PERKIN 1

Biosynthesis of phytotoxin neovasinin and its related metabolites, neovasipyrones A and B and neovasifuranones A and B, in the phytopathogenic fungus *Neocosmospora vasinfecta*

Toshio Furumoto, Takashi Hamasaki and Hiromitsu Nakajima *

Department of Agricultural Chemistry, Faculty of Agriculture, Tottori University, Koyama, Tottori 680-8553, Japan

Received (in Cambridge) 26th October 1998, Accepted 24th November 1998

To clarify the biosynthetic origins of phytotoxin neovasinin **1** and its biogenetically related metabolites, neovasipyrones A **2** and B **3** and neovasifuranones A **6** and B **7**, sodium [1,2-$^{13}C_2$]-, [1-^{13}C]- and [2-^{13}C]-acetate and L-[*S*-$^{13}CH_3$]methionine were fed to cultures of *Neocosmospora vasinfecta* NHL2298 in separate experiments. The labelling patterns of these metabolites indicated the same origin, *viz*. biosynthesis from a hexaketide chain plus five C_1 units. The absolute stereochemistries of C-7 and C-8 in neovasifuranones A **6** and B **7** were established by chemical reactions, and the stereochemical relationships among the metabolites of the fungus supported the contention that the metabolites have the same biosynthetic origin. L-[*S*-$^{13}C^2H_3$]Methionine was fed to the fungus to determine the oxidation level of the C_1 units introduced during biosynthesis. The labelling patterns indicated that in their biosynthesis the common intermediates are aldehydic, not alcoholic, compounds. The conversion of neovasifuranone A aldehyde **9** to neovasifuranone A **6** *in vivo* and *in vitro* is also evidence of this.

Introduction

Neocosmospora vasinfecta E. F. Smith is a phytopathogen which causes root- and fruit-rot and seedling damping-off in the Malvaceae, Leguminosae, Piperaceae and Cucurbitaceae.[1] During our investigation of phytopathogenic fungi as sources and/or metabolism models of novel phytotoxic secondary metabolites,[2,3] we found that a strain (NHL2298) of *N. vasinfecta* produces neovasinin **1** that is phytotoxic to soybean,[4] a host plant of this fungus.[5] The fungus also produces neovasipyrones A **2** and B **3** and neovasifuranones A **6** and B **7**, biogenetically related to neovasinin **1**.[6] The absolute stereochemistries of compounds **1**–**3** were determined unambiguously by X-ray analysis and chemical reactions. Those of the furanones **6** and **7** were deduced from comparisons of the coupling constants obtained in the ^{1}H NMR spectra and from biogenetic considerations. These metabolites have the following structural features: the carbon skeletons and stereochemistries of the asymmetric carbon atoms C-6 and C-10 of compounds **2** and **3** are identical with those of neovasinin **1**. Compounds **2**, **3**, **6** and **7** have a common side chain.

Neovasinin **1** is a fungal metabolite bearing a unique bicyclic unit, 2*H*,5*H*-pyrano[4,3-*b*]pyran-2-one, and probably is biosynthesised from a polyketide chain. Its biosynthetic origin, however, has not been verified because of the low incorporation of labelled acetate into it. To our knowledge, there are only a few reports of compounds that have this bicyclic unit; chlamydosporol,[7,8] isochlamydosporol,[8] *O*-methylisochlamydosporol[8] and multiforisin C.[9] Although the last two compounds are artefacts, multiforisin C seems to be formed from an aldehydic compound, multiforisin A. The biosynthesis of chlamydosporol and isochlamydosporol from the alcoholic compound, chlamydospordiol, was deduced from its ability to produce these metabolites and from their chemical structures, but detailed biosynthetic investigations have not been made.[8,10] In a previous report, we proposed the formation of neovasinin **1** from the alcoholic metabolites, neovasipyrones A **2** and B **3**, on the basis of their chemical structures, which were not examined.[6] In addition, the phytotoxic activity of **1** would be due to the bicyclic unit because **1** and neovasifuranones A **6** and B **7** are phytotoxic, whereas pyrones **2** and **3** are not. We therefore undertook experiments to clarify the biosynthetic pathway of neovasinin **1**, in particular the mechanism for bicyclic-unit formation, and the biogenetic relationship of neovasinin **1** to neovasipyrones A **2** and B **3** and neovasifuranones A **6** and B **7**. The incorporation patterns of ^{13}C-labelled precursors into these metabolites and the stereochemistries of furanones **6** and **7** showed that the metabolites have the same origin, *viz*. biosynthesis from a hexaketide plus five C_1 units. Moreover, on the basis of the labelling patterns of $^{13}C^2H_3$-labelled methionine and the conversion of candidates for the intermediates *in vivo* and *in vitro*, compounds **2** and **3** are not intermediates of neovasinin **1** and the common intermediates are aldehydic compounds.

Results and discussion

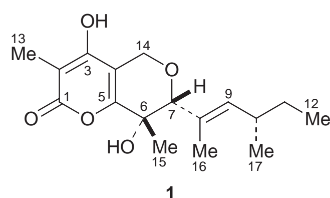
We first conducted ^{13}C-labelled sodium acetate and methionine incorporation experiments to confirm the polyketide origin of neovasinin **1**. A labelled acetate incorporation experiment using malt–sucrose–peptone medium supplemented with unlabelled methionine was unsuccessful because of the low incorporation (<0.2% specific incorporation,[11] data not shown) of labelled acetate into neovasinin **1**. This probably was owing to dilution of the fed labelled acetate by the endogenous unlabelled acetate of the fungus. Various media and carbon sources were tested to improve incorporation. The carbon economy of the cell could be controlled and improved by the use of malt–glycerol–peptone medium (~1% specific incorporation). Incorporation experiments with ^{13}C-labelled acetate therefore were done with this improved medium. In contrast, labelled methionine incorporation studies carried out with conventional malt–sucrose–peptone medium were successful. Results of the incorporation experiments with ^{13}C-labelled precursors are given in Table 1 and Scheme 1.

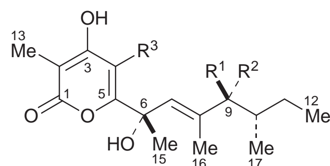
The ^{13}C NMR spectrum of neovasinin **1** enriched with sodium [1,2-$^{13}C_2$]acetate indicated that six intact acetate units were incorporated into the molecule (C-1 to C-12).[12] Six carbon atoms (C-1, C-3, C-5, C-7, C-9 and C-11) were enriched by

Table 1 ^{13}C NMR data[a] for neovasinin **1**, neovasipyrones A **2** and B **3** and neovasifuranones A **6** and B **7** enriched with ^{13}C-labelled precursors

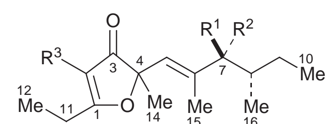
	δ_C					
Carbon	**1**	**2**	**3**	**6**	**7**	J_{CC} (Hz)[b] **1**
1	164.3■	164.9	164.7	194.0	189.5	76.3
2	98.9●	100.6	100.7	114.1	111.9	76.3
3	161.8■	167.7	167.5	208.2	205.6	60.4
4	107.6●	110.4	110.7	91.0	88.5	60.4
5	155.7■	163.5	163.3	124.4	123.7	53.6
6	68.2●	75.5	75.2	146.0	143.5	53.6
7	87.7■	132.7	134.7	82.3	82.1	50.4
8	131.1●	140.3	140.0	39.5	37.5	50.4
9	136.2■	80.8	83.2	28.1	24.4	43.0
10	33.2●	38.9	38.9	12.7	11.1	43.0
11	29.9■	27.6	26.1	24.2	22.5	35.2
12	11.9●	12.4	11.9	11.6▲	10.6▲	35.2
13	9.1▲	9.1▲	9.1▲	53.2▲	53.4▲	
14	61.7▲	58.8▲	58.7▲	25.2▲	23.9▲	
15	20.6▲	28.7▲	28.1▲	14.8▲	12.8▲	
16	13.3▲	13.8▲	12.4▲	15.2▲	15.6▲	
17	20.5▲	14.7▲	16.4▲			

[a] Recorded at 67.8 MHz in DMSO-d_6 for **1**, in acetone-d_6 for **2** and **3**, in methanol-d_4 for **6** and in $CDCl_3$ for **7**. [b] Obtained for **1** enriched with [1,2-$^{13}C_2$]acetate. ■: from [1-^{13}C]acetate (the range of relative enrichment observed, 1.8–2.6), ●: from [2-^{13}C]acetate (1.7–2.6), ▲: from [$^{13}CH_3$]methionine (9.1–28.8).

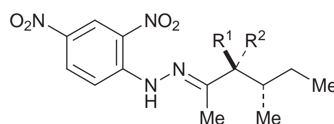




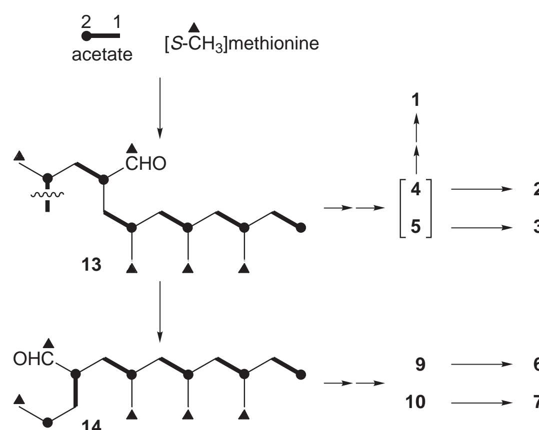
2 R¹ = H, R² = OH, R³ = CH₂OH (C-14)
3 R¹ = OH, R² = H, R³ = CH₂OH
4 R¹ = H, R² = OH, R³ = CHO
5 R¹ = OH, R² = H, R³ = CHO



6 R¹ = H, R² = OH, R³ = CH₂OH (C-13)
7 R¹ = OH, R² = H, R³ = CH₂OH
8 R¹R² = O, R³ = CH₂OCOC(Me)₃
9 R¹ = H, R² = OH, R³ = CHO
10 R¹ = OH, R² = H, R³ = CHO



11 R¹ = H, R² = OAc
12 R¹ = OAc, R² = H



Scheme 1 Proposed biosynthetic pathways to neovasinin **1**, neovasipyrones A **2** and B **3** and neovasifuranones A **6** and B **7**.

sodium [1-^{13}C]acetate, and the other six carbon atoms (C-2, C-4, C-6, C-8, C-10 and C-12) were enriched by sodium [2-^{13}C]acetate. The incorporation of L-[S-$^{13}CH_3$]methionine indicates that the remaining five carbon atoms (C-13 to C-17) were derived from the C_1 unit. The labelling patterns of neovasinin **1** show that it is biosynthesised from a linear hexaketide chain plus five C_1 units. In the incorporation experiments done with the labelled acetate, neither neovasipyrones A **2** and B **3** nor neovasifuranones A **6** and B **7** were detected in the culture broth. In the experiment with ^{13}C-labelled methionine, however, both of these metabolites and neovasinin **1** were obtained. Assignments of ^{13}C NMR resonances of compounds **3** and **7**, some of which were ambiguous,[6] were confirmed by two-dimensional NMR experiments (H,H- and C,H-COSY) (Table 1). In the ^{13}C NMR spectra of pyrones **2** and **3** enriched with ^{13}C-labelled methionine, five of the signals (C-13 to C-17) had enhanced intensities greater than the natural abundance. Five of the carbon signals (C-12 to C-16) of enriched furanones **6** and **7** also were enhanced. This suggests that the biosynthetic origin of neovasipyrones A **2** and B **3** and neovasifuranones A **6** and B **7** is the same as that of neovasinin **1** and that furanones **6** and **7** lose one carbon atom from the hexaketide chain during their biosynthesis.

The chemical reactions of neovasifuranones A **6** and B **7** next were investigated to establish their stereochemistries.[13] Neovasifuranone A **6** was protected partially with a pivaloyl group then oxidised with DMSO and acetic anhydride[14] to give the 7-oxo derivative **8**, which was identical with the derivative from neovasifuranone B **7**, evidence that furanones **6** and **7** differ only in their stereochemistry at C-7. Acetylation of diol **6** gave its diacetate. Oxidative cleavage of the diacetate with ruthenium trichloride and sodium metaperiodate,[15] followed by derivatisation of the product with 2,4-dinitrophenylhydrazine, afforded

the 2,4-dinitrophenylhydrazone derivative of one diastereoisomer of 3-acetoxy-4-methylhexan-2-one, compound **11**. The same treatment of diol **7** gave the 2,4-dinitrophenylhydrazone derivative of another diastereoisomeric 3-acetoxy-4-methylhexan-2-one, compound **12**. Similar reactions of neovasipyrones A **2** and B **3**, whose respective absolute stereochemistries were established by X-ray analysis and chemical reactions, gave the same products, (3*R*,4*S*)- and (3*S*,4*S*)-3-acetoxy-4-methylhexan-2-one 2,4-dinitrophenylhydrazone, **11** and **12**. This indicates that the configurations of C-7 and C-8 in furanone **6** are (*R*,*S*) and in furanone **7** (*S*,*S*). The stereochemistries of these metabolites support the supposition that these metabolites have the same biosynthetic origin.

The methylene carbon atom at C-14 in neovasinin **1**, which is derived from the C_1 unit, is involved in the formation of the pyran ring in the bicyclic unit. In general, *C*-methylation occurs by direct nucleophilic displacement of the *S*-methyl group in *S*-adenosyl-L-methionine before release of the completed polyketide from the synthase complex.[16] The hydroxymethyl carbon atoms in neovasipyrones A **2** and B **3** and neovasifuranones A **6** and B **7** also are derived from the C_1 unit and therefore would be of the same biosynthetic origin as the methylene carbon atom in neovasinin **1**. To determine the oxidation level at C-14 in compounds **1**–**3** and at C-13 in furanones **6** and **7** during their biosynthesis, we used L-[*S*-$^{13}C^2H_3$]methionine as the precursor in the incorporation experiment.[17] Deuterium-induced α-shifted signals in the ^{13}C NMR spectra of the enriched metabolites were used to determine the number of deuterium atoms retained.[12] In the ^{13}C NMR spectrum of neovasinin **1** enriched with this precursor, the natural signal of C-14 showed a triplet (*J* 22 Hz) 0.30 ppm upfield of the C-14 signal. Similar upfield-shifted triplets were present in the ^{13}C NMR spectra of compounds **2** (0.32 ppm), **3** (0.32 ppm), **6** (0.30 ppm) and **7** (0.32 ppm). The multiplicity and magnitudes of these α-shifted signals are consistent with the retention of one deuterium atom on the methylene carbon atom at C-14 in compounds **1**–**3** and at C-13 in furanones **6** and **7**,[12] evidence that the C_1 units introduced at these sites are oxidised to the aldehyde level during biosynthesis. The biosynthetic intermediate of these metabolites therefore appears to be an aldehydic rather than a hydroxymethyl compound.

To investigate this possibility, we conducted *in vivo* and *in vitro* conversion experiments with aldehydic and alcoholic compounds. Unfortunately, synthesis and isolation of aldehydic compounds of the neovasipyrones were unsuccessful. Oxidation of neovasifuranone A **6** with manganese dioxide,[18] however, gave its aldehydic compound, named neovasifuranone A aldehyde **9**. In the replacement culture experiment, the fungus converted aldehyde **9** to the alcohol **6**, whereas compound **6** was not converted to aldehyde **9**, nor were neovasipyrones A **2** and B **3** converted to neovasinin **1**. Enzymic conversion was performed with a cytosol fraction prepared from mycelia of *N. vasinfecta* NHL2298. When this fraction was incubated with aldehyde **9** in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH), only compound **6** was formed [Fig. 1(a)]. When, however, it was incubated with aldehyde **9** in the presence of reduced nicotinamide adenine dinucleotide (NADH) or in the absence of the coenzyme, aldehyde **9** was not converted to alcohol **6** [Fig. 1(b)]. No reverse reaction (from **6** to **9**) occurred under various conditions with the oxidised form of the coenzyme and a different buffer, indicative that the enzyme(s) catalyses the reduction of the aldehyde group in neovasifuranone A aldehyde **9** to the hydroxymethyl group and specifically requires NADPH as the primary electron donor.

On the basis of the above findings, we propose the biosynthetic pathways of neovasinin **1**, neovasipyrones A **2** and B **3** and neovasifuranones A **6** and B **7** to be as shown in Scheme 1. These metabolites originate from a hexaketide chain plus five C_1 units. Oxidation of one of the five C_1 units introduced to

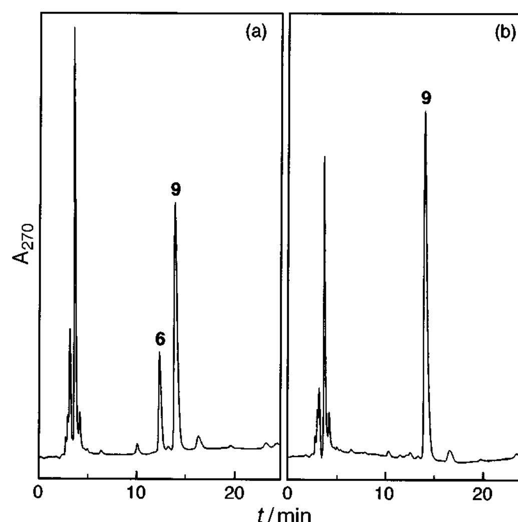


Fig. 1 HPLC chromatograms of the products derived from the enzymic conversion of neovasifuranone A aldehyde **9** to neovasifuranone A **6**; (a) in the presence of NADPH and (b) in the absence of the coenzyme.

the hexaketide chain produces the aldehydic intermediate **13**, the common biosynthetic intermediate of these metabolites. Reduction of an aldehyde group in common intermediate **13** leads to the production of pyrones **2** and **3**, and cyclisation leads to the production of neovasinin **1**. Decarboxylation of intermediate **13** generates aldehydic intermediate **14**. Compounds **6** and **7** respectively are biosynthesised through the formation of neovasifuranones A aldehyde **9** and B aldehyde **10** from intermediate **14** and the subsequent reductions of aldehydes **9** and **10**.

Elsewhere, we proposed the direct conversion of neovasipyrones A **2** and B **3** to neovasinin **1** on the basis of their chemical structures,[6] but our present findings exclude this hypothesis. Recently, we isolated and characterised neovasipyridones A–F produced by the same fungus (*N. vasinfecta* NHL2298).[19] Except for the N-alkyl groups, their carbon networks agree with those of neovasifuranones A **6** and B **7**. Moreover, the variety of N-alkyl groups suggests that Schiff bases formed from alkylamines and an aldehyde group of intermediate **14** are involved in the biosynthesis of the neovasipyridones, further evidence for the existence of an aldehydic intermediate. The direct biosynthetic intermediates of neovasipyrones A **2** and B **3** therefore are aldehydic compounds, named neovasipyrones A aldehyde **4** and B aldehyde **5**, derived from intermediate **13**. Neovasinin **1** also would be formed from aldehydes **4** and **5** *via* certain metabolic steps.

Experimental

General procedure

NMR spectra were recorded with a JEOL JNM GX-270 FT NMR spectrometer at 270 MHz for ^{1}H and 67.8 MHz for ^{13}C. All NMR chemical shifts were referenced to the signal of the deuterated solvent used ($CDCl_3$, δ_H 7.26, δ_C 77.0; acetone-d_6, δ_C 30.3; methanol-d_4, δ_C 49.8; DMSO-d_6, δ_C 39.5). *J*-Values are given in Hz. Mass spectra were obtained with a JEOL AX-505 spectrometer. IR spectra were measured with a JASCO FT/IR-7000 spectrometer, and UV spectra with a Shimadzu UV-2200 UV-Vis recording spectrophotometer. Optical rotations were determined with a Horiba SEPA-200 high sensitive polarimeter, and $[a]_D$-values are given in 10^{-1} deg cm^2 g^{-1}. All the labelled precursors were purchased from Sigma Chemical Company. Daisogel IR-60 was the silica gel used for column chromatography. Preparative TLC (PLC) was done on Merck Kieselgel 60 HF_{254} glass plates (20 × 20 × 0.05 cm).

Fungal material

The strain NHL2298 of *Neocosmospora vasinfecta* E. F. Smith var. *africana* (von Arx) Cannon et Hawksworth used in this study was a gift in 1983 from Dr S. Udagawa of the National Institute of Hygienic Sciences (present address: Nodai Research Institute, Tokyo University of Agriculture, Tokyo, Japan) and has been maintained on potato–dextrose–agar medium.

Incorporation of labelled acetates

The fungus was grown without shaking at 24 °C in a 500 cm^3 conical flask containing liquid medium (100 cm^3 × 20) composed of glycerol (10 g dm^{-3}), peptone (3 g dm^{-3}) and the extract from 50 g dm^{-3} of malt and water. Sodium [1,2-$^{13}C_2$]-, [1-^{13}C]- or [2-^{13}C]-acetate (99 atom% ^{13}C; 200 mg per day) and unlabelled L-methionine (100 mg per day) dissolved in sterilised water (20 cm^3) were supplied to the culture every 24 h from day 5 to day 9. Three days after the last supply of labelled acetate, the culture filtrate was acidified to pH 2.0 with HCl, then was treated with EtOAc (4 × 500 cm^3). The EtOAc extracts were dried over Na_2SO_4, concentrated under reduced pressure and treated with 1 M $NaHCO_3$ (2 × 0.5 vol.). The $NaHCO_3$ solution obtained was acidified to pH 2.0 with HCl and treated with EtOAc (4 × 1 vol.) to give the acidic EtOAc-soluble (AE) fraction. This fraction was subjected to silica gel partition column chromatography (20 g; impregnated with 12 cm^3 of 0.1 M HCO_2H; 2.1 × 13 cm). The column was developed successively with 100-cm^3 portions of 10, 20, 30, 40 and 50% EtOAc in hexane saturated with 0.1 M HCO_2H. The 20 and 30% fractions were combined and concentrated. The residue was purified by PLC [acetone–$CHCl_3$–AcOH (10:90:1); quadruple development] to afford neovasinin **1** (~14 mg).

Incorporation of labelled methionines

The fungus was grown without shaking at 24 °C in a 500 cm^3 conical flask containing liquid medium (100 cm^3 × 10) composed of sucrose (50 g dm^{-3}), peptone (3 g dm^{-3}) and the extract from 100 g dm^{-3} of malt and water. L-[*S*-$^{13}CH_3$]-Methionine (99 atom% ^{13}C; 100 mg per day) or L-[*S*-$^{13}C^2H_3$]-methionine (99 atom% ^{13}C, 99 atom% ^{2}H; 50 mg per day) dissolved in sterilised water (10 cm^3) was supplied to the culture every 24 h from day 8 to day 12. Nine (^{13}C-labelled methionine) or seven days ($^{13}C^2H_3$-labelled methionine) after the last supply of labelled methionine, the culture filtrate was acidified to pH 2.0 with HCl, then was treated with EtOAc (4 × 500 cm^3). The EtOAc extracts were dried over Na_2SO_4, concentrated under reduced pressure and washed with 1 M $NaHCO_3$ (2 × 0.5 vol.), to give the neutral EtOAc-soluble (NE) fraction. The $NaHCO_3$ washings were acidified to pH 2.0 with HCl and treated with EtOAc (4 × 1 vol.), to afford the AE fraction. The AE fraction was subjected to silica gel partition column chromatography as described above. The 20 and 30% fractions were purified by PLC [acetone–$CHCl_3$–AcOH (10:90:1); quadruple development], to give neovasinin **1** (^{13}C-labelled: 63 mg, $^{13}C^2H_3$-labelled: 91 mg). The 40 and 50% fractions were purified by PLC [acetone–$CHCl_3$–AcOH (25:75:1); quadruple development] to give neovasipyrones A **2** (^{13}C-labelled: 47 mg, $^{13}C^2H_3$-labelled: 61 mg) and B **3** (^{13}C-labelled: 18 mg, $^{13}C^2H_3$-labelled: 22 mg). The NE fraction was purified by PLC [acetone–$CHCl_3$ (1:3); triple development] giving neovasifuranones A **6** (^{13}C-labelled: 11 mg, $^{13}C^2H_3$-labelled: 10 mg) and B **7** (^{13}C-labelled: 6 mg, $^{13}C^2H_3$-labelled: 7 mg).

7-Oxo derivative 8

Pivaloyl chloride (25 mm^3) was added to a solution of neovasifuranone A **6** (20.5 mg) in pyridine (10 mm^3) and CH_2Cl_2 (0.5 cm^3), and the solution was stirred for 8 h at 20 °C. The reaction mixture was diluted with water (1 cm^3), stirred for 1 h, then was diluted with EtOAc (20 cm^3). The resulting solution was washed successively with 1 M $NaHCO_3$ (2 × 10 cm^3) and brine (2 × 10 cm^3), dried over Na_2SO_4, and concentrated to dryness under reduced pressure. A solution of the residue (26.7 mg) in dry DMSO (0.3 cm^3) and Ac_2O (0.2 cm^3) was stirred for 18 h at 20 °C. EtOH (1.5 cm^3) was added to the reaction mixture, and the solution was stirred for 1 h. The reaction mixture then was diluted with water (10 cm^3) and treated with EtOAc (4 × 10 cm^3). The EtOAc solution obtained was washed with brine (2 × 15 cm^3), dried over Na_2SO_4, then was concentrated to dryness. The residue was purified by preparative HPLC [COSMOSIL $5C_{18}$-AR (10 × 250 mm); 1.0 cm^3 min^{-1}; MeOH–water (9:1)] to give 7-oxo derivative **8** (6.4 mg, 24%) as an oil, $[\alpha]_D^{22}$ −40.3 (*c* 0.3, EtOH); λ_{max}(EtOH)/nm 235 (log ε/dm^3 mol^{-1} cm^{-1} 4.01) and 266 (3.97); ν_{max}(KBr)/cm^{-1} 1730, 1676, 1626, 1462, 1406, 1281, 1204, 1150 and 1051; δ_H($CDCl_3$) 0.80 (3H, t, *J* 7.4, 10-H_3), 1.05 (3H, d, *J* 6.8, 16-H_3), 1.16 (9H, s, Me of pivaloyl), 1.25 (3H, t, *J* 7.6, 12-H_3), 1.35 (1H, m, 9-H), 1.57 (3H, s, 14-H_3), 1.62 (1H, m, 9-H), 1.96 (3H, d, *J* 1.3, 15-H_3), 2.72 (2H, q, *J* 7.6, 11-H_2), 3.08 (1H, ddq, *J* 6.8, 6.8 and 6.8, 8-H), 4.74 (2H, s, 13-H_2) and 6.44 (1H, dq, *J* 0.5 and 1.3, 5-H); *m/z* (CI) 365 (MH^+, 37%) and 263 (100).

Neovasifuranone B **7** (19.8 mg) was subjected to the procedure used for diastereoisomer **6** and gave 7-oxo derivative **8** (3.2 mg, 13%), $[\alpha]_D^{22}$ −39.7 (*c* 0.3, EtOH).

(3*R*,4*S*)- and (3*S*,4*S*)-3-Acetoxy-4-methylhexan-2-one 2,4-dinitrophenylhydrazone, 11 and 12

A solution of neovasipyrone A **2** (52.0 mg) in pyridine (0.2 cm^3), Ac_2O (0.1 cm^3) and CH_2Cl_2 (0.4 cm^3) was stirred for 22 h at rt. Water (10 cm^3) was added, and the solution was stirred for 1 h, then was treated with EtOAc (4 × 10 cm^3). The EtOAc solution obtained was washed with brine (2 × 25 cm^3), dried over Na_2SO_4, then was dried completely under reduced pressure. A solution of the residue (71.0 mg), $RuCl_3$ (5 mg) and $NaIO_4$ (130 mg) in CCl_4 (1 cm^3), MeCN (1 cm^3) and water (1.5 cm^3) was stirred vigorously for 1 h at rt. This reaction mixture was added to EtOAc (20 cm^3), and the solution obtained was washed with brine (4 × 10 cm^3). A solution of 2,4-dinitrophenylhydrazine (160 mg) in EtOH (1 cm^3) and conc. H_2SO_4 (0.5 cm^3) was added to the EtOAc solution, and the whole was stirred for 40 min at rt. The reaction mixture was then washed with brine (3 × 10 cm^3), dried over Na_2SO_4 and concentrated to dryness. The residue was purified by PLC [acetone–hexane (1:19); quintuple development], to afford (3*R*,4*S*)-3-acetoxy-4-methylhexan-2-one 2,4-dinitrophenylhydrazone **11** (28.5 mg, 51%) as a yellow oil, $[\alpha]_D^{20}$ +24.9 (*c* 0.3, EtOH); ν_{max}(KBr)/cm^{-1} 1744, 1618, 1595, 1518, 1339 and 1235; δ_H($CDCl_3$) 0.95 (3H, t, *J* 7.3), 0.99 (3H, d, *J* 6.6), 1.22 (1H, m), 1.43 (1H, m), 1.91 (1H, m), 2.04 (3H, s), 2.14 (3H, s), 5.26 (1H, d, *J* 7.0), 7.95 (1H, d, *J* 9.5), 8.33 (1H, ddd, *J* 9.5, 2.6 and 0.5), 9.13 (1H, d, *J* 2.6) and 11.05 (1H, br s); *m/z* (EI) 352 (M^+, 67%), 292 (45), 263 (91), 253 (56), 207 (45), 191 (37) and 59 (100).

Neovasipyrone B **3** (52.0 mg) was subjected to the procedure used for diastereoisomer **2**, to give (3*S*,4*S*)-3-acetoxy-4-methylhexan-2-one 2,4-dinitrophenylhydrazone **12** (22.4 mg, 40%) as a yellow oil, $[\alpha]_D^{20}$ +14.0 (*c* 0.3, EtOH); ν_{max}(KBr)/cm^{-1} 1742, 1618, 1595, 1518, 1339 and 1233; δ_H($CDCl_3$) 0.90 (3H, d, *J* 6.8), 0.95 (3H, t, *J* 7.3), 1.24 (1H, m), 1.59 (1H, m), 1.91 (1H, m), 2.04 (3H, s), 2.12 (3H, s), 5.19 (1H, d, *J* 8.2), 7.96 (1H, d, *J* 9.5), 8.32 (1H, ddd, *J* 9.5, 2.6 and 0.5), 9.11 (1H, d, *J* 2.6) and 11.03 (1H, br s); *m/z* (EI) 352 (M^+, 36%), 292 (22), 263 (43), 253 (30), 207 (25), 191 (20) and 59 (100).

A solution of neovasifuranone A **6** (52.3 mg) in pyridine (0.2 cm^3), Ac_2O (0.1 cm^3) and CH_2Cl_2 (0.4 cm^3) was stirred for 22 h at rt. Water (5 cm^3) was added to the reaction mixture, and the whole was stirred for 1 h, then was treated with EtOAc (4 × 5 cm^3). The EtOAc solution obtained was washed with brine (2 × 10 cm^3), dried over Na_2SO_4, and concentrated to dryness.

A solution of the residue (64.0 mg), $RuCl_3$ (2 mg) and $NaIO_4$ (160 mg) in CCl_4 (0.5 cm^3), MeCN (0.5 cm^3) and water (0.75 cm^3) was stirred vigorously for 12 h at 28 °C. This reaction mixture was combined with EtOAc (10 cm^3), and the solution was washed successively with 1 M $NaHCO_3$ (2 × 5 cm^3) and brine (2 × 5 cm^3). A solution of 2,4-dinitrophenylhydrazine (180 mg) in EtOH (1 cm^3) and conc. H_2SO_4 (0.2 cm^3) was added to this EtOAc solution, and the whole was stirred for 40 min at 28 °C. The reaction mixture then was washed with brine (3 × 10 cm^3), dried over Na_2SO_4, and concentrated to dryness. The residue was purified by PLC to give 2,4-dinitrophenylhydrazone **11** (33.1 mg, 51%), $[\alpha]_D^{20}$ +26.2 (*c* 0.3, EtOH).

Neovasifuranone B **7** (54.8 mg) was subjected to the procedure used for diastereoisomer **6**, to afford 2,4-dinitrophenylhydrazone **12** (30.6 mg, 45%), $[\alpha]_D^{20}$ +14.8 (*c* 0.3, EtOH).

Neovasifuranone A aldehyde 9

A suspension of neovasifuranone A **6** (10 mg) and MnO_2 (200 mg) in dry benzene (3 cm^3) was stirred for 8 h at 28 °C. The reaction mixture was diluted with water-saturated EtOAc, then was filtered through silica gel (Kieselgel 60 HF_{254}), after which the filtrate was concentrated to dryness. The residue was purified by PLC [acetone–hexane (3:7); double development] to give compound **9** (2.7 mg, 27%) as an oil, $[\alpha]_D^{22}$ −165 (*c* 0.1, EtOH); λ_{max}(EtOH)/nm 202 (log ε/dm^3 mol^{-1} cm^{-1} 3.90), 234 (4.05) and 271 (4.03); ν_{max}(KBr)/cm^{-1} 3439, 1717, 1680, 1570, 1462, 1366, 1262 and 1047; δ_H($CDCl_3$) 0.84 (3H, d, *J* 6.8, 16-H_3), 0.91 (3H, t, *J* 7.3, 10-H_3), 1.14 (1H, m, 9-H), 1.28 (3H, t, *J* 7.6, 12-H_3), 1.33 (1H, m, 9-H), 1.52 (1H, m, 8-H), 1.59 (3H, s, 14-H_3), 1.72 (3H, d, *J* 1.2, 15-H_3), 3.03 (1H, dq, *J* 15.2 and 7.6, 11-H), 3.10 (1H, dq, *J* 15.2 and 7.6, 11-H), 3.82 (1H, br d, *J* 5.8, 7-H), 5.53 (1H, dq, *J* 1.2 and 1.2, 5-H) and 9.80 (1H, s, 13-H); *m*/*z* (EI) 280 (M^+, 8%), 262 (21), 223 (23), 205 (10), 195 (14), 167 (21), 149 (15), 127 (40), 97 (100), 71 (14) and 57 (36).

Conversion of neovasifuranone A aldehyde 9 to neovasifuranone A 6[2,20]

Replacement culture. The fungus was grown without shaking at 24 °C in a test-tube (10 × 75 mm) containing liquid medium (0.5 cm^3 × 5) composed of sucrose (50 g dm^{-3}), peptone (3 g dm^{-3}), malt extract (20 g dm^{-3}; DIFCO) and water. After 8 days of incubation the medium was replaced with 0.1 M potassium phosphate buffer (pH 7.0), and the fungus was cultured for 2 days. The buffer then was replaced with fresh phosphate buffer containing neovasifuranone A aldehyde **9** (10 µg per test tube), and the fungus was cultured for 4 days. EtOAc was used to extract the converted products from the culture filtrate. The products were detected by HPLC analysis [COSMOSIL $5C_{18}$-AR (4.6 × 150 mm); MeOH–water–MeCN–AcOH (50:40:10:0.5); 0.5 cm^3 min^{-1}; 270 nm].

Preparation of the cytosol fraction. The fungus was grown without shaking at 24 °C for 8 days in a 500 cm^3 conical flask containing liquid medium (100 cm^3 × 10) composed of sucrose (50 g dm^{-3}), peptone (3 g dm^{-3}), the extract from 100 g dm^{-3} of malt, L-methionine (0.3 g dm^{-3}) and water. All subsequent procedures were done at 0 to 5 °C, and 50 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM dithiothreitol and 6 µM leupeptin was the buffer, unless otherwise stated. Mycelial mats obtained after the culture broth was filtered were washed successively with distilled water and buffer. The washed mycelia (69 g, wet weight) were ground with sea sand (24 g) and buffer (140 cm^3) in a mortar with a pestle. The homogenate was centrifuged at 800 × *g* for 10 min then at 12,000 × *g* for 15 min, to give a cell-free extract, which was centrifuged at 105,000 × *g* for 1.5 h. The resulting supernatant was used as the cytosol fraction. This fraction, to which glycerol (final concentration, 25%) was added, was stored at −80 °C until used. Protein content (1.1 mg cm^{-3}) was estimated by the method of Bradford[21] with bovine serum albumin as the standard.

Enzymic conversion. The reaction mixture in a 1.5 cm^3 microtube consisted of the buffer (33 mm^3), 1.5 mM neovasifuranone A aldehyde **9** (2 mm^3 in MeOH), 20 mM NADPH (5 mm^3 in the buffer) and the cytosol fraction (10 mm^3). It was incubated at 30 °C for 30 min with shaking (100 rpm), and its products were extracted with water-saturated EtOAc (3 × 100 mm^3). These products were detected by the method used in the replacement culture experiment.

Acknowledgements

We thank Dr S. Udagawa of Tokyo University of Agriculture for providing the fungus, and Mr K. Kato, Mr T. Miyatani and Ms S. Tamaki of this laboratory for their technical assistance.

References

1 K. H. Domsch, W. Gams and T.-H. Anderson, *Compendium of Soil Fungi*, Academic Press, London, 1980, vol. 1, p. 509.
2 H. Nakajima, K. Isomi, T. Hamasaki and M. Ichinoe, *Tetrahedron Lett.*, 1994, **35**, 9597; H. Nakajima, Y. Toratsu, Y. Fujii, M. Ichinoe and T. Hamasaki, *Tetrahedron Lett.*, 1998, **39**, 1013.
3 H. Nakajima, R. Matsumoto, Y. Kimura and T. Hamasaki, *J. Chem. Soc., Chem. Commun.*, 1992, 1654; H. Nakajima, H. Fujimoto, R. Matsumoto and T. Hamasaki, *J. Org. Chem.*, 1993, **58**, 4526; H. Nakajima, K. Fukuyama, H. Fujimoto, T. Baba and T. Hamasaki, *J. Chem. Soc., Perkin Trans. 1*, 1994, 1865.
4 H. Nakajima, K. Nishimura, T. Hamasaki, Y. Kimura and S. Udagawa, *Agric. Biol. Chem.*, 1987, **51**, 2831; H. Nakajima, K. Fukuyama, Y. Kimura and T. Hamasaki, *Biosci., Biotechnol. Biochem.*, 1992, **56**, 1148.
5 *Compendium of Soybean Diseases*, ed. J. B. Sinclair, The American Phytopathological Society, Minnesota, 2nd edn., 1982, p. 35.
6 T. Furumoto, K. Fukuyama, T. Hamasaki and H. Nakajima, *Phytochemistry*, 1995, **40**, 745.
7 J. F. Grove and P. B. Hitchcock, *J. Chem. Soc., Perkin Trans. 1*, 1991, 997.
8 M. Solfrizzo, A. Visconti, M. E. Savard, B. A. Blackwell and P. E. Nelson, *Mycopathologia*, 1994, **127**, 95.
9 H. Fujimoto, Y. Satoh, M. Nakayama, T. Takayama and M. Yamazaki, *Chem. Pharm. Bull.*, 1995, **43**, 547.
10 A. Visconti, M. Solfrizzo, A. Fruchier and J. W. ApSimon, *J. Nat. Prod.*, 1994, **57**, 695.
11 E. I. Graziani and R. J. Andersen, *J. Am. Chem. Soc.*, 1996, **118**, 4701.
12 T. J. Simpson, *Chem. Soc. Rev.*, 1987, **16**, 123.
13 T. Furumoto, T. Hamasaki and H. Nakajima, *Tetrahedron Lett.*, 1997, **38**, 5523.
14 J. D. Albright and L. Goldman, *J. Am. Chem. Soc.*, 1965, **87**, 4214.
15 P. H. J. Carlsen, T. Katsuki, V. S. Martin and K. B. Sharpless, *J. Org. Chem.*, 1981, **46**, 3936.
16 M. Yamazaki and S. Shibata, *Chem. Pharm. Bull.*, 1966, **14**, 96; M. W. Steward and N. M. Packter, *Biochem. J.*, 1968, **109**, 1; R. H. Carter, M. J. Garson and J. Staunton, *J. Chem. Soc., Chem. Commun.*, 1979, 1097; L. Colombo, C. Gennari and C. Scolastico, *J. Chem. Soc., Chem. Commun.*, 1979, 492.
17 C. N. Lewis, J. Staunton and D. C. Sunter, *J. Chem. Soc., Perkin Trans. 1*, 1988, 747.
18 D. J. Pasto and C. R. Johnson, *Organic Structure Determination*, Prentice-Hall, NJ, 1969, p. 364.
19 H. Nakajima, K. Shimomura, T. Furumoto and T. Hamasaki, *Phytochemistry*, 1995, **40**, 1643; 1996, **43**, 1015.
20 K. Yabe, Y. Ando and T. Hamasaki, *Appl. Environ. Microbiol.*, 1988, **54**, 2101; K. Yabe, Y. Matsuyama, Y. Ando, H. Nakajima and T. Hamasaki, *Appl. Environ. Microbiol.*, 1993, **59**, 2486.
21 M. M. Bradford, *Anal. Biochem.*, 1976, **72**, 248.

Paper 8/08295B