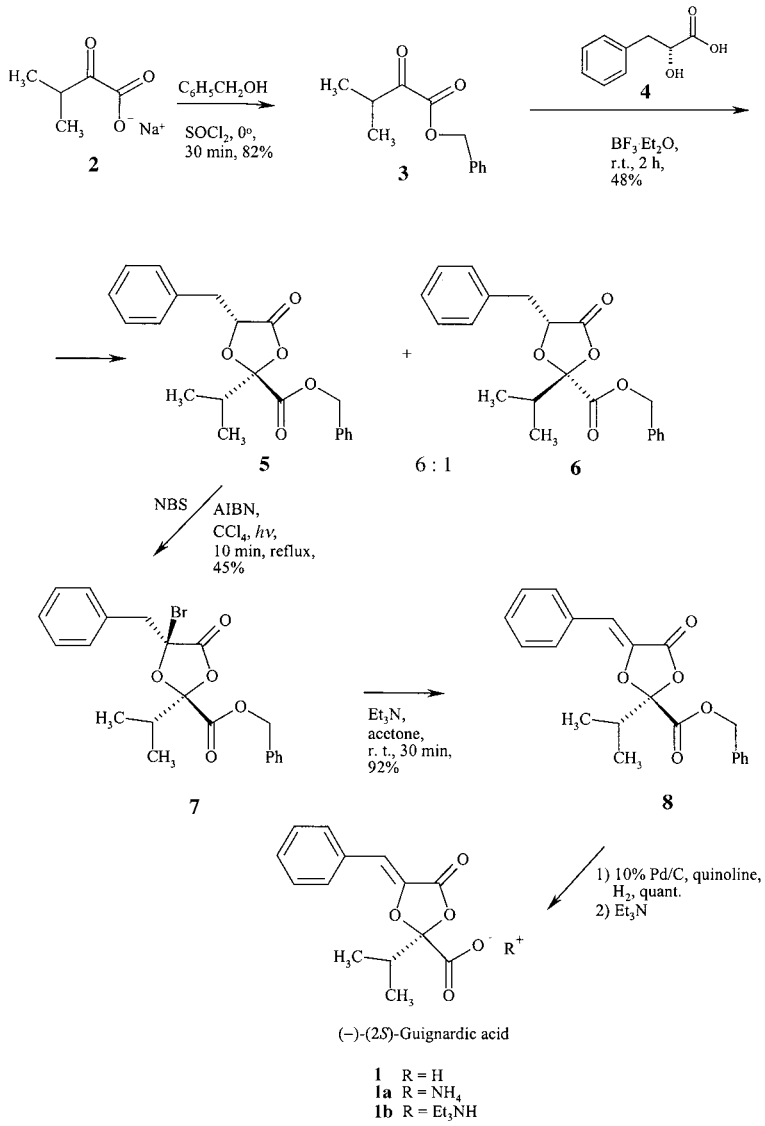
The ^{13}C-NMR spectrum of **1a** confirms the presence of two C=O groups at δ(C) 166.5 and 164.6, one olefinic methin CH at δ(C) 104.0, and two quaternary C-atoms of which one is an olefinic (δ(C) 139.1) and the other a quaternary acetal C-atom (δ(C) 111.0). The lack of spin-spin coupling of the signal at δ(H) 6.22 (*s*) is evidence that the corresponding olefinic CH group is located between two quaternary C-atoms and is part of a conjugated, exocyclic C=C bond. The signals for one methine C-atom (δ(C) 31.6 and δ(H) 2.62 (*sept.*), together with the signals for two Me groups (δ(C) 16.4 and 15.1 ppm and the *d* at δ(H) 0.92 and 0.95, resp.) indicate the presence of an isopropyl moiety in **1a**. The *d* at δ(H) 7.66 and two *t* at δ(H) 7.38 and 7.27 in the ^{1}H-NMR spectrum of **1a** are typical of a mono-substituted benzene ring. The structure elucidation of (−)-guignardic acid (**1**) was further supported by 2D NMR experiments (DEPT, HSQC, and HMBC). In addition, the presence of the strong UV absorption band at 301 nm, typical for the (*E*)-cinnamic acid chromophore is evidence for the (*Z*)-configuration of the exocyclic double bond of **1** [9][10].

To confirm the structural conclusions described above and to establish its absolute configuration, (−)-(*S*)-guignardic acid (**1**) was asymmetrically synthesized, as shown in *Scheme 1*. Starting from benzyl 3-methyl-2-oxobutanoate (**3**) (obtained from the commercially available sodium 3-methyl-2-oxobutanoate (**2**) and phenylmethanol in the presence of $SOCl_2$) and commercially available (2*R*)-2-hydroxy-3-phenylpropanoic acid (**4**) in the presence of $BF_3 \cdot Et_2O$ [11], the 6:1 mixture of the diastereoisomeric dioxolanones **5** and **6** was prepared in 48% yield similarly to a published procedure. The mixture was separated by CC. The (2*S*,5*R*)-configuration of **5** was established by a NOESY experiment (NOE between Me_2CH and $PhCH_2$−C(5)). Similarly, the (2*R*,5*R*)-configuration was deduced for the diastereoisomer **6** (NOE between Me_2CH and H−C(5)). According to a procedure used for similar compounds [12], diastereoisomer **5** was refluxed for 10 min in the presence of *N*-bromosuccinimide (NBS) and a catalytic amount of 2,2′-azobis[isobutyronitrile] (AIBN) in CCl_4 under irradiation with white light to give the brominated derivative **7** in 45% yield. The NOESY experiment of **7** confirmed its (2*R*,4*S*)-configuration (NOE between both Me of Me_2CH and arom. H-atoms of $PhCH_2$−C(5)). Thus, the bromination of **5** at C(5) proceeded in a strictly stereoselective manner with retention of configuration. The elimination of HBr from compound **7** carried out in acetone in the presence of Et_3N, similarly to a published procedure [12], yielded ester **8** quantitatively. By selective catalytic hydrogenolysis in the presence of *Lindlar* catalyst, compound **8** was transformed to (−)-(*S*)-guignardic acid (**1**) almost quantitatively. Because of its instability under acidic conditions, the synthetically prepared **1** was isolated as its triethylammonium salt **1b**. The synthetic triethylammonium (−)-(*S*)-guignardate (**1b**) was identical to the natural ammonium salt **1a** by TLC, HPLC (t_R), UV, and chiroptical properties (*Fig.*), including its absolute configuration[1]).

---

[1]) The differences between the ^{1}H-NMR chemical shifts of the natural (isolated as ammonium salt **1a**) and synthetic (−)-(*S*)-guignardic acid (prepared as triethylammonium salt **1b**) are a function of the different cationic counterparts.

Scheme 1



To the best of our knowledge, (–)-(*S*)-guignardic acid (**1**) is the first identified member of a new class of natural compounds. Obviously, the biogenetic precursors of **1** are 3-methyl-2-oxobutanoic acid (dimethylpyruvic acid; **9**) and 2-oxo-3-phenylpropanoic acid (phenylpyruvic acid; **10**), which are products of the oxidative deamination of the amino acids valine and phenylalanine, respectively. The proposed biogenetic pathway of these compounds is shown in *Scheme 2*. The existence of other combinations of naturally occurring keto acids derived from deaminated α-amino

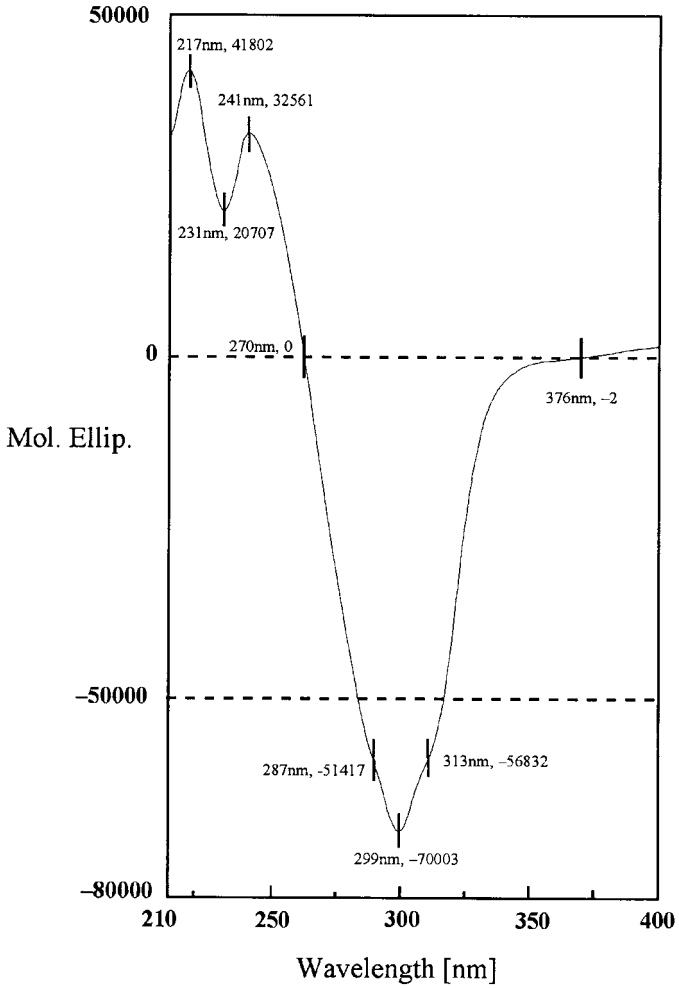
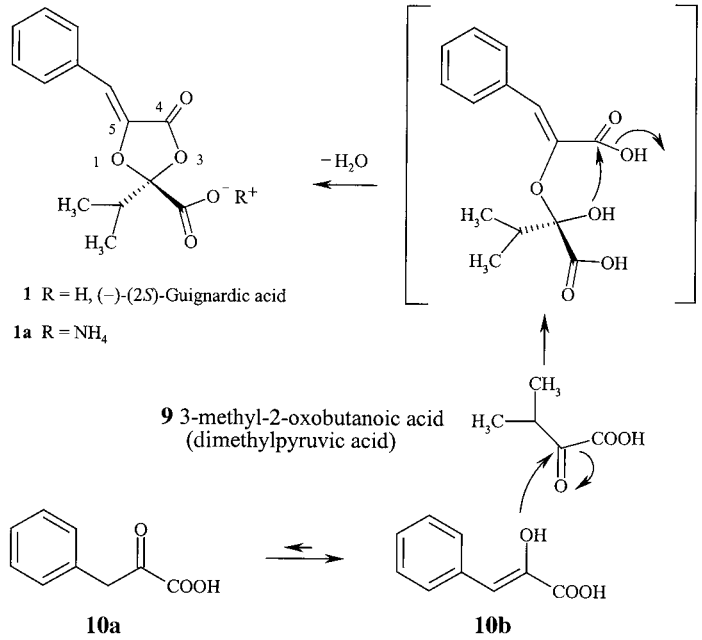


Figure. 1. *CD Spectrum of (−)-(2S)-guignardic acid ammonium salt* (**1a**)

acids is likely. It is known that phenylpyruvic acid exists mainly in its enol-tautomer form **10b**. Obviously the tautomerism of phenylpyruvic acid makes it a favored reaction partner in the formation of such natural dioxolanones.

The authors are grateful to Mr. *Martin Binder* of our NMR Department for the measurements and assistance during the interpretation of the NMR spectra of the natural guignardic acid and to Drs. *Laurent Bigler* and *Aycil Yurdakul* for the MS measurements. This study was supported in part by a *Grant-in-Aid for Scientific Research* from the *Brazilian National Council for Scientific and Technological Research* (*CNPq*), the *Swiss National Science Foundation*, and the *Dr. Helmut Legerlotz-Stiftung*.

Scheme 2



Experimental Part

General. TLC: *Merck* precoated plates, silica gel *60 F_{254}*; detection by UV (254 or 366 nm). CC: silica gel *60 F*254 (70–230 mesh) from *Merck*. UV Spectra: *Perkin-Elmer 555* spectrophotometer. IR Spectra: *Perkin-Elmer 297* infrared spectrometer; in cm^{-1}. NMR: *Bruker AMX-600* (600 MHz), *AC-300*, or *AMX-300*; (D_6)DMSO or $CDCl_3$; chemical shifts δ in ppm, Me_4Si as internal standard, *J* in Hz, ESI-MS and ESI-MS/MS: *Finnigan TSQ-700* mass spectrometer.

Fungal Strain. *Guignardia* sp. was isolated as endophyte from surface-sterilized leaves of the *Spondias mombin* L. tree as previously described [13]. Our fungal culture produced both sexual and asexual stages (*Phyllosticta*). This fungus is still under investigation. Apparently, it is a widespread species, which has been isolated repeatedly from other unrelated host plant families. It had been subjected to molecular studies, showing identical internal transcribed spacer (ITS) sequences to *Phyllosticta telopeae*, which is available in the GeneBank (accession number AF 312015). The isolate is preserved, lyophilized, and deposited in the fungal culture collection of the Mycology Department (IOC) at *FIOCRUZ* (Brazil).

Extraction and Isolation. The AcOEt extract from the broth of a 14-day fermentation in malt extract (20 g/l malt extract, 1 g/l peptone, and 20 g/l glucose) was evaporated and the residue purified by prep. TLC (silica gel, $CHCl_3$/MeOH/25% aq. NH_3 soln. 78 : 19 : 3). Eight UV-absorbing bands were scraped from the plates and eluted with MeOH/$CHCl_3$ 4 : 1. From *Fr. 2* (R_f 0.2), (−)-(*S*)-guignardic acid was isolated by prep. HPLC (*Nucleosil-C_8* column (7 μm, 10 × 250 mm; *Macherey-Nagel*, Oensingen, Switzerland), flow rate 5 ml min^{-1}, linear gradient from 100% H_2O to 100% MeOH in 20 min, then 5 min at 100% MeOH).

(−)-Ammonium (2S,5Z)-2-(1-Methylethyl)-4-oxo-5-(phenylmethylene)-1,3-dioxolane-2-carboxylate = (−)-(2S)-Guignardic Acid Ammonium Salt; **1a**). Yellowish amorphous solid. $[\alpha]_D = -56.5$ ($c = 0.20$, MeOH). IR ($CHCl_3$): 3404 (br., OH), 1783*s* (C=O, lactone), 1666*s* (C=O, acid), 1646*s* (C=C, conj.), 1360*m* (C–O). ^{1}H-NMR ((D_6)DMSO): 7.66 (*d*, *J* = 7.4, 2 arom. H); 7.38 (*t*, *J* = 7.7, 2 arom. H); 7.27 (*t*, *J* = 7.4, 1 arom. H); 6.62

(*s*, 1 H); 2.62 (*sept.*, $J = 6.8$, 1 H); 0.95 (*d*, $J = 6.9$, 1 Me); 0.92 (*d*, $J = 6.9$, 1 Me). ^{13}C-NMR ((D$_6$)DMSO): 166.5 (C=O); 164.6; 139.1; 111.0; 133.3; 129.1; 128.8; 128.1; 104.0; 31.6; 15.1; 16.4. ESI-MS: 261 ([$M-H$]$^-$, [$C_{14}H_{14}O_5-H$]$^-$). ESI-MS/MS (–DAU 261, 8 eV): 261 (36, [$M-H$]$^-$), 217 (2, [$M-H-CO_2$]$^-$), 189 (100, [$M-H-CO_2-CO$]$^-$).

Phenylmethyl 3-Methyl-2-oxobutanoate (**3**). To a suspension of sodium 3-methyl-2-oxobutanoate (**2**; 3.4 g) in phenylmethanol (12 ml), $SOCl_2$ (2.9 ml) was added dropwise at 0°. The mixture was stirred at 0° for 30 min. After addition of H_2O (20 ml), the mixture was stirred for 10 min and then extracted with AcOEt. The org. layer was washed with aq. Na_2CO_3 soln. and H_2O and evaporated. The residual oil was purified by CC (silica gel, hexane, then hexane/THF 10:0.5): **3** (3.7 g, 82%). Colorless oil. TLC (hexane/THF 19:1): R_f 0.4. ^{1}H-NMR ($CDCl_3$): 7.5–7.25 (*m*, 5 arom. H); 5.28 (*s*, PhCH_2O); 3.24 (*m*, $J = 6.9$, Me_2CH); 1.14 (*d*, $J = 6.9$, Me_2CH). ^{13}C-NMR ($CDCl_3$): 197.74 (C=O); 161.60 (ester, C=O); 134.53 (arom. quat. C); 128.59, 128.43, 128.28, 127.66 (arom. C); 67.58 (COOCH$_2$Ph); 37.07 (Me_2CH); 16.98 (Me). EI-MS: 205 (3, [$M-H$]$^+$), 91 (100, $C_7H_7^+$).

*Phenylmethyl (2*S*,5*R*)-2-(1-Methylethyl)-4-oxo-5-(phenylmethyl)-1,3-dioxolane-2-carboxylate* (**5**). A mixture of commercially available (2*R*)-2-hydroxy-3-phenylpropanoic acid (**4**; 2 g, 0.012 mol), **3** (1.27 g, 0.006 mol), and 48% $BF_3 \cdot Et_2O$ (6 ml) was stirred for 2 h at r.t., then diluted with CH_2Cl_2, and carefully transferred by small portions to a separatory funnel containing aq. Na_2CO_3 soln. After every portion, the funnel was carefully shaken until the liberation of CO_2 was finished. The aq. phase was extracted once more with CH_2Cl_2, the combined org. extract washed with H_2O and evaporated, and the residue dissolved in toluene (5 ml) and Et_3N (1.5 ml) and purified by CC (silica gel, hexane, then hexane/THF 10:0.5): **5** (1 g, 48%). Colorless oil. TLC (hexane/THF 19:1): R_f 0.3 (**6**: R_f 0.27). $[\alpha]_D = +12$ ($c = 2.38$, $CHCl_3$). ^{1}H-NMR ($CDCl_3$): 7.45–7.15 (*m*, 10 arom. H); 5.21 (*s*, PhCH_2O); 4.67, 4.66 (2*d*, $J = 6.8$, 4.2, H–C(5)); 3.21, 3.2 (2*d*, $J = 14.7$, 4.2, 1 H, PhCH_2CH); 3.07, 3.04 (2*d*, $J = 14.7$, 6.8, 1 H, PhCH_2CH); 2.37 (*m*, $J = 6.9$, Me_2CH); 0.81 (*d*, $J = 6.9$, Me_2CH). ^{13}C-NMR ($CDCl_3$): 171.5, 166.7 (2 C=O); 135.21, 134.56 (2 arom. quat. C); 129.56, 128.59, 128.41, 128.10, 127.04 (arom. C); 107.98 (C(2)); 75.75 (C(5)); 67.75 (COOCH$_2$Ph); 36.79 (PhCH$_2$CH); 31.63 (Me_2CH); 15.17, 14.62 (Me_2CH). EI-MS: 313 (40, [$M-CH_2$=CHCH$_2$]$^+$), 219 (28, [$M-$PhCH$_2$OCO]$^+$), 191 (20, [$M-$PhCH$_2$OCO – C=O]$^+$), 91 (100, $C_7H_7^+$).

*Phenylmethyl (2*R*,4*S*)-4-Bromo-2-(1-methylethyl)-5-oxo-4-(phenylmethyl)-1,3-dioxolane-2-carboxylate* (**7**). To a soln. of **5** (0.86 g, 0.0024 mol) in CCl_4 (5 ml), NBS (0.44 g, 0.0027 mol) and AIBN (12 mg) were added. The pale yellow suspension was refluxed for 10 min under irradiation with white light (normal-pressure 200-W lamp, placed at *ca.* 30 cm from the reaction flask) to give a colorless suspension[2]). The mixture was diluted with CCl_4, the insoluble succinimide removed by filtration, and the filtrate washed with sat. aq. $NaHCO_3$ soln. and evaporated. The residual oil was dissolved in a few ml of hexane containing a few drops of THF and separated by CC (hexane/THF 10:0.5): **7** (470 mg, 45%)[3]). Colorless oil. TLC (hexane/THF 19:1): R_f 0.4. $[\alpha]_D = -63$ ($c = 0.88$, $CHCl_3$). ^{1}H-NMR ($CDCl_3$): 7.45–7.15 (*m*, 10 arom. H); 5.27 (*s*, PhCH_2O); 3.81 (*s*, PhCH_2CBr); 1.99 (*m*, $J = 6.9$, Me_2CH); 0.7 (*d*, $J = 6.9$, 3 H, Me_2CH); 0.36 (*d*, $J = 6.9$, 3 H, Me_2CH). ^{13}C-NMR ($CDCl_3$): 165.66, 165.49 (2 C=O); 134.15, 133.10 (2 arom. quat. C); 128.79, 128.67, 128.56, 128.50, 127.98 (arom. C); 107.96 (C(2)); 88.45 (CBr); 68.36 (COOCH$_2$Ph); 46.32 (PhCH$_2$CBr); 33.51 (Me_2CH); 15.37, 13.66 (Me_2CH). EI-MS: 352 (4, [$M-$HBr]$^+$), 297 (20, [$M-$PhCH$_2$OCO – H]$^+$), 217 (20, [$M-$PhCH$_2$OCO – HBr]$^+$), 91 (100, $C_7H_7^+$).

*Phenylmethyl (2*S*,5*Z*)-2-(1-Methylethyl)-4-oxo-5-(phenylmethylene)-1,3-dioxolane-2-carboxylate* (**8**). To a soln. of **7** (100 mg) in acetone (4 ml), Et_3N (1 ml) was added at r.t. The mixture was stirred for 2 h (→ crystals of $Et_3N \cdot HBr$). The solvent was evaporated, the residue dissolved in toluene, the insoluble $Et_3N \cdot HBr$ filtered off, and the filtrate separated by CC (hexane, then hexane/THF 50:1): **8** (80 mg, quant.). Colorless, glass-like residue. TLC (hexane/THF 19:1): R_f 0.4. $[\alpha]_D = -142$ ($c = 0.59$, $CHCl_3$). ^{1}H-NMR ($CDCl_3$): 7.8–7.15 (*m*, 10 arom. H); 6.49 (*s*, PhCH=C); 5.26 (*s*, PhCH_2O); 2.68 (*m*, $J = 6.9$, Me_2CH); 1.04 (*d*, $J = 6.9$, Me_2CH). ^{13}C-NMR ($CDCl_3$): 165.17, 152.75 (2 C=O); 135.66, 134.43, 132.15 (2 arom. + 1 olef. quat. C); 129.79, 128.96, 128.65, 128.53, 127.90 (arom. C); 109.59 (olef. C); 108.4 (C(2)); 68.02 (COOCH$_2$Ph); 32.72 (Me_2CH); 15.12, 14.36 (Me_2CH). CI-MS: 370 (100, [$M+NH_3+H$]$^+$), 352 (10, $M^{+\cdot}$).

*Triethylammonium (2*S*,5*Z*)-2-(1-Methylethyl)-4-oxo-5-(phenylmethylene)-1,3-dioxolane-2-carboxylate* (**1b**). To a suspension of 10% Pd/C (35 mg) in toluene (2.5 ml), a 0.25% soln. of quinoline (0.67 ml) in toluene and a soln. of **8** in toluene (100 mg in 2 ml) were added. The suspension was stirred 2 h at r.t. under H_2.

---

[2]) Sometimes, the bromination reaction worked poorly; the introduction of a few drops of diluted Br_2 soln. in CCl_4 to the starting suspension helped in these cases.

[3]) Because of additional bromination of **5** at the benzyl ester CH_2 group and following hydrolysis, some benzaldehyde was formed, which was eluted just before.

Then, Et_3N (0.5 ml) was added, the catalyst filtered off, and the filtrate evaporated (bath temp. $< 45°$): **1b** (96 mg, 93%). Viscous, colorless oil. TLC ($CHCl_3$/MeOH/25% aq. NH_3 soln. 8:2:0.3): R_f 0.2. $[\alpha]_D = -92$ ($c = 1.8$, MeOH). ^{1}H-NMR ((D_6)DMSO): 7.69 (d, $J = 7.6$, 2 arom. H); 7.6–7.2 (m, 3 arom. H); 6.3 (s, 1 olef. H); 3.15 (q, $J = 7.3$, $(MeCH_2)_3N$); 2.72 (m, $J = 6.9$, Me_2CH); 1.23 (t, $J = 7.3$, $(MeCH_2)_3N$); 1.05 (t, $J = 6.9$, Me_2CH). ^{13}C-NMR ((D_6)DMSO): 166.43, 163.83 (2 C=O); 138.02, 132.83 (2 arom. quat. C); 129.55, 128.95, 128.53, 128.06 (arom. C); 110.09 (C(2)); 104.84 (olef. CH); 45.21 ($(MeCH_2)_3N$); 31.35 (Me_2CH); 15.76, 14.73 (Me_2CH); 8.26 ($(MeCH_2)_3N$). ESI-MS: 102 (100, Et_3NH^+), 364 (20, $[M + Et_3N + H]^+$), 386 (40, $[M + Et_3N + Na]^+$).

REFERENCES

[1] J. Corthout, L. Pieters, M. Claeys, D. A. Vanden Berghe, A. J. Vlietinck, *Phytochemistry* **1991**, *30*, 1129.
[2] O. Petrini, J. Stone, F. E. Carroll, *Can. J. Bot*. **1982**, *60*, 789.
[3] J. A. Johnson, N. J. Whitney, *Can. J. Bot*. **1989**, *67*, 723.
[4] A. Leuchtmann, O. Petrini, L. Petrini, G. C. Carroll, *Mycol. Res.* **1992**, *96*, 287.
[5] R. Sakai, R. Sato, H. Niki, S. Sakamura, *Plant Cell Physiol*. **1970**, *11*, 907.
[6] S. Sakamura, J. Ito, R. Sakai, *Agr. Biol. Chem*. **1971**, *35*, 105.
[7] L. A. Callhoun, J. A. Findlay, J. D. Miller, N. J. Whitney, *Mycol. Res.* **1992**, *96*, 281.
[8] K. F. Rodrigues, M. Hesse, C. Werner, *J. Basic Microbiol*. **2000**, *40*, 261.
[9] H.-J. Brunk, H. Fritz, E. Hädicke, *Chem. Ber*. **1983**, *116*, 2165.
[10] R. Ramage, G. J. Griffiths, F. E. Shutt, J. N. A. Sweeney, *J. Chem. Soc., Perkin Trans. 1*, **1984**, 1531.
[11] M. Farines, J. Soulier, *Bull. Soc. Chim. Fr.* **1970**, 332.
[12] G. Schwenker, K. Stiefvater, *Arch. Pharm. (Weinheim)* **1991**, *324*, 307.
[13] K. F. Rodrigues, G. J. Samuels, *J. Basic Microbiol*. **1999**, *39*, 131.

Received June 20, 2001