Determination of the absolute configuration of the fragments composing the phytotoxin phomalide

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Abstract: The absolute configurations of the residues constituting phomalide (**1**) were determined on the products resulting from acidic hydrolysis. The configurations of the amino acids Val and Leu were determined as (*S*) and (*R*), respectively, by chiral GC analysis (Chirasil-Val capillary column), employing *N*-trifluoroacetyl (TFA) amino acid methyl ester derivatives. The configurations of the hydroxy acids O-Phe and O-Leu were determined as (*S*) by ^{1}H NMR of the Mosher's esters of the isopropyl ester derivatives. The method described allowed for the unambiguous assignment of the absolute configuration of the α-amino and α-hydroxy acid residues composing phomalide (**1**).

Key words: absolute configuration, depsipeptide, Mosher's ester.

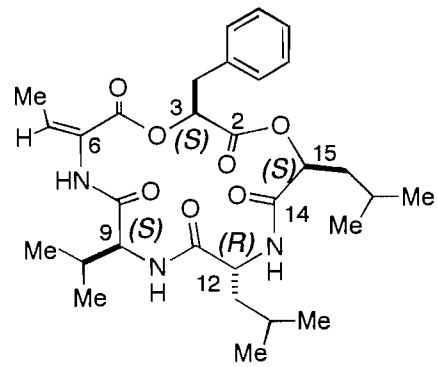
Résumé : En se basant sur les produits de son hydrolyse acide, on a déterminé les configurations absolues des divers résidus formant le phomalide (**1**). Grâce à une analyse par CG chirale (colonne capillaire Chirasil-Val) sur les dérivés *N*-trifluoroacétyles (TFA) des esters méthyliques des acides aminés, on a déterminé que les configurations absolues des acides aminés Val et Leu sont respectivement (*S*) et (*R*). Faisant appel à la RMN du ^{1}H des esters isopropyliques des esters de Mosher, on a déterminé que les configurations des acides hydroxylés O-Phe et O-Leu sont (*S*). La méthode décrite permet d'attribuer sans ambiguïté la configuration des acides α-aminés et α-hydroxylés formant le phomalide (**1**).

Mots clés: configuration absolue, depsipeptide, ester de Mosher.

[Traduit par la rédaction]

The depsipeptide phomalide (**1**) is a host-selective toxin produced by the blackleg fungus (*Leptosphaeria maculans* (Desm.) Ces. et de Not., asexual stage *Phoma lingam* (Tode ex Fr.) Desm.) of brassicas (1). Diverse phytotoxic depsipeptides are produced by phytopathogenic fungi, as for example the destruxins from *Alternaria brassicae* (2) and AM-toxins from *A. mali* (3). Phomalide is, however, an unusual depsipeptide in that it has a 15-membered ring composed of five residues; more common depsipeptides, and cyclic peptides as well, incorporate typically either four or more than five residues (4). Remarkably, phomalide is produced by the blackleg fungus in liquid cultures only for a short period (24–60 h); older fungal cultures produce nonselective phytotoxins (epipolythiodioxopiperazines) such as sirodesmin PL (**2**).[2] This unusually short production period was attributed to an inhibitory effect of sirodesmin PL (**2**) on the biosynthesis of phomalide.

The structure of phomalide (**1**) was assigned from analysis of spectroscopic data, including HMBC and HMQC (1). The



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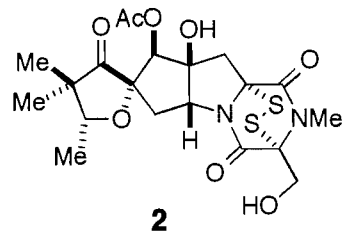

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configuration of the double bond of the rare amino acid dehydrothreonine (DhThr) residue was assigned as (*E*) based on NOE experiments on phomalide (1); chemical degradation was required to establish the absolute configuration of the four stereogenic centers. However, due to the small quantities of compound obtained from fungal cultures and the need for its

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[2] For a recent review of phytotoxins produced by the blackleg fungus see ref. 5.

biological evaluation, the use of chemical degradation presented a significant dilemma. Additionally, while the absolute configuration of the amino acid residues valine (Val) and leucine (Leu) present in phomalide (**1**) could be determined by microscale chiral GC analysis of the derivatized hydrolysis products (6), the determination of the absolute configuration of the hydroxy acid residues 3-phenyllactic acid (O-Phe) and leucic acid (O-Leu) required the development of specific methodology. The determination of the absolute configurations of the four stereogenic centers present in phomalide (**1**) is now reported here.

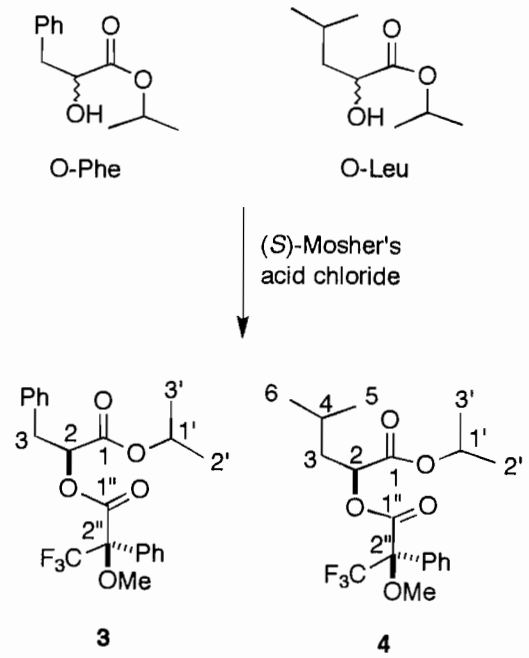
Results and discussion

The absolute configurations of the residues composing phomalide (**1**) were determined by analysis of the products resulting from acidic hydrolysis. The configurations of the amino acids Val and Leu were determined as (*S*) and (*R*), respectively, by chiral GC analysis (Chirasil-Val capillary column), employing the *N*-trifluoroacetyl (TFA) amino acid methyl ester derivatives. The configurations of the hydroxy acids O-Phe and O-Leu were determined as (*S*) by ^{1}H NMR analysis of the Mosher's esters of the isopropyl ester derivatives, as described below.

Initially, efforts were made to determine the absolute configuration of the two hydroxy residues by chiral GC analysis of the corresponding methyl, 2-propyl, and 3-pentyl ester derivatives (7). Although chiral GC analysis of the 2-propyl ester of racemic O-Leu acid indicated the presence of the enantiomeric pair (confirmed by chiral GC–MS), similar analysis could not resolve the 2-propyl, the 3-pentyl, or the methyl esters of racemic O-Phe acid (8).[3] Next, different diastereomeric derivatives of 2-propyl esters of racemic O-Phe and O-Leu were prepared to identify a suitable derivative for assignment of the absolute configuration. Thus, esterification of the O-Phe and O-Leu 2-propyl esters with (*S*)-2-methoxy-2-phenylacetyl chloride and with (*S*)-2-methoxy-2-trifluoromethyl-2-phenylacetyl chloride (Mosher's acid chloride, MPTACl) gave diastereomeric mixtures, which were analyzed by TLC and ^{1}H NMR. The two pairs of diastereomeric products resulting from reaction with the (*S*)-MPTACl were clearly distinguishable by ^{1}H NMR spectroscopy, and separable by TLC. Although the diastereomeric products resulting from reaction of 2-propyl (*R,S*)-O-Leu ester with (*S*)-2-methoxy-2-phenylacetyl chloride could be separated by TLC, those from 2-propyl (*R,S*)-O-Phe ester were inseparable. Therefore, determination of the configurations of the O-Leu and O-Phe residues present in phomalide proceeded employing derivatization of the 2-propyl esters with (*S*)-MPTACl.[4]

Phomalide was hydrolyzed by heating in aqueous HCl in a sealed tube, and the reaction mixture was extracted with Et_2O. Both the Et_2O extract (containing hydroxy acids) and the remaining aqueous phase (containing amino acids) were concentrated to dryness. The amino acid fraction was methylated with CH_2N_2, followed by acetylation with TFAA. The resulting mixture was analyzed by chiral GC and the retention times compared with authentic standards of the (*S*), (*R*), and racemic *N*-TFA amino acid methyl ester derivatives. The hydroxy acid fraction was first esterified with 2-propanol, followed by reaction with the (*S*)-MPTACl (9). The ^{1}H NMR spectrum of the reaction mixture showed characteristic proton signals due to the MeO groups at δ 3.33 and 3.53 (q, J = 1.0 Hz). These two signals allowed the unambiguous assignment of the configurations; since the signal at δ 3.33 could only be due to the derivative of (*S*)-O-Phe (**3**, cf. experimental section), the signal at δ 3.53 had to result from the (*S*)-O-Leu derivative (**4**). Analysis of the remaining proton signals of the mixture confirmed these assignments; for example, the two doublets at δ 0.92 and 0.91 (J = 6.2 Hz) due to H-5/H-6 confirmed the presence of the (*S*)-O-Leu derivative (cf. δ 0.82 and 0.80 for the (*R*)-O-Leu derivative). Furthermore, the coupling constants for the proton signal at δ 5.34 (dd, J = 10.0, 3.7 Hz, H-2) corroborated the presence of the (*S*)-O-Phe derivative (**3**) (cf. J = 8.2, 4.2 Hz, H-2, for the (*R*) isomer). These assignments have now been confirmed by the total synthesis of phomalide (10).

Scheme 1. Absolute configuration of phomalide fragments.



In summary, a method for determining the absolute configuration of small amounts of depsipeptides was developed. The method described allowed for the unambiguous assignment of the absolute configuration of the α-hydroxy acid residues composing phomalide (**1**). This method may also be applicable to other hydroxy acids of various depsipeptides.

Experimental

General

All chemicals were purchased from Aldrich Chemical Company, Inc., Madison, Wis., or Sigma Chemical Company,

[3] It appears that chiral GC methods are less likely to resolve α-hydroxy acids than α-aminoacids; see for example ref. 8.

[4] Note that the *R,S* descriptor for the absolute configuration of MPTACl is opposite to that of the precursor acid or the derived ester because the presence of the chlorine atom changes the relative Cahn–Ingold–Prelog priorities.

St. Louis, Mo. All solvents were HPLC grade and used as such, unless indicated otherwise. Preparative TLC: (Merck, Kieselgel 60 F_{254}), 20 × 20 cm × 0.25 mm; analytical TLC (Merck, Kieselgel 60 F_{254}, aluminum sheets) 5 × 2 cm × 0.2 mm; compounds were visualized by exposure to UV light and by dipping the plates in a 5% aqueous (w/v) phosphomolybdic acid solution containing a trace of ceric sulfate and 4% (v/v) H_2SO_4, followed by heating at 200°C.

GC analysis was carried out with a Hewlett–Packard 5880 gas chromatograph, equipped with a chiral capillary column (Chirasil-Val® column, Alltech, 25 m × 0.32 mm id, film thickness 0.2 μm), with He as carrier gas (flow 30 cm/s, measured at 50°C); detector at 250°C; injector at 220°C; oven temperature 70–200°C, at 4°C min^{-1}; samples were injected in split mode (ca. 1:30) with CH_2Cl_2 as solvent. NMR spectra were recorded on a Bruker AMX 500 or AM 360 spectrometer; for ^1H (360 MHz), δ values were referenced to $CHCl_3$ (7.24 ppm) and for ^{13}C (125.8 MHz) referenced to $CDCl_3$ (77.0 ppm). Mass spectra (MS) were obtained on a VG 70-250 SEQ hybrid mass spectrometer or a Finnigan Mat model 4500 mass spectrometer (electron impact (EI), or chemical ionization (CI) with ammonia as carrier gas), employing a solids probe in both cases.

Hydrolysis of phomalide

Degassed 6 M HCl (1 mL) was added to phomalide (**1**) (4 mg, 0.007 mmol) and the mixture was heated in a sealed tube at 110°C for 20 h. The reaction mixture was diluted with distilled H_2O (5 mL) and extracted with Et_2O (4 × 5 mL). The Et_2O extract, containing the hydroxy acids, was dried, filtered through a cotton plug, concentrated, and derivatized as described below. The aqueous extract, containing the amino acids, was concentrated to dryness and derivatized as described below.

Derivatization of hydrolysis products: preparation of *N*-trifluoroacetyl methyl esters of α-amino acids

One third of the residue from the aqueous extract (containing amino acid mixture) obtained from hydrolysis of phomalide was dissolved in MeOH (200 μL) and excess ethereal CH_2N_2 (1 mL) was added. After 15 min, the reaction mixture was concentrated (rotary evaporator), dissolved in CH_2Cl_2 (200 μL), and a solution of TFAA in CH_2Cl_2 (10 μL of a 25% solution, v/v) was added (6). After 1 h at room temperature, the reaction mixture was concentrated with a slow stream of N_2. The *N*-trifluoroacetyl amino acid methyl ester derivatives were analyzed by chiral GC employing a chiral capillary column.

Derivatization of hydrolysis products: preparation of 2-propyl Mosher's esters of α-hydroxy acids

The residue from the Et_2O extract (containing hydroxy acid mixture) obtained from hydrolysis of phomalide (above) was dissolved in 2-propanol (200 μL) containing 10% AcCl (v/v) in a ReactiVial and heated at 110°C for 15 min. The reaction mixture was concentrated (rotary evaporator), the residue was dissolved in $CHCl_3$ (300 μL), and Et_3N (4 μL, 0.03 mmol) and DMAP (4 mg, 0.033 mmol) were added. This solution was added to the neat (*S*)-MPTACl (6.5 mg, 0.026 mmol) (9). After 10 min the reaction mixture was concentrated (rotary evaporator) and analyzed by TLC and ^1H NMR.

Preparation of *N*-trifluoroacetyl methyl esters of Val and Leu

The *N*-trifluoroacetyl methyl esters (*N*-TFA) of both (*R*)-valine, (*R*)-leucine, and of both (*R,S*)-valine and (*R,S*)-leucine were prepared by a modification of an established method (6). Typically, to the amino acid (7.7 μmol) solution in MeOH (200 μL), a solution of CH_2N_2 in Et_2O (1.5 mL) was added at room temperature. After 30 min, the reaction mixture was concentrated (rotary evaporator) and the residue was dissolved in 25% TFAA in CH_2Cl_2 (v/v). Subsequent evaporation of the TFAA solvent with a gentle N_2 stream yielded the *N*-TFA methyl esters of the amino acids. GC analysis of the amino acid derivatives dissolved in CH_2Cl_2 and of the standards purchased from Sigma were carried out as described above; under those conditions the retention times were the following: *N*-TFA-(*R*)-Val Me ester, 5.6 min; *N*-TFA-(*S*)-Val Me ester, 6.0 min; *N*-TFA-(*R*)-Leu Me ester, 10.1 min; *N*-TFA-(*S*)-Leu Me ester, 9.2 min.

Preparation of Mosher's esters of 2-propyl O-Leu and 2-propyl O-Phe

The 2-propyl esters of hydroxy acids (*R*)-O-Leu, (*R*)-O-Phe, (*R,S*)-O-Leu, and (*R,S*)-O-Phe were prepared by a modification of an established method (7). Typically, a solution of the hydroxy acid (0.028 mmol) in AcCl–2-propanol (10:90, v/v, 200 μL) was heated to 110°C for 60 min. After concentration (rotary evaporator), Et_3N (15 μL) and DMAP (1 mg) were added to the reaction mixture dissolved in $CHCl_3$ (500 μL); this solution was added to the neat acid chloride of the (*R*) Mosher's acid ((*R*)-2-methoxy-2-phenyl-2-trifluoromethylacetic acid), prepared according to published procedure (9). The diastereomeric products (ca. 90% yield) obtained from derivatization of (*R,S*) O-Phe were separated by prep TLC (hexane–EtOAc, 10:1, v/v; multiple development); the diastereomeric products obtained from derivatization of (*R,S*) O-Leu were separated by prep TLC (hexane–EtOAc, 20:1, v/v; multiple development).

*1′-Methylethyl (*R*)-2-[(*R*)-2″-methoxy-2″-phenyl-2″-trifluoromethyl] acetoxy-3-phenylpropanoate*: ^1H NMR (360 MHz, $CDCl_3$) δ: 7.50–7.05 (m, 10 H's), 5.32 (dd, J = 8.2, 4.2 Hz, H-2), 5.05 (sept, J = 6.3 Hz, H-1′), 3.53 (q, J = 1.0 Hz, OMe), 3.18 (dd, J = 14.4, 4.2 Hz, H-3), 3.09 (dd, J = 14.4, 8.2 Hz, H-3), 1.20 (d, J = 6.3 Hz, H-2′/H-3′), 1.17 (d, J = 6.3 Hz, H-3′/H-2′); ^{13}C NMR (90.5 MHz, $CDCl_3$) δ: 168.0, 166.1, 135.1, 132.0, 129.4 (2 C's) 129.3 (2 C's), 128.33 (2 C's), 128.27 (2 C's), 127.4, 127.0, 123.0 (q, J_{C-F} = 289 Hz), 84.4 (q, $^2J_{C-F}$ = 28 Hz) 74.9, 69.8, 55.6, 37.1, 21.6, 21.6; CIMS (NH_3) *m/z*: (relative abundance): 442 (M + 18, 100); EIMS *m/z*: 189 (100), 148 (23).

*1′-Methylethyl (*S*)-2-[(*R*)-2″-methoxy-2″-phenyl-2″-trifluoromethyl] acetoxy-3-phenylpropanoate (**3**)*: ^1H NMR (360 MHz, $CDCl_3$) δ: 7.33–7.19 (m, 10 H's), 5.34 (dd, J = 10.0, 3.7 Hz, H-2), 5.06 (sept, J = 6.2 Hz, H-1′), 3.33 (q, J = 1.0 Hz, OMe), 3.28 (dd, J = 14.6, 3.7 Hz, H-3), 3.08 (dd, J = 14.6, 10.0 Hz, H-3), 1.22 (d, J = 6.2 Hz, H-2′), 1.21 (d, J = 6.2 Hz, H-3′); ^{13}C NMR (90.5 MHz, $CDCl_3$) δ: 167.9, 166.1, 135.5, 131.9, 129.5 (2 C's), 129.4 (2 C's), 128.6 (2 C's), 128.2 (2 C's), 127.5, 127.2, 123.1 (q, J_{C-F} = 289 Hz), 84.5 (q, $^2J_{C-F}$ = 28 Hz), 75.2, 69.8, 55.3, 37.1, 21.6 (2 C's); CIMS (NH_3) *m/z* (relative abun-

dance): 442 (M + 18, 100); EIMS *m/z* (relative abundance): 189 (100), 148 (20).

1′-Methylethyl (R)-2-[(R)-2″-methoxy-2″-phenyl-2″-trifluoromethyl] acetoxy-4-methylpentanoate: ^{1}H NMR (360 MHz, $CDCl_3$) δ: 7.68–7.63 (m, 2H); 7.39–7.36 (m, 3H); 5.08 (dd, J = 10.0, 3.5 Hz, H-2), 5.09 (sept, J = 6.3 Hz, H-1′), 3.64 (q, J = 1.0 Hz, OCH_3), 1.80 (m, 1H), 1.58 (m, 2H), 1.27 (d, J = 6.3 Hz, H-2′/H-3′), 1.23 (d, J = 6.3 Hz, H-3′/H-2′), 0.82 (d, J = 6.5 Hz, H-5/H-6), 0.80 (d, J = 6.5 Hz, H-6/H-5); ^{13}C NMR (90.5 MHz, $CDCl_3$) δ: 169.4, 166.4, 132.2, 129.6, 128.3 (2 C′s), 127.5 (2 C′s), 123.3, (q, J_{C-F} = 289 Hz), 84.5 (q, $^2J_{C-F}$ = 28), 73.0, 69.5, 55.7, 39.5, 24.2, 21.7, 21.7, 20.9; CIMS (NH_3) *m/z* (relative abundance): 408 (M + 18, 100); EIMS *m/z* (relative abundance): 189 (100), 115 (62).

*1′-Methylethyl (S)-2-[(R)-2″-methoxy-2″-phenyl-2″-trifluoromethyl] acetoxy-4-methyl pentanoate (**4**)*: ^{1}H NMR (360 MHz, $CDCl_3$) δ: 7.56–7.52 (m, 2 H′s), 7.40–7.37 (m, 3 H′s), 5.11 (dd, J = 10.0, 3.8 Hz, H-2), 5.06 (sept, J = 6.3 Hz, H-1′), 3.53 (q, J = 1.0 Hz, OCH_3), 1.84 (m, H-3), 1.73 (m, H-4), 1.65 (m, H-3), 1.24 (d, J = 6.3 Hz, H-2′/H-3′), 1.23 (d, J = 6.3 Hz, H-3′/H-2′), 0.92 (d, J = 6.2 Hz, H-5/H-6), 0.91 (d, J = 6.2 Hz, H-6/H-5); ^{13}C NMR (90.5 MHz, $CDCl_3$) δ: 169.1, 166.4, 131.7, 129.6, 128.3 (2 C′s), 127.8 (2 C′s), 123.2 (q, J_{C-F} = 289 Hz), 84.6 (q, $^2J_{C-F}$ = 28), 73.2, 69.4, 55.4, 39.5, 24.4, 23.0, 21.6 (2 C′s), 21.2; CIMS (NH_3) *m/z* (relative abundance): 408 (M + 18, 100); EIMS *m/z* (relative abundance): 189 (100), 115 (60).

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