Biosynthetic study of the phytotoxin neovasinin, produced by the phytopathogenic fungus *Neocosmospora vasinfecta*

（植物病原菌 *Neocosmospora vasinfecta* の生産する植物毒素 neovasinin の生合成研究）

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CHAPTER 1

General Introduction

The components of all plants, animals, and microorganisms are biosynthesized by enzyme reactions. The study of metabolism, a fundamental and vital procedure in living things, has provided us detailed understanding of the processes involved. A complex network of enzyme-catalyzed reactions, beginning with the photosynthetic reaction of carbon dioxide and water, is now evident that produces diverse compounds called primary metabolites (e.g., monomers such as amino acids, sugars, nucleotides, acetyl coenzyme A, and mevalonic acid, as well as polymers such as proteins, polysaccharides, lipids, and nucleic acids) [1]. This intricate network of vital biochemical reactions is referred to as primary metabolism. Several criteria distinguish secondary metabolites from primary metabolites. Secondary metabolites have restricted distributions that often are characteristic of individual genera, species, or strains. Moreover, they are formed from primary metabolites via specialized pathways. Unlike secondary metabolites, primary metabolites have a broad distribution and are common organic compounds in all living things. In particular, these latter metabolites are intimately involved in essential life processes. Although secondary metabolites probably are important to the organism that produces them, they are nonessential for life, and their roles are not apparent [2].

Secondary metabolites are biosynthesized from a small number of primary metabolites: acetyl coenzyme A (acetyl CoA), mevalonic acid, amino acids, sugars, and shikimic acid [3, 4]. In particular, acetyl CoA is an important intermediate of secondary metabolite biosynthesis because almost all other intermediates are directly or indirectly produced from it. The classification of secondary metabolites is based on biogenesis: terpenoids from isoprene units, polyketides from acetate units, metabolites from the shikimic acid pathway, alkaloids, and miscellaneous metabolites.

Polyketide-derived compounds abound in both procaryotes and eucaryotes; for example, in actinomycetes and fungi [5]. Polyketides are secondary metabolites that represent a large group of secondary natural products. They also are one of the major

sources of antibiotics, mycotoxins, plant growth regulators, pigments, enzyme inhibitor, and so on. Findings of a large number of previous investigations support the hypothesis that the mechanism of polyketide biosynthesis from short chain fatty acids, such as acetate, is closely related to that of fatty acid biosynthesis and is catalyzed by a multienzyme complex. In 1907 Collie [6] suggested that the [CH_2-CO] group could be made to yield a very large number of natural products by means of the simplest reactions. No biochemical support of his idea was provided until 1953 when Birch et al. [7] reported from the isotopic labeling patterns of several fungal metabolites that the biosynthesis of numerous secondary metabolites is correlated with repeating [CH_2-CO] units based on the acetate. Polyketide chain growth differs from fatty acid biosynthesis at several points (Fig. 1.1) [8]. In fatty acid biosynthesis, the addition of the next acetate unit (i.e., malonyl CoA) occurs together with fatty acid synthase (FAS) after reduction of the preceding unit is completed. In polyketide biosynthesis, however, this addition may occur with polyketide synthase (PKS)

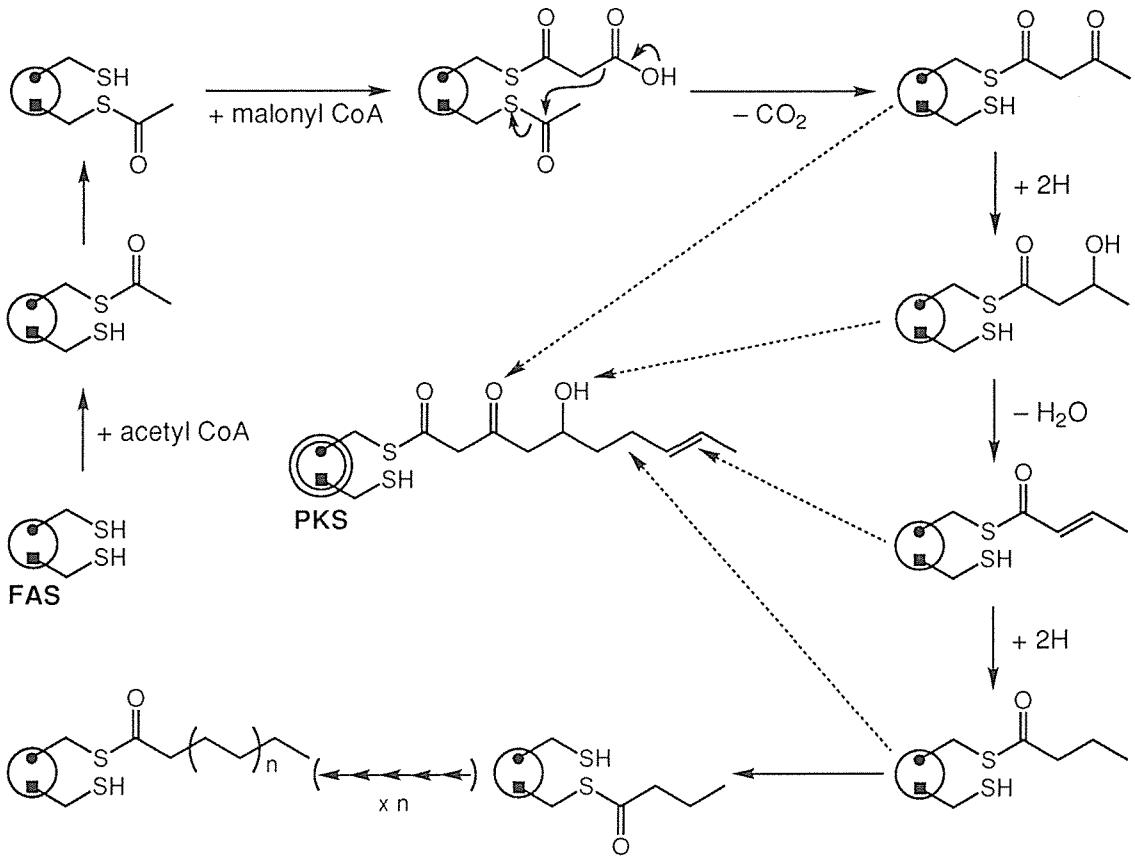

Fig. 1.1. Mechanisms of fatty acid and polyketide formation.

before the reduction, leading to the incorporation of double bonds and hydroxyl and carbonyl groups into the growing chain; formation of aromatic, ether, or lactone ring system; and retention of acetate-derived oxygen atoms in the metabolites [9–11]. Another difference is addition of such moieties as methyl and acetyl groups, terpene chains, amino acids, and/or sugar residues to the polyketide metabolites [5]. *C*-methylation, in particular, occurs before the release of the completed polyketide from the PKS enzyme [12–15]. In general, polyketides have the components of between four and ten acetate units. A large number of tetra-, penta-, hepta-, and octaketides are known, whereas tri-, hexa-, nona-, and decaketides are less common [4, 5].

The genus *Neocosmospora* belongs to the Sphaeriales, family Hypocreaceae, and occurs as a mild parasite on a wide range of host plants in tropical areas [16, 17]. Its frequent occurrence on soil or rhizosphere suggests that it may be a widely distributed root-infecting fungus. *Neocosmospora vasinfecta* E. F. Smith is a pathogen which causes root- and fruit-rot, and seedling damping-off in the Malvaceae, Leguminosae, Piperaceae, Cucurbitaceae, etc., including pepper, groundnuts, soybean, beans, coconuts, *Albizzia*, *Crotalaria* spp., and others. Soybean, *Glycine max* L., an important crop for food, feed, and industrial uses, suffers Neocosmospora stem rot by this fungus [18]. The primary symptom is reddish to dark brown discoloration of the pith and xylem with occasional chlorosis and defoliation of the lower leaves. This fungus also damages pea plants and cress seedlings by its excretion of the phytotoxins javanicin and fusarubin which also have antifungal activity [16].

Neovasinin (Fig. 1.2) was isolated by Nakajima et al. from the culture filtrate of the strain (NHL2298) of *Neocosmospora vasinfecta* E. F. Smith var. *africana* (von Arx) Cannon et Hawksworth (Fig. 1.3), obtained from soil in Johannesburg (Republic of South Africa) [19]. It is phytotoxic to soybean, a host plant of this fungus [20]. Its structure has been deduced by spectroscopic methods and from a key reaction [19]. Its absolute stereochemistry has been established by X-ray analysis and from a degradation reaction [20]. A metabolite related to neovasinin, neovasinone (Fig. 1.2), also has been isolated from the same fungus [21].

Neovasinin is a new type of fungal metabolite that has a unique bicyclic unit, 2*H*,5*H*-

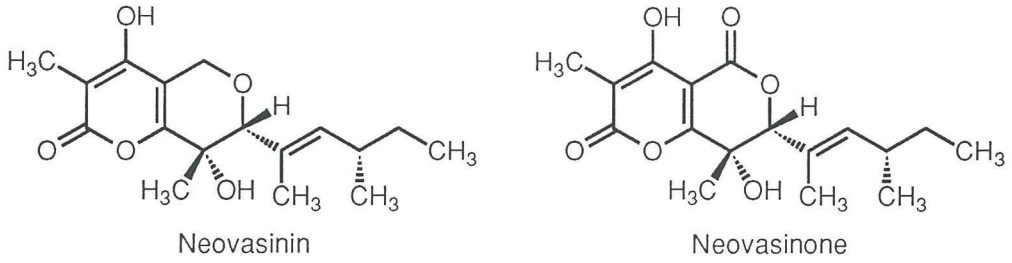

Fig. 1.2. Structures of neovasinin and neovasinone.

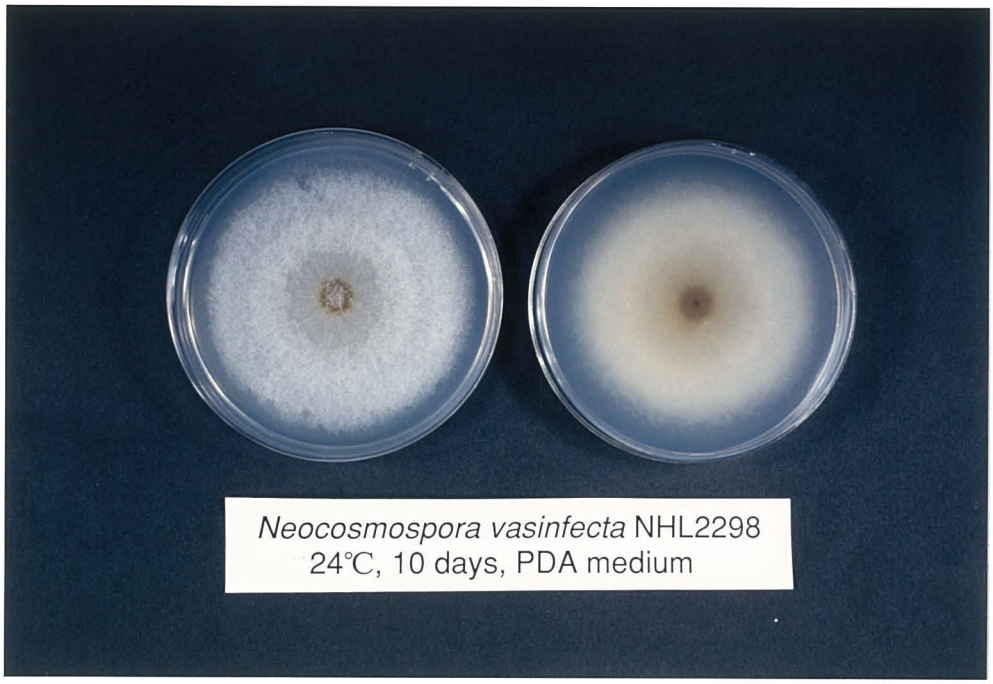

Fig. 1.3. *Neocosmospora vasinfecta* E. F. Smith, strain NHL2298 grown on potato–dextrose–agar medium at 24°C for 10 days.

pyrano[4,3-*b*]pyran-2-one, and that probably is biosynthesized from a hexaketide and five C_1 units. There are a few reports on metabolites that have this bicyclic unit; chlamydosporol [22], isochlamydosporol [23], and multiforisin C [24], but no biosynthetic investigations of these metabolites have been made [25]. In this research therefore my intention was to clarify the biosynthetic pathway of neovasinin, in particular the formation of the bicyclic unit.

In chapter 2, the isolation and structure determination of neovasipyrones A and B, neovasifuranones A and B, and vasinfectins A and B are described. In chapter 3, the isolation and structure determination of neovasipyridones A–F are described. In chapter 4, the incorporation of ^{13}C-labeled precursors into neovasinin, neovasipyrones, and neovasifuranones is described. The incorporation of [*S*-$^{13}C^{2}H_3$]-L-methionine into neovasinin, neovasipyrones, and neovasifuranones, as well as the replacement culture and enzymatic conversion of neovasifuranone A aldehyde to neovasifuranone A also are reported.

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CHAPTER 2

Isolation and Structure Determination of Neovasipyrones, Neovasifuranones, and Vasinfectins

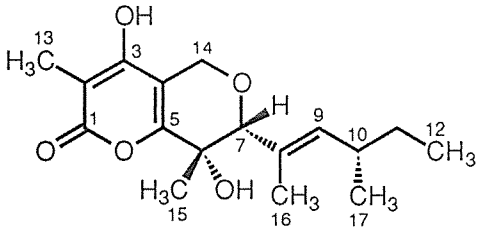
Introduction

Neocosmospora vasinfecta E. F. Smith is a pathogen which causes root- and fruit-rot and seedling damping-off in various plants [1]. *N. vasinfecta* NHL2298 produces neovasinin (**1**) which is phytotoxic to soybean, a host plant of this fungus [2, 3]. Compound **1** is a new type of fungal metabolite that has a unique bicyclic unit, 2*H*,5*H*-pyrano[4,3-*b*]pyran-2-one, and that probably is biosynthesized from a hexaketide and five C_1 units. To explore its biosynthetic pathway, in particular the formation of the bicyclic unit, the isolation of the metabolites biogenetically related to neovasinin (**1**), produced by this fungus, was attempted. The isolation and structures of six new metabolites, named neovasipyrones A (**2a**) and B (**2b**), neovasifuranones A (**5a**) and B (**5b**), and vasinfectins A (**9a**) and B (**9b**) are described in this chapter.

Results and Discussion

N. vasinfecta NHL2298 produced neovasipyrones (**2**), neovasifuranones (**5**), and vasinfectins (**9**) in trace amounts on the conventional malt–peptone–sucrose medium, which had been used for production of neovasinin (**1**). Addition of L-methionine to this medium, according to the report on the biosynthesis of secondary metabolites in another fungus [4], stimulated a dramatic production of neovasipyrones (**2**), neovasifuranones (**5**), and vasinfectins (**9**) as well as **1**. In this study I adopted this improved culture condition.

Neovasipyrones A (**2a**) and B (**2b**) in the acidic ethyl acetate extract of *N. vasinfecta* culture filtrate were purified by silica gel partition, silica gel, and Sephadex LH-20 column chromatography, successively. The separation of **2a** from **2b** was achieved by preparative thin-layer chromatography (TLC) to afford **2a** and **2b** both as pure compounds in



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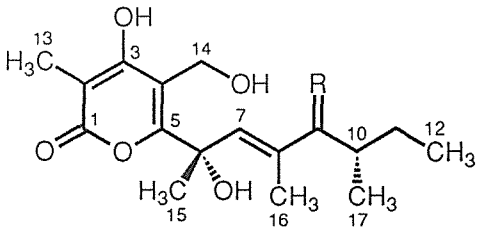
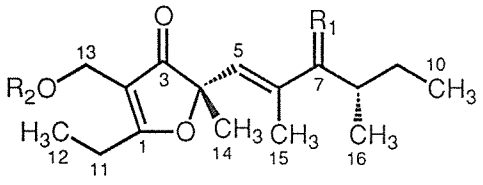
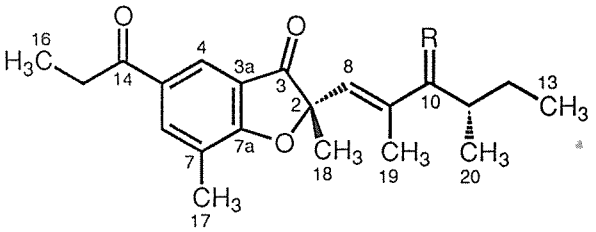


2a R = α-OH, β-H
2b R = α-H, β-OH
3 R = O



5a R_1 = α-OH, β-H, R_2 = H
5b R_1 = α-H, β-OH, R_2 = H
6 R_1 = α-OAc, β-H, R_2 = Ac
7 R_1 = O, R_2 = $COC(CH_3)_3$



9a R = α-OH, β-H
9b R = α-H, β-OH
10 R = α-OAc, β-H
11 R = O

respective yields of 72 and 31 mg/L. Neovasifuranones A (**5a**) and B (**5b**) in the neutral ethyl acetate extract were purified by silica gel and Sephadex LH-20 column chromatography, successively. The separation of **5a** from **5b** also was achieved by preparative TLC to afford **5a** and **5b** both in a pure state in respective yields of 3.0 and 1.9 mg/L. Vasinfectins A (**9a**) and B (**9b**) in the neutral ethyl acetate extract were purified by silica gel column chromatography and preparative TLC, successively. The separation of **9a** from **9b** was achieved by reversed phase high performance liquid chromatography (HPLC) to afford **9a** and **9b** both as pure compounds in respective yields of 0.48 and 0.50 mg/L.

Neovasipyrones A and B.

Neovasipyrone A (**2a**) had a molecular formula of $C_{17}H_{26}O_6$ [high resolution fast atom bombardment mass spectrometry (HRFABMS) and ^{13}C NMR data]. The ^{13}C NMR spectrum of **2a** (Table 2.1) showed 17 resonances, five of which were due to methyls, two

Table 2.1. NMR data (^{1}H, 270.05 MHz and ^{13}C, 67.80 MHz) for neovasipyrones A (**2a**) and B (**2b**) in acetone-d_6.

Position	**2a**		**2b**	
	δ_C	δ_H	δ_C	δ_H
1	164.9		164.7	
2	100.6		100.7	
3	167.7		167.5	
4	110.4		110.7	
5	163.5		163.3	
6	75.5		75.2	
7	132.7	5.74 *dq* (1.1, 1.2)	134.7	5.69 *dq* (0.7, 1.3)*
8	140.3		140.0	
9	80.8	3.66 *dd* (1.1, 5.8)	83.2	3.54 *br. d* (8.6)
10	38.9	1.46 *m*	38.9	1.40 *m*
11	27.6	1.05 *m*	26.1	1.08 *m*
		1.35 *m*		1.71 *m*
12	12.4	0.83 *t* (7.7)	11.9*	0.82 *t* (7.4)
13	9.1	1.81 *s*	9.1	1.82 *s*
14	58.8	4.80 *d* (13.1)	58.7	4.85 *d* (13.4)
		4.88 *d* (13.1)		4.88 *d* (13.4)
15	28.7	1.54 *s*	28.1	1.55 *s*
16	13.8	1.43 *d* (1.2)	12.4*	1.39 *d* (1.3)
17	14.7	0.81 *d* (6.9)	16.4	0.70 *d* (6.7)

^{1}H NMR data represent chemical shift, multiplicity (*J* in Hz).
* Assignments may be interchanged.

to methylenes, three to methines, and seven to quaternary carbons from a distortionless enhancement by polarization transfer (DEPT) experiment [5], indicative of four hydroxyls in **2a**. The five ^{13}C resonances (δ_C 100.6, 110.4, 163.5, 164.9, and 167.7), together with the ultraviolet (UV) absorption at 291 nm and the infrared (IR) absorption bands (3373, 1663, and 1567 cm^{-1}), indicated a trisubstituted 4-hydroxy-2*H*-pyran-2-one moiety in **2a** [2, 6, 7]. Two ^{13}C resonances (δ_C 132.7 and 140.3) were attributed to two olefinic carbons

in a trisubstituted double bond. The structure of **2a** was unambiguously deduced from analysis of ^{1}H-^{1}H correlation spectroscopy (H,H-COSY) [8, 9], C,H-COSY [10, 11], long-range C,H-COSY (COLOC) [10, 11], and nuclear Overhauser effect (NOE) difference spectral data. The COLOC spectrum of **2a** was especially helpful in the assignment of the structure. The significant correlations in the COLOC spectrum are summarized in Fig. 2.1.

The structure of the side chain (C-6 to C-12) was deduced from H,H-COSY and COLOC data. The H,H-COSY data allowed the construction of a 1-hydroxy-2-methylbutyl group. Additionally, the olefinic proton, 7-H (δ_H 5.74), had the allylic correlations with both the hydroxymethine proton, 9-H (δ_H 3.66), and the methyl protons, 16-H_3 (δ_H 1.43), indicative of the attachment of a 1-hydroxy-2-methylbutyl group and a methyl group (C-16) to the olefinic carbon, C-8, in the double bond. The stereochemistry around the double bond was assigned as *E* on the basis of NOE data. Irradiation of the signal of the olefinic proton, 7-H (δ_H 5.74), caused NOE enhancement on the signal of the hydroxymethine, 9-H (δ_H 3.66), but had no effect on the signal of the methyl protons, 16-H_3 (δ_H 1.43). The connection of C-6 and C-7 was deduced from COLOC data as follows. First, the attachment of the methyl group, C-15, to the hydroxy quaternary carbon, C-6, was quite obvious from ^{1}H and ^{13}C NMR data. The two carbons, C-6 and C-7, had correlations with the methyl protons, 15-H_3, and in addition, C-6 had a correlation with the olefinic proton, 7-H. Thus the establishment of the structure of the side chain was

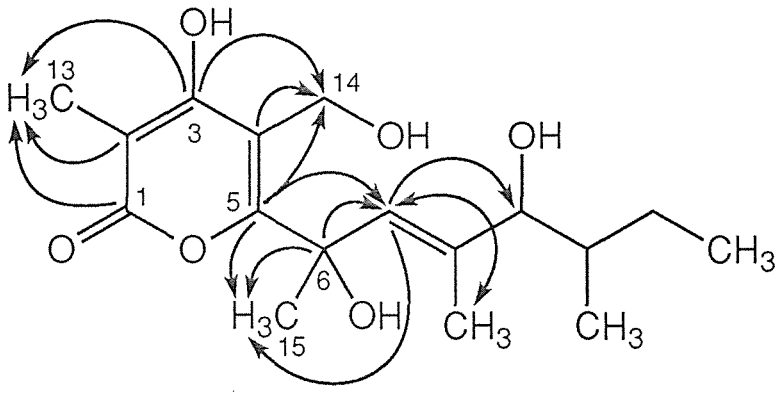


Fig. 2.1. Key long-range couplings detected in the COLOC experiment of neovasipyrone A (**2a**).

completed. The structure of the 4-hydroxy-2*H*-pyran-2-one portion was then established through the analysis of COLOC data. Three carbons, C-1, C-2, and C-3, were correlated with the methyl protons, 13-H_3, and also three carbons, C-3, C-4, and C-5, were correlated with the hydroxymethyl protons, 14-H_2. The carbon, C-5, had correlations with both the olefinic proton, 7-H, and the methyl protons, 15-H_3. These correlations allowed the complete assignment of the 4-hydroxy-2*H*-pyran-2-one portion, and the entire structure therefore was established for **2a**.

Neovasipyrone B (**2b**) had a molecular formula of $C_{17}H_{26}O_6$ (HRFABMS and ^{13}C NMR data) and the same structure as neovasipyrone A (**2a**) from their NMR data comparison (Table 2.1), that is, they are diastereomer of each other. Oxidation of **2a** with Jones reagent [12] gave a 9-oxo derivative (**3**), which was identical with the derivative from **2b**. This indicate that **2a** and **2b** are different only in the stereochemistry at C-9. To establish their stereochemistry by X-ray analysis, crystallization of **2a** was attempted, but was unsuccessful. Fortunately, **2b** was crystallized from acetone–benzene to afford plates containing one equivalent of benzene, and the crystals were adequate for X-ray analysis. The molecular structure of **2b** is shown in Fig. 2.2. The relative configuration of **2b** was found to be (6*S**,9*S**,10*S**), and thus that of **2a** was (6*S**,9*R**,10*S**). Degradation of **2a** was carried out to establish its absolute stereochemistry. The reaction of **2a** with osmium tetroxide (OsO_4) and sodium metaperiodate ($NaIO_4$) [13], followed by treatment with 2,4-

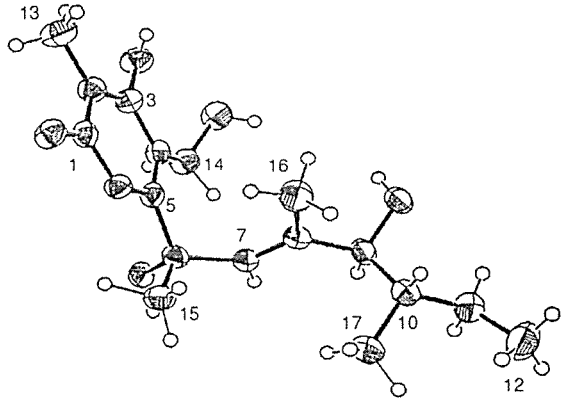


Fig. 2.2. Perspective view of neovasipyrone B (**2b**) drawn by ORTEP-II [27].

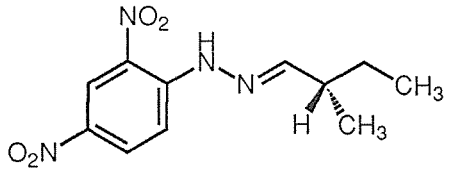


Fig. 2.3. Structure of 2,4-dinitrophenylhydrazone of (*S*)-2-methyl-1-butanal (**4**).

dinitrophenylhydrazine, afforded the 2,4-dinitrophenylhydrazone of 2-methyl-1-butanal (**4**, Fig. 2.3). Its optical rotation (+24.7°) was identical with that of the synthetic 2,4-dinitrophenylhydrazone of (*S*)-2-methyl-1-butanal (**4**, +23.3°) [3], indicative that the configuration of C-10 in **2a** is *S*. Consequently, the absolute stereochemistry of **2a** is (6*S*,9*R*,10*S*), and that of **2b** is (6*S*,9*S*,10*S*).

Neovasifuranones A and B.

Neovasifuranone A (**5a**) had a molecular formula of $C_{16}H_{26}O_4$ [high resolution electron ionization mass spectrometry (HREIMS) and ^{13}C NMR data]. The ^{13}C NMR spectrum of **5a** (Table 2.2) showed 16 resonances, five of which were due to methyls, three to methylenes, three to methines, and five to quaternary carbons, indicative of two hydroxyls in **5a**. The four ^{13}C resonances (δ_C 91.0, 114.1, 194.0, and 208.2), together with the UV absorption at 268 nm and the IR absorption bands (1688 and 1611 cm^{-1}), indicated a tetrasubstituted 2,3-dihydrofuran-3-one portion in **5a** [14–16]. Two ^{13}C resonances (δ_C 124.4 and 146.0) were attributed to two olefinic carbons in a trisubstituted double bond. The structure of **5a** was unambiguously deduced from analysis of H,H-COSY, C,H-COSY, COLOC, and NOE data in the same manner as in the structural elucidation of neovasipyrone A (**2a**). The significant correlations in the COLOC spectrum are summarized in Fig. 2.4. Acetylation of **5a** afforded a diacetyl derivative (**6**), in the ^1H NMR spectrum of which two proton resonances associated with a hydroxymethine and a hydroxymethyl were shifted downfield (from δ_H 3.68 to 4.92 and from δ_H 4.20 to 4.64 and 4.66), indicative that the two carbons, C-7 and C-13, in **5a** bear free hydroxyl groups.

The structure of the side chain (C-5 to C-10) was deduced to be 3-hydroxy-2,4-dimethyl-1-hexenyl from H,H-COSY and COLOC data. The stereochemistry of the

Table 2.2. NMR data (^{1}H, 270.05 MHz and ^{13}C, 67.80 MHz) for neovasifuranones A (**5a**) in CD_3OD and B (**5b**) in $CDCl_3$.

Position	**5a**		**5b**	
	δ_C	δ_H	δ_C	δ_H
1	194.0		189.5	
2	114.1		111.9	
3	208.2		205.6	
4	91.0		88.5	
5	124.4	5.45 *dq* (1.0, 1.1)	123.7	5.44 *dq* (1.2, 1.0)
6	146.0		143.5	
7	82.3	3.68 *dd* (1.0, 6.5)	82.1	3.66 *br. d* (7.7)
8	39.5	1.49 *m*	37.5	1.50 *m*
9	28.1	1.08 *m*	24.4*	1.10 *m*
		1.38 *m*		1.66 *m*
10	12.7	0.90 *t* (7.3)	11.1†	0.89 *t* (7.3)
11	24.2	2.69 *dq* (14.6, 7.6)	22.5*	2.62 *dq* (13.9, 7.5)
		2.75 *dq* (14.6, 7.6)		2.67 *dq* (13.9, 7.5)
12	11.6	1.25 *t* (7.6)	10.6†	1.25 *t* (7.5)
13	53.2	4.20 *s*	53.4	4.31 *s*
14	25.2	1.47 *s*	23.9	1.51 *s*
15	14.8	1.70 *d* (1.1)	12.8†	1.73 *d* (1.0)
16	15.2	0.84 *d* (6.7)	15.6	0.73 *d* (6.8)

^{1}H NMR data represent chemical shift, multiplicity (*J* in Hz).

*, † Assignments may be interchanged.

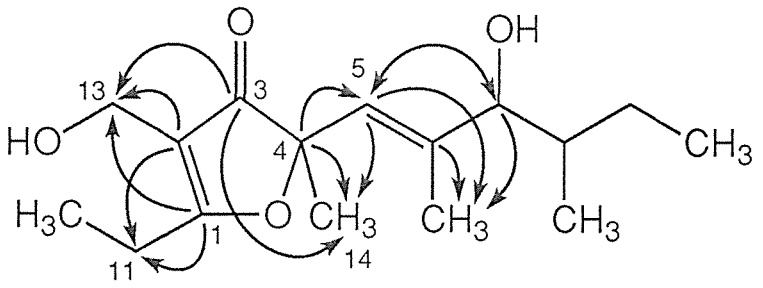


Fig. 2.4. Key long-range couplings detected in the COLOC experiment of neovasifuranone A (**5a**).

double bond was assigned as *E* on the basis of NOE data. Irradiation of the signal of the olefinic proton, 5-H (δ_H 5.45), caused NOE enhancement on the signal of the hydroxymethine, 7-H (δ_H 3.68), but had no effect on the signal of the methyl protons, 15-H_3 (δ_H 1.70). A methyl, an ethyl, and a hydroxymethyl group in **5a** were deduced from ^1H NMR data. The substitution pattern around the 2,3-dihydrofuran-3-one moiety was established on the basis of COLOC data. The three carbons, C-1, C-2, and C-3, were correlated with the hydroxymethyl protons, 13-H_2, and the two carbons, C-1 and C-2, were correlated with the methylene protons, 11-H_2. The three carbons, C-3, C-4, and C-5, had correlations with the methyl protons, 14-H_3, and furthermore, the carbon, C-4, had a correlation with the olefinic proton, 5-H. These correlations allowed the complete assignment of **5a**, and the entire structure was established.

Neovasifuranone B (**5b**) had a molecular formula of $C_{16}H_{26}O_4$ (HREIMS and ^{13}C NMR data) and the same structure as neovasifuranone A (**5a**) from their NMR data comparison (Table 2.2), that is, they are diastereomer of each other. The compound **5a** was treated with pivaloyl chloride and pyridine [17] and then oxidized with dimethyl sulfoxide (DMSO) and acetic anhydride (Ac_2O) [18] to afford 7-oxo derivative (**7**), which was identical with the derivative from **5b**. This indicate that **5a** and **5b** are different only in the stereochemistry at C-7. The stereochemistries of C-7 and C-8 in neovasifuranones (**5**) were determined by chemical reactions. Acetylation of **5a** with Ac_2O and pyridine gave **6**. The reaction of **6** with ruthenium trichloride ($RuCl_3$) and $NaIO_4$ [19], followed by treatment of the product with 2,4-dinitrophenylhydrazine, afforded the 2,4-dinitrophenylhydrazone of 3-acetoxy-4-methyl-2-hexanone (**8a**, Fig. 2.5). The same treatments of **5b** gave **8b** (Fig. 2.5). Similar reactions of neovasipyrones A (**2a**) and B

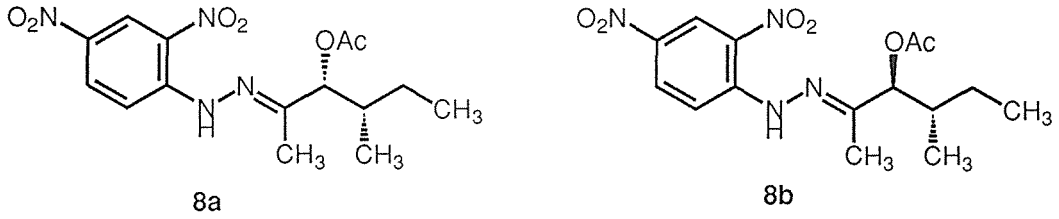


Fig. 2.5. Structures of 2,4-dinitrophenylhydrazone of 3-acetoxy-4-methyl-2-hexanone (**8a** and **8b**).

(**2b**), whose absolute stereochemistries were established by X-ray analysis and chemical reactions, respectively gave the same products, **8a** and **8b**. The configurations of C-7 and C-8 in **5a** therefore are (*R*,*S*), and in **5b** are (*S*,*S*). The stereochemistry of C-4 has yet to be resolved.

Vasinfectins A and B.

The ^{13}C NMR (Table 2.3) and HREIMS spectra of vasinfectin A (**9a**) gave the molecular formula $C_{21}H_{28}O_4$ (eight unsaturations). The ^{13}C NMR spectrum of **9a** had 21 resonances: six owing to methyl groups, two to methylenes, five to methines, and eight to quaternary carbons from its DEPT data. Acetylation of **9a** afforded a monoacetyl derivative (**10**), in the ^1H NMR spectrum of which proton resonance associated with a hydroxymethine was shifted downfield (from δ_H 3.77 to 4.99), indicative that the carbon, C-10, in **9a** bear free hydroxyl group. The ^1H and ^{13}C NMR spectra (Table 2.3) of **9a** indicate the presence of two carbonyl groups, three sp^2 methines, and five sp^2 quaternary carbons; therefore, **9a** contains two rings in its structure. The structural fragments of **9a** shown in Fig. 2.6 were deduced from H,H-COSY, C,H-COSY, COLOC experiments, and a consideration of chemical shift. The stereochemistry of the olefinic bond (C-8 and C-9) was confirmed by NOE data. Irradiation of the methine proton, 10-H (δ_H 3.77), caused NOE enhancement of the olefinic proton signal, 8-H (δ_H 5.56), and irradiation of the 8-H proton induced NOE enhancement of the 10-H signal. The geometry of this double bond

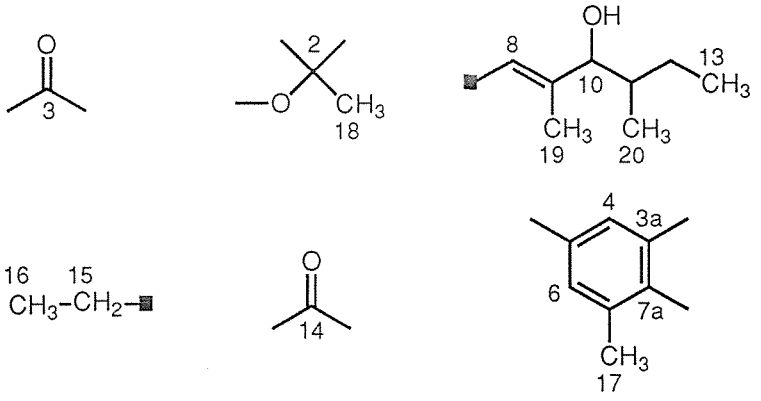


Fig. 2.6. Structural fragments of vasinfectin A (**9a**).

Table 2.3. NMR data (^{1}H, 270.05 MHz and ^{13}C, 67.80 MHz) for vasinfectins A (**9a**) and B (**9b**) in $CDCl_3$.

Position	**9a**		**9b**	
	δ_C	δ_H	δ_C	δ_H
2	90.8		90.7	
3	202.4		202.3	
3a	118.8		118.8	
4	123.1	8.07 *d* (1.8)	123.1	8.10 *d* (1.8)
5	131.3		131.2	
6	137.9	8.11 *dq* (1.8, 0.9)	138.0	8.14 *dq* (1.8, 0.9)
7	124.1		124.2	
7a	172.6		172.5	
8	122.6	5.56 *dq* (1.1, 1.1)	123.4	5.55 *dq* (1.2, 1.2)
9	144.0		144.1	
10	80.7	3.77 *br. d* (6.1)	82.1	3.68 *br. d* (7.7)
11	37.4	1.50 *m*	37.4	1.51 *m*
12	26.3	1.10 *m*	24.3	1.11 *m*
		1.34 *m*		1.66 *m*
13	11.6	0.88 *t* (7.3)	11.1	0.88 *t* (7.3)
14	198.9		199.0	
15	31.5	2.94 *q* (7.3)	31.5	2.97 *q* (7.3)
16	8.4	1.19 *t* (7.3)	8.4	1.21 *t* (7.3)
17	14.3	2.33 *br. s*	14.3	2.36 *br. s*
18	24.0	1.59 *s*	24.0	1.62 *s*
19	13.7	1.66 *d* (1.1)	12.9	1.68 *d* (1.2)
20	13.6	0.82 *d* (6.8)	15.6	0.72 *d* (6.8)

^{1}H NMR data represent chemical shift, multiplicity (*J* in Hz).

therefore was established as the *E* configuration. The connections of these structural fragments were deduced from the long-range ^{13}C–^{1}H correlation detected in the COLOC spectrum (Fig. 2.7). The three carbon resonances, C-2, C-3, and C-8, were correlated with the 18-H_3 signal, indicative that C-2 bound with C-3 and C-8. The connection of C-14 with C-5 and C-15 was shown by correlation of the carbon resonance for C-14 with the 4-

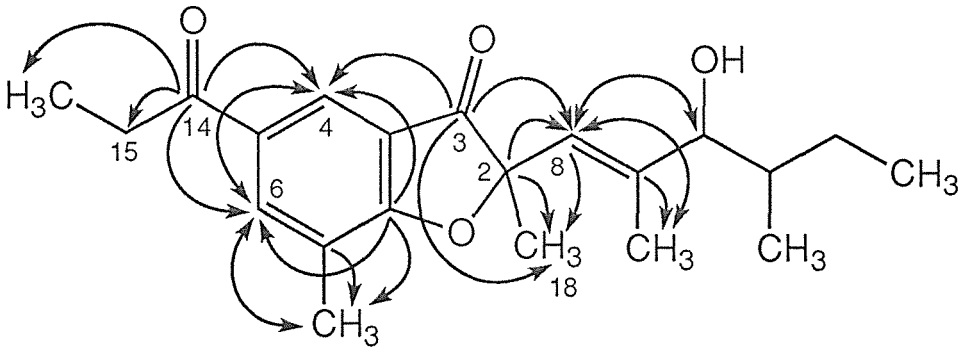


Fig. 2.7. Key long-range couplings detected in the COLOC experiment of vasinfectin A (**9a**).

H, 6-H, and 15-H_2 signals. The C-3 signal was correlated with the 4-H signal, indicative of a bond between C-3 and C-3a. The unsaturation of **9a** and chemical shifts of the aromatic carbon, C-7a (δ_C 172.6), indicated a bond between C-7a and the oxygen atom on C-2. The chemical shifts of the signals produced by the benzofuranone ring carbons in the ^{13}C NMR spectrum of **9a**, especially those of the furanone ring carbons (C-2, C-3, C-3a, and C-7a), are consistent with the shifts reported for other benzofuranone compounds [20–22]. Consequently, the planar structure for **9a** was established.

Vasinfectin B (**9b**) had the same molecular formula, $C_{21}H_{28}O_4$, as vasinfectin A (**9a**), and its spectral features are very similar to those of **9a**. All ^1H and ^{13}C NMR signals were assigned as shown in Table 2.3 by results of H,H-COSY and C,H-COSY experiments. The geometry of its double bond, *E*, was confirmed by NOE data. These findings suggested that **9b** is a diastereomer of **9a**. Oxidation of **9a** with DMSO and Ac_2O gave a 10-oxo derivative (**11**), which was identical with the derivative from **9b**. Thus **9b** is a diastereomer of **9a** differing only in its stereochemistry at C-10. The stereochemistries of C-10 and C-11 in vasinfectins (**9**) were determined by the same method as used for neovasifuranones (**5**). Acetylation of **9a** with Ac_2O and pyridine gave **10**. The reaction of **10** with $RuCl_3$ and $NaIO_4$, followed by treatment of the product with 2,4-dinitrophenylhydrazine, afforded **8a** (Fig. 2.5). The same treatments of **9b** gave **8b**. The configurations of C-10 and C-11 in **9a** therefore are (*R*,*S*), and in **9b** are (*S*,*S*). The stereochemistry of C-2 has yet to be resolved. However, the absolute stereochemistries of

neovasinin (**1**) and neovasipyrones (**2**) suggest that the stereochemistry of the remaining asymmetric carbon in neovasifuranones (**5**) and vasinfectins (**9**) would be *S* as shown in the figure.

Neovasipyrones (**2**), neovasifuranones (**5**), and vasinfectins (**9**) have the same side chain, but they have different ring system; respectively, α-pyrone, 3(2*H*)-furanone, and 3(2*H*)-benzofuranone. In addition, these metabolites were isolated from the same fungus, each as a mixture of two diastereomers with respect to the hydroxymethine carbon on the side chain. It is noteworthy that this fungus produces three types of biogenetically related diastereomers simultaneously, probably by the enzyme-catalyzed reactions [23, 24].

Preliminary leaf spot bioassay indicated that neovasifuranones (**5**) and vasinfectins (**9**) were phytotoxic to soybean, but neovasipyrones (**2**) were not. Neovasinin (**1**) caused necrosis at 2 μg per plant on soybean leaves; compounds **5** caused no symptom at 2 μg per plant, but caused necrosis at 5 μg per plant. Compounds **9** caused necrosis at 2 μg per plant, but the symptom size caused by **9** were fairly smaller than the size caused by **1** and **5**.

Experimental Section

General procedure.

NMR spectra were recorded on a JEOL JNM GX-270 FT NMR spectrometer or a JEOL JNM GX-400 FT NMR spectrometer. All NMR chemical shifts were referenced against the deuterated solvent used ($CDCl_3$, δ_H 7.26, δ_C 77.0; Me_2CO-d_6, δ_H 2.00, δ_C 30.3; CD_3OD, δ_H 3.30, δ_C 49.8). Mass spectra were obtained with a JEOL DX-300 spectrometer or a JEOL AX-505 spectrometer. In FABMS, glycerol was used as matrix. In EIMS, the ionization voltage was 70 eV. 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. Circular dichroism (CD) spectra were measured with a JASCO J-720 spectrophotometer. Daisogel IR-60 was the silica gel used for the column chromatography. Preparative TLC was carried out on Merck Kieselgel 60 HF_{254} glass

plates (20 x 20 x 0.05 cm).

Fungal material.

Strain NHL2298 of *Neocosmospora vasinfecta* E. F. Smith var. *africana* (von Arx) Cannon et Hawksworth used in this study was a gift from Dr. Shun-ichi Udagawa (National Institute of Hygienic Sciences, Tokyo) in 1983 and was maintained on potato dextrose agar.

Fermentation and extraction.

The fungus was grown in 500-mL conical flasks (50) containing liquid medium (200 mL/flask) made up of sucrose (50 g/L), peptone (3 g/L), the extract from 100 g/L on malt, L-methionine (0.3 g/L), and water, without shaking at 24°C for 21 days in the dark. The culture filtrate was acidified to pH 2.0 with HCl and extracted with EtOAc (3 x 7 L). The EtOAc extracts were dried over sodium sulfate (Na_2SO_4), concentrated, and washed with 1 M $NaHCO_3$ (2 x 0.5 vol.) to afford the neutral EtOAc-soluble (NE) fraction. The $NaHCO_3$ washings were acidified to pH 2.0 with HCl and extracted with EtOAc (3 x 1 vol.) to afford the acidic EtOAc-soluble (AE) fraction.

Isolation of neovasipyrones A (2a) and B (2b).

The residue (5.7 g) from AE fraction was purified by silica gel partition column chromatography (300 g, impregnated with 180 mL of 0.1 M HCO_2H, 4.6 i.d. x 44 cm). Elution was performed with each 1.5 L (5 x 300 mL) of 10, 20, 30, 40, and 50% EtOAc in *n*-hexane saturated with 0.1 M HCO_2H. Fractions 15–21 were combined and concentrated. The residue (1.9 g) was subjected to silica gel column chromatography (180 g, 3.2 i.d. x 44 cm). The column was developed with Me_2CO–C_6H_6–AcOH (40 : 60 : 0.5), and each 100 mL of eluate was collected as one fraction. The combined fractions (5–10, 1.6 g) were then subjected to Sephadex LH-20 column chromatography (3.2 i.d. x 135 cm) using $CHCl_3$–MeOH (1 : 1) as the solvent. Each 10 mL of eluate was collected as one fraction, and fractions 60–80, which contained both **2a** and **2b**, were combined. The separation of **2a** from **2b** was achieved by preparative TLC [Me_2CO–$CHCl_3$–AcOH (25 :

75 : 1), quadruple development] to afford 720 mg of **2a** (lower R_f band) and 310 mg of **2b** (higher R_f band).

Isolation of neovasifuranones A (5a) and B (5b).

The combined NE fractions (5.9 g) from five cultivations (5 x 10 L of medium) were purified by silica gel column chromatography (140 g, 3.6 i.d. x 26 cm). The column was developed successively with 900 mL each of 2, 5, 10, 20, 30, 40, and 50% Me_2CO in *n*-hexane. The fraction (1.1 g), eluted with 30% Me_2CO in *n*-hexane, was contained neovasifuranones (**5**) and subjected to Sephadex LH-20 column chromatography (3.2 i.d. x 123 cm) using MeOH as the solvent. Each 10 mL of eluate was collected as one fraction, and fractions 60–69 were combined and concentrated. The residue (850 mg) was further purified by preparative TLC [Me_2CO–$CHCl_3$ (1 : 3), triple development] to afford **5a** (152 mg, lower R_f band) and **5b** (97 mg, higher R_f band).

Isolation of vasinfectins A (9a) and B (9b).

The 10% fraction (270 mg), eluted from silica gel column chromatography of NE fractions, was subjected to preparative TLC [Me_2CO–$CHCl_3$ (1 : 19)]. The residue (70 mg) containing vasinfectins (**9**) was purified by reversed phase HPLC [COSMOSIL $5C_{18}$-AR (10 i.d. x 250 mm), 75% aq. MeOH, 1.5 mL/min] to afford **9a** (24 mg, R_t 42.0 min) and **9b** (25 mg, R_t 38.7 min).

Neovasipyrone A (2a).

Oil. $[\alpha]_D^{20}$ –235.4° (*c* 1.0, EtOH). UV (EtOH) λ_{max} nm (log ε): 209 (4.29), 291 (3.90). IR (film) ν_{max} cm^{-1}: 3373, 2963, 1663, 1567, 1414, 1244, 988. ^1H and ^{13}C NMR spectral data: Table 2.1. FABMS *m/z* (rel. int.): 327 $[M+H]^+$ (39), 291 (35), 249 (26), 205 (36), 191 (40), 179 (33), 109 (58), 43 (100); exact mass calcd for $C_{17}H_{27}O_6$ 327.1808, found 327.1791.

Neovasipyrone B (2b).

Oil. $[\alpha]_D^{20}$ –124.0° (*c* 1.0, EtOH). UV (EtOH) λ_{max} nm (log ε): 209 (4.25), 291

(3.87). IR (film) ν_{max} cm^{-1}: 3373, 2967, 1680, 1565, 1419, 1245, 1010. ^{1}H and ^{13}C NMR spectral data: Table 2.1. FABMS *m/z* (rel. int.): 327 $[M+H]^+$ (30), 291 (12), 249 (16), 205 (15), 179 (12), 109 (18), 106 (26), 74 (44), 42 (100); exact mass calcd for $C_{17}H_{27}O_6$ 327.1808, found 327.1804.

Crystal data for neovasipyrone B (2b).

$C_{17}H_{26}O_6 \cdot C_6H_6$, M = 404.5, orthorhombic, space group $P2_12_12_1$, a = 24.773(4), b = 11.525(2), c = 7.961(1) Å, V = 2272.9(4) $Å^3$ (cell parameters by least squares from the setting angles of 24 reflections), μ(Cu Kα) = 6.5 cm^{-1}, Z = 4, D_x = 1.18 g cm^{-3}, $F(000)$ = 872, T = 293 K.

X-ray crystal structure analysis.

Intensity data were measured on a Rigaku four-circle diffractometer using Ni-filtered Cu Kα radiation (λ = 1.5418 Å) and a rotating anode generator. A crystal of dimensions 0.6 x 0.4 x 0.2 mm was used. The ω–2θ scan mode was employed, with background measurement at each end of the scan. Intensities of 1952 unique reflections were measured to $2\theta_{max}$ = 120° in the range $0 \le h \le 27$, $0 \le k \le 12$, $0 \le l \le 8$, with ω scan width of 0.9° + 0.15° tan θ, a scan speed of 4° min^{-1}, and background counting time of 5 sec. No significant change was observed in the intensities of the 3 standard reflections measured every 100 reflections. Intensity data were corrected only for Lorentz and polarization effects. The structure was determined by direct methods using SHELX 86 [25] and refined by block diagonal least squares [26] on F using atomic scattering factors from *International Tables for X-ray Crystallography* (1974). All H atoms for **2b** molecule were located in the difference Fourier map, but those for the C_6H_6 molecule were invisible in the map. The oxygen and carbon atoms were refined anisotropically and the H atoms isotropically. Strongest reflection, 0 2 0, was omitted from the refinement. Weighting scheme used in the final stage of refinement was $w = [\sigma(F_O)^2 + 0.02F_O + 0.001F_O^2]^{-1}$. The residual electron densities in the final difference Fourier map ranged from –0.19 to 0.19 $eÅ^{-3}$. The final R and R_w were 0.061 and 0.080, respectively, for 1728 unique reflections with $F_O > 2\sigma(F_O)$. Atomic coordinates, temp. factors, and bond lengths and angles have

been deposited at the Cambridge Crystallographic Data Centre.

Oxidation of neovasipyrones A (2a) and B (2b).

A solution composed of chromium trioxide (CrO_3, 500 mg), conc. H_2SO_4 (0.5 mL), and H_2O (1.5 mL) was prepared, and 50 μL was added to a stirred solution of **2a** (36.0 mg) in 1.5 mL of Me_2CO at 0°C. After 2 min, 20 μL of *iso*-PrOH was added to the reaction mixture. After 1 min, the reaction mixture was diluted with 30 mL of H_2O, and the solution was extracted with EtOAc (3 x 30 mL). The EtOAc solution was washed with brine, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was subjected to preparative TLC [Me_2CO–$CHCl_3$–AcOH (20 : 80 : 1)] to afford 6.4 mg of compound **3** as an oil. $[\alpha]_D^{22}$ –96° (*c* 1.0, EtOH). ^{1}H NMR (270 MHz, $CDCl_3$): δ 0.85 (3H, *t*, *J* = 7.0 Hz, H-12), 1.05 (3H, *d*, *J* = 7.0 Hz, H-17), 1.38 (1H, *m*, H-11), 1.59 (3H, *s*, H-15), 1.63 (1H, *m*, H-11), 1.76 (3H, *br. s*, H-16), 1.87 (3H, *s*, H-13), 3.11 (1H, *ddq*, *J* = 7.0, 7.0, 7.0 Hz, H-10), 4.63 (1H, *d*, *J* = 13.5 Hz, H-14), 4.95 (1H, *d*, *J* = 13.5 Hz, H-14), 6.73 (1H, *br. s*, H-7). EIMS *m/z* (rel. int.): 324 $[M]^+$ (3), 222 (8), 194 (42), 182 (51), 140 (100), 57 (33). FABMS *m/z* (rel. int.): 325 $[M+H]^+$ (100), 307 (15), 291 (11), 249 (13), 223 (19), 183 (25), 57 (37). Compound **2b** (31.9 mg) was subjected to the same procedure as for **2a** to afford **3** (5.4 mg), $[\alpha]_D^{22}$ –83° (*c* 1.0, EtOH).

2,4-Dinitrophenylhydrazone of 2-methyl-1-butanal (4) from 2a.

A solution of **2a** (118 mg), OsO_4 (5 mg), and $NaIO_4$ (221 mg) in dioxane (2 mL), H_2O (1.5 mL), and pyridine (0.1 mL) was stirred for 12 hr at room temperature. The reaction mixture was diluted with 20 mL of EtOAc, and the EtOAc solution was washed with brine (4 x 10 mL). A solution of 2,4-dinitrophenylhydrazine (308 mg) in EtOH (1.5 mL) and conc. H_2SO_4 (1 mL) was added to the EtOAc solution at 0°C, and the reaction mixture was stirred for 20 min. The reaction mixture was washed with brine (2 x 10 mL), dried over Na_2SO_4, and concentrated to dryness. The residue was purified with silica gel flash column chromatography [Wakogel FC-40, EtOAc–*n*-hexane (1 : 9)] and preparative HPLC [Whatman Partisil 5-ODS-2 (8.0 i.d. x 150 mm), 1.0 mL/min, 85% aq. MeOH] to afford 5.5 mg of **4** as a yellow solid. The spectroscopic data were in accordance with

those reported previously. $[\alpha]_D^{22}$ +24.7° (*c* 0.3, $CHCl_3$).

Neovasifuranone A (5a).

Oil. $[\alpha]_D^{22}$ −111.2° (*c* 0.4, EtOH). UV (EtOH) λ_{max} nm (log ε): 202 (3.91), 268 (3.98). IR (film) ν_{max} cm^{-1}: 3405, 2965, 1688, 1611, 1445, 1408, 1277, 1096, 1053, 1017. ^1H and ^{13}C NMR spectral data: Table 2.2. EIMS *m/z* (rel. int.): 282 $[M]^+$ (5), 264 (13), 246 (8), 235 (7), 225 (19), 207 (79), 189 (17), 151 (49), 121 (28), 111 (62), 109 (100), 57 (63); exact mass calcd for $C_{16}H_{26}O_4$ 282.1831, found 282.1837.

Neovasifuranone B (5b).

Oil. $[\alpha]_D^{22}$ −102.3° (*c* 0.4, EtOH). UV (EtOH) λ_{max} nm (log ε): 202 (3.93), 268 (4.01). IR (film) ν_{max} cm^{-1}: 3356, 2965, 1688, 1611, 1464, 1412, 1277, 1115, 1051, 1011. ^1H and ^{13}C NMR spectral data: Table 2.2. EIMS *m/z* (rel. int.): 282 $[M]^+$ (9), 264 (12), 246 (5), 235 (3), 225 (14), 207 (90), 189 (15), 151 (48), 121 (21), 111 (68), 109 (100), 57 (62); exact mass calcd for $C_{16}H_{26}O_4$ 282.1831, found 282.1831.

Diacetyl neovasifuranone A (6).

A solution of neovasifuranone A (**5a**, 4.8 mg) in pyridine (0.1 mL) and Ac_2O (50 μL) was allowed to stand for 12 hr at room temperature. The reaction mixture was added to H_2O (2.5 mL), and the solution was stirred for 5 hr. The products were extracted with EtOAc (3 x 3 mL). The EtOAc solution was dried over Na_2SO_4 and concentrated to dryness under reduced pressure. The residue was purified by preparative TLC [Me_2CO–C_6H_6 (1 : 4), double development] to afford **6** (5.1 mg) as an oil. UV (EtOH) λ_{max} nm (log ε): 202 (4.02), 266 (4.13). IR (film) ν_{max} cm^{-1}: 2969, 1742, 1709, 1626, 1464, 1412, 1373, 1236, 1022. ^1H NMR (400 MHz, $CDCl_3$): δ 0.73 (3H, *d*, *J* = 6.6 Hz, H-16), 0.82 (3H, *t*, *J* = 7.2 Hz, H-10), 1.04 (1H, *m*, H-9), 1.16 (3H, *t*, *J* = 7.5 Hz, H-12), 1.25 (1H, *m*, H-9), 1.41 (3H, *s*, H-14), 1.57 (1H, *m*, H-8), 1.67 (3H, *d*, *J* = 1.3 Hz, H-15), 1.96 (3H, *s*, Me of Ac), 2.00 (3H, *s*, Me of Ac), 2.59 (1H, *dq*, *J* = 14.7, 7.5 Hz, H-11), 2.62 (1H, *dq*, *J* = 14.7, 7.5 Hz, H-11), 4.64 (1H, *d*, *J* = 12.2 Hz, H-13), 4.66 (1H, *d*, *J* = 12.2 Hz, H-13), 4.92 (1H, *br. d*, *J* = 6.3 Hz, H-7), 5.35 (1H, *dq*, *J* = 1.3, 1.3 Hz, H-5). EIMS *m/z* (rel. int.): 366

$[M]^+$ (3), 306 (18), 249 (100), 217 (57), 207 (67), 179 (34), 151 (19), 138 (38), 109 (28).

Oxidation of neovasifuranones A (5a) and B (5b).

To a solution of **5a** (20.5 mg) in pyridine (10 μL) and CH_2Cl_2 (0.5 mL), pivaloyl chloride (25 μL) was added, and the solution was stirred for 8 hr at 20°C. The reaction mixture was diluted with H_2O (1 mL), stirred for 1 hr, and then diluted with EtOAc (20 mL). The solution was washed with 1 M $NaHCO_3$ (2 x 10 mL) and brine (2 x 10 mL), 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 mL) and Ac_2O (0.2 mL) was stirred for 18 hr at 20°C. EtOH (1.5 mL) was added to the reaction mixture, and the solution was stirred for 1 hr. The reaction mixture was diluted with H_2O (10 mL), and the solution was extracted with EtOAc (4 x 10 mL). The EtOAc solution was washed with brine (2 x 15 mL), dried over Na_2SO_4, and concentrated to dryness under reduced pressure. The residue was purified by preparative HPLC [COSMOSIL $5C_{18}$-AR (10 i.d. x 250 mm), 1.0 mL/min, 90% aq. MeOH] to afford compound **7** (6.4 mg) as an oil. $[\alpha]_D^{22}$ –40.3° (*c* 0.3, EtOH). UV (EtOH) λ_{max} nm (log ε): 235 (4.01), 266 (3.97). IR (film) ν_{max} cm^{-1}: 2969, 1730, 1676, 1626, 1462, 1406, 1281, 1204, 1150, 1051. ^1H NMR (270 MHz, $CDCl_3$): δ 0.80 (3H, *t*, *J* = 7.4 Hz, H-10), 1.05 (3H, *d*, *J* = 6.8 Hz, H-16), 1.16 (9H, *s*, Me of pivaloyl), 1.25 (3H, *t*, *J* = 7.6 Hz, H-12), 1.35 (1H, *m*, H-9), 1.57 (3H, *s*, H-14), 1.62 (1H, *m*, H-9), 1.96 (3H, *d*, *J* = 1.3 Hz, H-15), 2.72 (2H, *q*, *J* = 7.6 Hz, H-11), 3.08 (1H, *ddq*, *J* = 6.8, 6.8, 6.8 Hz, H-8), 4.74 (2H, *s*, H-13), 6.44 (1H, *dq*, *J* = 0.5, 1.3 Hz, H-5). Chemical ionization mass spectrometry (CIMS) *m/z* (rel. int.): 365 $[M+H]^+$ (37), 263 (100). Compound **5b** (19.8 mg) was subjected to the same procedure as for **5a** to afford **7** (3.2 mg), $[\alpha]_D^{22}$ –39.7° (*c* 0.3, EtOH).

Vasinfectin A (9a).

Oil. $[\alpha]_D^{20}$ –53° (*c* 0.5, EtOH). UV (EtOH) λ_{max} nm (log ε): 202 (4.04), 243 (4.55), 274 (3.97), 329 (3.54). IR (film) ν_{max} cm^{-1}: 3434, 1725, 1686, 1607, 1163. ^1H and ^{13}C NMR spectral data: Table 2.3. EIMS *m/z* (rel. int.): 344 $[M]^+$ (7), 326 (13), 287 (100), 258 (43), 231 (21), 191 (43), 161 (12), 57 (19); exact mass calcd for $C_{21}H_{28}O_4$ 344.1988, found

344.1983.

Vasinfectin B (9b).

Oil. $[\alpha]_D^{20}$ –41° (*c* 0.5, EtOH). UV (EtOH) λ_{max} nm (log ε): 202 (4.05), 243 (4.56), 274 (3.97), 329 (3.54). IR (film) ν_{max} cm^{-1}: 3499, 1726, 1686, 1607, 1163. ^1H and ^{13}C NMR spectral data: Table 2.3. EIMS *m/z* (rel. int.): 344 $[M]^+$ (5), 326 (10), 287 (100), 258 (34), 231 (15), 191 (34), 161 (10), 57 (15); exact mass calcd for $C_{21}H_{28}O_4$ 344.1988, found 344.1992.

Monoacetyl vasinfectin A (10).

A solution of vasinfectin A (**9a**, 11.2 mg) in pyridine (0.2 mL) and Ac_2O (0.1 mL) was stirred for 6 hr at room temperature. The reaction mixture was added to H_2O (5 mL), and the solution was extracted with EtOAc (4 x 5 mL). The EtOAc solution was concentrated to dryness under reduced pressure. The residue was purified by preparative HPLC [COSMOSIL $5C_{18}$-AR (10 i.d. x 250 mm), 2.0 mL/min, 80% aq. MeOH] to afford **10** (10.5 mg) as an oil. UV (EtOH) λ_{max} nm (log ε): 202 (4.02), 243 (4.57), 274 (3.95), 329 (3.56). IR (film) ν_{max} cm^{-1}: 1730, 1688, 1607, 1238. ^1H NMR (270 MHz, $CDCl_3$): δ 0.79 (3H, *d*, *J* = 6.7 Hz, H-20), 0.89 (3H, *t*, *J* = 7.3 Hz, H-13), 1.12 (1H, *m*, H-12), 1.22 (3H, *t*, *J* = 7.3 Hz, H-16), 1.32 (1H, *m*, H-12), 1.59 (3H, *s*, H-18), 1.64 (1H, *m*, H-11), 1.73 (3H, *d*, *J* = 1.2 Hz, H-19), 2.06 (3H, *s*, Me of Ac), 2.36 (3H, *br. s*, H-17), 2.97 (2H, *q*, *J* = 7.3 Hz, H-15), 4.99 (1H, *br. d*, *J* = 5.9 Hz, H-10), 5.51 (1H, *dq*, *J* = 1.2, 1.2 Hz, H-8), 8.10 (1H, *d*, *J* = 1.8 Hz, H-4), 8.14 (1H, *dq*, *J* = 1.8, 0.9 Hz, H-6). EIMS *m/z* (rel. int.): 386 $[M]^+$ (4), 326 (13), 300 (11), 287 (51), 258 (75), 218 (100), 191 (26), 161 (15), 127 (12), 57 (17).

Oxidation of vasinfectins A (9a) and B (9b).

A solution of **9a** (19.6 mg) in dry DMSO (0.3 mL) and Ac_2O (0.2 mL) was stirred for 18 hr at 20°C. EtOH (1.5 mL) was added to the reaction mixture, and the solution was stirred for 1 hr. The reaction mixture was added to H_2O (10 mL), and the solution was extracted with EtOAc (4 x 10 mL). The EtOAc solution was washed with brine (2 x 15

mL), dried over Na_2SO_4, and concentrated to dryness under reduced pressure. The residue was purified by preparative TLC [Me_2CO–*n*-hexane (1 : 9), triple development] to afford compound **11** (9.8 mg) as an oil. CD (EtOH) λ_{ext} nm ($\Delta\varepsilon$): 342 (+6.6), 311 (–3.5), 245 (–21.3). UV (EtOH) λ_{max} nm (log ε): 201 (3.94), 245 (4.63), 276 (3.91), 330 (3.56). IR (film) ν_{max} cm^{-1}: 1728, 1684, 1607, 1161. ^1H NMR (270 MHz, $CDCl_3$): δ 0.80 (3H, *t*, *J* = 7.4 Hz, H-13), 1.07 (3H, *d*, *J* = 6.8 Hz, H-20), 1.22 (3H, *t*, *J* = 7.3 Hz, H-16), 1.34 (1H, *m*, H-12), 1.63 (1H, *m*, H-12), 1.69 (3H, *s*, H-18), 2.01 (3H, *br. d*, *J* = 1.3 Hz, H-19), 2.39 (3H, *br. s*, H-17), 2.97 (2H, *q*, *J* = 7.3 Hz, H-15), 3.09 (1H, *ddq*, *J* = 6.8, 6.8, 6.8 Hz, H-11), 6.53 (1H, *q*, *J* = 1.3 Hz, H-8), 8.12 (1H, *d*, *J* = 1.8 Hz, H-4), 8.17 (1H, *dq*, *J* = 1.8, 0.9 Hz, H-6). EIMS *m/z* (rel. int.): 342 $[M]^+$ (45), 313 (67), 285 (34), 258 (54), 229 (91), 191 (19), 152 (100), 123 (86), 57 (59). Compound **9b** (20.2 mg) was subjected to the same procedure as for **9a** to afford **11** (10.7 mg), CD (EtOH) λ_{ext} nm ($\Delta\varepsilon$): 342 (+6.6), 312 (–3.6), 245 (–20.5).

2,4-Dinitrophenylhydrazone of 3-acetoxy-4-methyl-2-hexanone (8a) from 2a.

A solution of **2a** (52.0 mg) in pyridine (0.2 mL), Ac_2O (0.1 mL), and CH_2Cl_2 (0.4 mL) was stirred for 22 hr at room temperature. H_2O (10 mL) was added to the reaction mixture, and the solution was stirred for 1 hr. The reaction mixture was extracted with EtOAc (4 x 10 mL). The EtOAc solution was washed with brine (2 x 25 mL), dried over Na_2SO_4, and concentrated to dryness under reduced pressure. A solution of the residue (71.0 mg), $RuCl_3$ (5 mg), and $NaIO_4$ (130 mg) in CCl_4 (1 mL), CH_3CN (1 mL), and H_2O (1.5 mL) was stirred vigorously for 1 hr at room temperature. The reaction mixture was added to EtOAc (20 mL), and the solution was washed with brine (4 x 10 mL). A solution of 2,4-dinitrophenylhydrazine (160 mg) in EtOH (1 mL) and conc. H_2SO_4 (0.5 mL) was added to the EtOAc solution, and the solution was stirred for 40 min at room temperature. The reaction mixture was washed with brine (3 x 10 mL), dried over Na_2SO_4, and concentrated to dryness under reduced pressure. The residue was purified by preparative TLC [Me_2CO–*n*-hexane (1 : 19), quintuple development] to afford **8a** (28.5 mg) as a yellow oil. $[\alpha]_D^{20}$ +24.9° (*c* 0.3, EtOH). IR (film) ν_{max} cm^{-1}: 1744, 1618, 1595, 1518, 1339, 1235. ^1H NMR (270 MHz, $CDCl_3$): δ 0.95 (3H, *t*, *J* = 7.3 Hz), 0.99 (3H, *d*, *J* = 6.6

Hz), 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 Hz), 7.95 (1H, *d*, *J* = 9.5 Hz), 8.33 (1H, *ddd*, *J* = 9.5, 2.6, 0.5 Hz), 9.13 (1H, *d*, *J* = 2.6 Hz), 11.05 (1H, *br. s*). EIMS *m/z* (rel. int.): 352 $[M]^+$ (67), 292 (45), 263 (91), 253 (56), 207 (45), 191 (37), 59 (100).

2,4-Dinitrophenylhydrazone of 3-acetoxy-4-methyl-2-hexanone (8b) from 2b.

Compound **2b** (52.0 mg) was subjected to the same procedure as for **2a** to afford **8b** (22.4 mg) as a yellow oil. $[\alpha]_D^{20}$ +14.0° (*c* 0.3, EtOH). IR (film) ν_{max} cm^{-1}: 1742, 1618, 1595, 1518, 1339, 1233. ^1H NMR (270 MHz, $CDCl_3$): δ 0.90 (3H, *d*, *J* = 6.8 Hz), 0.95 (3H, *t*, *J* = 7.3 Hz), 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 Hz), 7.96 (1H, *d*, *J* = 9.5 Hz), 8.32 (1H, *ddd*, *J* = 9.5, 2.6, 0.5 Hz), 9.11 (1H, *d*, *J* = 2.6 Hz), 11.03 (1H, *br. s*). EIMS *m/z* (rel. int.): 352 $[M]^+$ (36), 292 (22), 263 (43), 253 (30), 207 (25), 191 (20), 59 (100).

Compound 8a from 5a and 8b from 5b.

A solution of **5a** (52.3 mg) in pyridine (0.2 mL), Ac_2O (0.1 mL), and CH_2Cl_2 (0.4 mL) was stirred for 22 hr at room temperature. H_2O (5 mL) was added to the reaction mixture, and the solution was stirred for 1 hr. The reaction mixture was extracted with EtOAc (4 x 5 mL). The EtOAc solution was washed with brine (2 x 10 mL), dried over Na_2SO_4, and concentrated to dryness under reduced pressure. A solution of the residue (64.0 mg), $RuCl_3$ (2 mg), and $NaIO_4$ (160 mg) in CCl_4 (0.5 mL), CH_3CN (0.5 mL), and H_2O (0.75 mL) was stirred vigorously for 12 hr at 28°C. The reaction mixture was added to EtOAc (10 mL), and the solution was washed with 1 M $NaHCO_3$ (2 x 5 mL) and brine (2 x 5 mL). A solution of 2,4-dinitrophenylhydrazine (180 mg) in EtOH (1 mL) and conc. H_2SO_4 (0.2 mL) was added to the EtOAc solution, and the solution was stirred for 40 min at 28°C. The reaction mixture was washed with brine (3 x 10 mL), dried over Na_2SO_4, and concentrated to dryness under reduced pressure. The residue was purified by preparative TLC [Me_2CO–*n*-hexane (1 : 19), quintuple development] to afford **8a** (33.1 mg), $[\alpha]_D^{20}$ +26.2° (*c* 0.3, EtOH). Compound **5b** (54.8 mg) was subjected to the same procedure as for **5a** to afford **8b** (30.6 mg), $[\alpha]_D^{20}$ +14.8° (*c* 0.3, EtOH).

Compound 8a from 9a and 8b from 9b.

A solution of **9a** (21.0 mg) in pyridine (0.2 mL), Ac_2O (0.1 mL), and CH_2Cl_2 (0.4 mL) was stirred for 20 hr at room temperature. H_2O (5 mL) was added to the reaction mixture, and the solution was stirred for 1 hr. The reaction mixture was extracted with EtOAc (4 x 5 mL). The EtOAc solution was washed with brine (2 x 10 mL), dried over Na_2SO_4, and concentrated to dryness under reduced pressure. A solution of the residue (23.8 mg), $RuCl_3$ (2 mg), and $NaIO_4$ (55 mg) in CCl_4 (0.5 mL), CH_3CN (0.5 mL), and H_2O (0.75 mL) was stirred vigorously for 12 hr at 28°C. The reaction mixture was added to EtOAc (10 mL), and the solution was washed with 1 M $NaHCO_3$ (2 x 5 mL) and brine (2 x 5 mL). A solution of 2,4-dinitrophenylhydrazine (60 mg) in EtOH (1 mL) and conc. H_2SO_4 (0.2 mL) was added to the EtOAc solution, and the solution was stirred for 40 min at 28°C. The reaction mixture was washed with brine (3 x 10 mL), dried over Na_2SO_4, and concentrated to dryness under reduced pressure. The residue was purified by preparative TLC [Me_2CO–*n*-hexane (1 : 19), quintuple development] to afford **8a** (7.9 mg), $[\alpha]_D^{20}$ +26.2° (*c* 0.3, EtOH). Compound **9b** (21.3 mg) was subjected to the same procedure as for **9a** to afford **8b** (5.7 mg), $[\alpha]_D^{20}$ +16.0° (*c* 0.3, EtOH).

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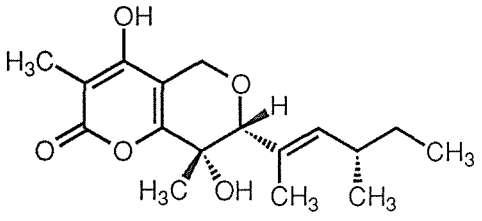
CHAPTER 3

Isolation and Structure Determination of Neovasipyridones

Introduction

In previous chapter, the structures of neovasipyrones A (**2a**) and B (**2b**), neovasifuranones A (**3a**) and B (**3b**), and vasinfectins A and B are shown. These metabolites have the same side chain and were isolated from the culture filtrate of *Neocosmospora vasinfecta* NHL2298. To obtain other metabolites related to neovasinin (**1**) [1, 2], isolation of the metabolites from the mycelia was then attempted. The isolation and structures of six new metabolites, named neovasipyridones A (**4a**), B (**4b**), C (**4c**), D (**4d**), E (**4e**), and F (**4f**) are described in this chapter.



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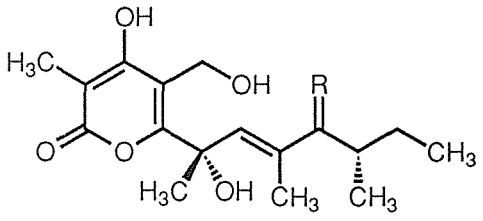
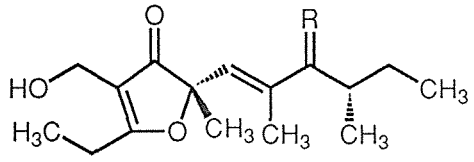
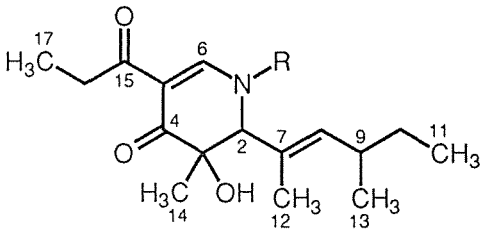


2a R = α-OH, β-H
2b R = α-H, β-OH



3a R = α-OH, β-H
3b R = α-H, β-OH



4a R = $^{18}CH_2CH(Me)^{22}CH_2^{21}Me$
4b R = $^{18}CH_2CH_2CH(Me)_2^{21,22}$
4c R = $^{18}CH_2CH(Me)_2^{20,21}$
4d R = $^{18}CH_2CH_2C_6H_5^{20-25}$
4e R = $^{18}CH_2^{19}Me$
4f R = $^{18}CH_2CH_2CH_2^{20}CONH_2$

Results and Discussion

Neovasipyridones A (**4a**), B (**4b**), C (**4c**), D (**4d**), E (**4e**), and F (**4f**) were isolated as oils with respective yields of 36, 23, 18, 35, 7.9, and 4.4 μg/g from air-dried mycelia of the fungus *N. vasinfecta* NHL2298 grown on a malt extract medium supplemented with peptone and L-methionine [3].

Table 3.1. ^{13}C NMR data for neovasipyridones A–E (**4a–e**) in $CDCl_3$.

C	**4a***	**4b***	**4c***	**4d***	**4e†**
2	76.7	76.1	76.3	76.5	76.7
3	71.1	70.9	71.0	70.9	70.8
4	191.7	191.6	191.7	191.7	191.7
5	104.9	104.8	104.8	104.9	104.8
6	158.8	158.7	158.1	158.1	157.8
7	127.6	127.7	127.5	127.7	127.7
8	140.1	140.0	140.1	140.1	139.9
9	34.0	34.0	34.0	34.0	34.0
10	30.1	30.1	30.1	30.0	30.1
11	11.8	11.8	11.8	11.8	11.9
12	13.1	13.0	13.1	12.9	13.1
13	20.6	20.6	20.6	20.7	20.7
14	28.8	28.6	28.9	28.6	28.5
15	197.6	197.6	197.6	197.5	197.7
16	34.9	34.8	34.9	34.8	34.9
17	8.5	8.6	8.5	8.5	8.6
18	61.2	53.6	62.7	56.0	50.1
19	33.7	37.6	27.2	35.3	14.3
20	26.4	25.7	19.8	136.5	
21	10.8	22.0	20.1	129.0	
22	16.8	22.5		128.6	
23				127.3	
24				128.6	
25				129.0	

* 67.8 MHz, † 100 MHz.

The ^{13}C NMR data (Table 3.1) and HRFAB mass spectrum of neovasipyridone A (**4a**) showed its molecular formula to be $C_{21}H_{35}NO_3$ (five unsaturations). The ^{13}C NMR spectrum of **4a** showed 21 resonances, seven of which were due to methyls, four to methylenes, five to methines, and five to quaternary carbons from a DEPT experiment [4], indicative that one of the protons in the molecule is bound to oxygen or nitrogen. The 2,3-dihydro-4-pyridinone portion in **4a** was indicated by two IR absorption bands at 1647 and 1580 cm^{-1}, three ^{13}C resonances at δ_C 191.7, 158.8, and 104.9, and a UV absorption at 322 nm [5, 6]. Two ^{13}C resonances at δ_C 127.6 and 140.1 were attributed to two olefinic carbons of a trisubstituted double bond. One ^{13}C resonance of δ_C 197.6 was characteristic of a ketonic carbonyl carbon conjugated to a double bond. The structure of **4a** was unambiguously deduced from analysis of H,H-COSY [7, 8], C,H-COSY [9, 10], COLOC [9, 10], and NOE data. The significant correlations in the COLOC spectrum are summarized in Fig. 3.2.

The partial structure A (Fig. 3.1) was deduced from H,H-COSY and COLOC data. The structure of the side chain (C-7 to C-11), deduced to be 1,3-dimethyl-1-pentenyl from H,H-COSY, was confirmed by COLOC data. The bonds between C-2 and C-7, and between C-2 and C-3, were deduced from COLOC data. The allylic methyl carbon, C-12, was correlated with the methine proton, 2-H, indicative of the attachment of a 1,3-dimethyl-1-pentenyl group to the methine carbon, C-2. Furthermore, the oxygen-bearing quaternary carbon, C-3, was correlated both with the methine proton, 2-H, and the methyl protons, 14-H_3, and the methyl carbon, C-14, was in turn correlated with the methine proton, 2-H. These correlations indicated that the methyl carbon, C-14, is bound to the oxygen-bearing quaternary carbon, C-3, which is attached to the methine carbon, C-2.

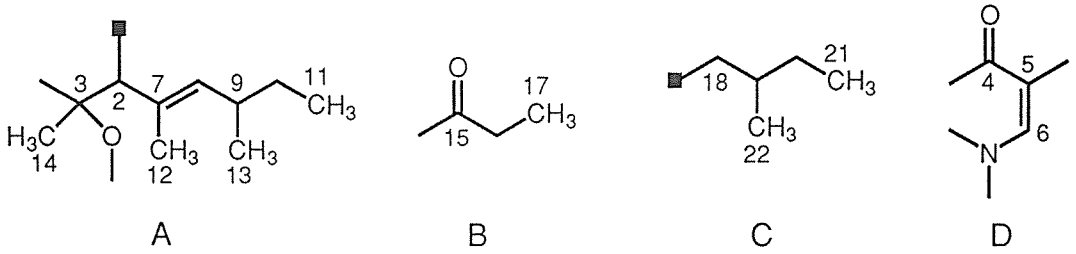


Fig. 3.1. Structural fragments of neovasipyridone A (**4a**).

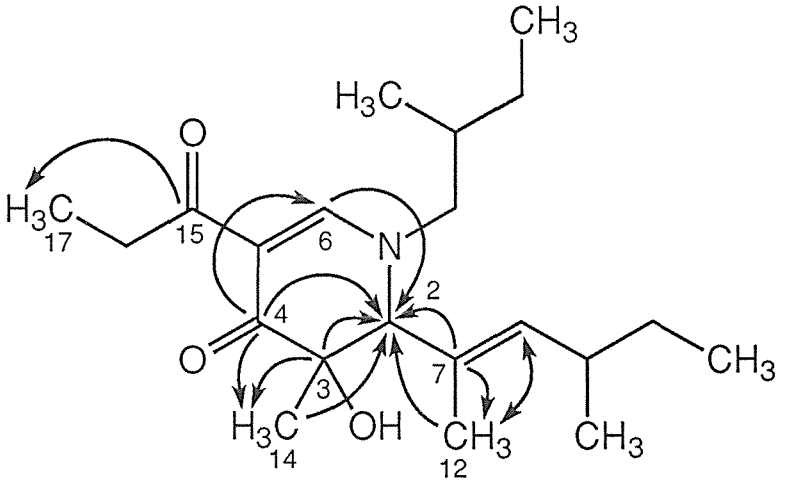


Fig. 3.2. Key long-range couplings detected in the COLOC experiment of neovasipyridone A (**4a**).

The stereochemistry of the double bond is *E* based on the NOE data. Irradiation of the methine proton, 2-H (δ_H 3.82), caused NOE enhancement of the signal of the olefinic proton, 8-H (δ_H 5.12), and irradiation of the olefinic proton, 8-H, induced NOE enhancement of the signal of the methine proton, 2-H. The partial structure A therefore was established. The partial structure B (Fig. 3.1) also was deduced from H,H-COSY and COLOC data. An ethyl group (C-16 and C-17) was indicated by the ^{1}H NMR (Table 3.2) and H,H-COSY data. Attachment of the ethyl group to a ketonic carbonyl carbon, C-15, was deduced from the COLOC correlation of the carbonyl carbon, C-15, to the methyl protons, 17-H_3, thus giving the partial structure B. The partial structure C (Fig. 3.1) was shown to be 2-methylbutyl from the ^{1}H NMR and H,H-COSY data. The partial structure D (Fig. 3.1) was deduced from the IR, UV, and ^{13}C NMR data as described above.

These four partial structures A–D were combined on the basis of COLOC data and a consideration of chemical shift. The carbonyl carbon, C-4, in partial structure D had COLOC correlations with the methyl protons, 14-H_3, and with the methine proton, 2-H, both in partial structure A, indicative of a bond between C-3 and C-4. The COLOC correlation of the olefinic carbon, C-6, in partial structure D to the methine proton, 2-H, in partial structure A and the chemical shift of the methine carbon, C-2 (δ_C 76.7), were in agreement with there being a bond between C-2 and the nitrogen atom. The finished

Table 3.2. ^{1}H NMR data (270.05 MHz) for neovasipyridones A–F (**4a–f**) in $CDCl_3$.

H	**4a**	**4b**	**4c**	**4d**	**4e**	**4f**
2	3.82 *s*	3.80 *s*	3.80 *s*	3.78 *s*	3.81 *s*	3.85 *s*
6	8.21 *s*	8.25 *s*	8.20 *s*	8.23 *s*	8.29 *s*	8.26 *s*
8	5.12 *dq* (9.5, 0.9)	5.12 *dq* (10.8, 1.2)	5.13 *dq* (9.5, 1.0)	5.14 *dq* (9.7, 1.1)	5.12 *dq* (10.3, 1.1)	5.16 *br. d* (8.6)
9	2.28 *m*	2.25 *m*	2.28 *m*	2.23 *m*	2.28 *m*	2.20 *m*
10	1.10–1.40 *m*	1.10–1.42 *m*	1.07–1.40 *m*	1.08–1.42 *m*	1.10–1.40 *m*	1.10–1.40 *m*
11	0.83 *t* (7.6)	0.82 *t* (7.5)	0.81 *t* (7.5)	0.82 *t* (7.6)	0.83 *t* (7.0)	0.83 *t* (7.6)
12	1.43 *d* (0.9)	1.41 *d* (1.2)	1.41 *d* (1.0)	1.39 *d* (1.1)	1.44 *d* (1.1)	1.43 *br. s*
13	0.93 *d* (6.8)	0.91 *d* (6.8)	0.91 *d* (6.8)	0.95 *d* (6.5)	0.92 *d* (6.5)	0.92 *d* (6.3)
14	1.38 *s*	1.34 *s*	1.37 *s*	1.15 *s*	1.36 *s*	1.36 *s*
16	2.86 *dq* (17.1, 7.2)	2.84 *dq* (16.5, 7.5)	2.84 *dq* (17.0, 7.0)	2.78 *dq* (17.2, 7.6)	2.84 *dq* (17.3, 7.6)	2.87 *dq* (17.1, 7.5)
	2.98 *dq* (17.1, 7.2)	2.97 *dq* (16.5, 7.5)	2.97 *dq* (17.0, 7.0)	2.87 *dq* (17.2, 7.6)	2.98 *dq* (17.3, 7.6)	2.94 *dq* (17.1, 7.5)
17	1.10 *t* (7.2)	1.09 *t* (7.5)	1.08 *t* (7.0)	1.07 *t* (7.6)	1.08 *t* (7.6)	1.09 *t* (7.5)
18	3.10 *dd* (13.5, 8.6)	3.29 *dd* (13.5, 7.5)	2.94 *dd* (14.0, 9.0)	3.57 *dd* (14.0, 7.6)	3.42 *q* (7.6)	3.30–3.55 *m*
	3.23 *dd* (13.5, 5.8)	3.39 *dd* (13.5, 7.0)	3.28 *dd* (14.0, 5.2)	3.65 *dd* (14.0, 8.1)		
19	1.80 *m*	1.60 *m*	2.03 *m*	2.92 *m*	1.34 *t* (7.6)	2.20–2.41 *m*
20	1.25 *m*	1.60 *m*	0.97 *d* (6.8)			2.20–2.41 *m*
21	0.95 *t* (7.2)	0.95 *d* (6.5)	0.98 *d* (6.8)	7.18–7.20 *m*		
22	0.98 *d* (6.3)	0.96 *d* (6.5)		7.23–7.38 *m*		
23				7.23–7.38 *m*		
24				7.23–7.38 *m*		
25				7.18–7.20 *m*		
NH_2						5.48 *br. s*

^{1}H NMR data represent chemical shift, multiplicity (*J* in Hz).

combination of partial structure A and D gave an integrated partial structure with a 2,3-dihydro-4-pyridinone ring. The chemical shift of the ketonic carbonyl carbon, C-15 (δ_C 197.6), in partial structure B indicated bonding of the carbonyl carbon, C-15, to the olefinic carbon, C-5, on the 2,3-dihydro-4-pyridinone ring. The chemical shifts of the methylene carbon (C-18, δ_C 61.2) and the methylene protons [18-Ha (refers to the methylene proton that resonates in the upper field), δ_H 3.10; 18-Hb (lower field), δ_H 3.23] indicated a bond between C-18 and the nitrogen atom. This was confirmed by the NOE observations. Irradiation of 2-H proton caused NOE enhancement of the signal of one of the methylene protons (18-Hb), and irradiation of 6-H proton caused NOE enhancement of another methylene proton (18-Ha). Finally, the one remaining hydrogen atom was bonded to the oxygen atom on C-3 to give the complete structure of **4a**. NOE enhancement of the signal of the methyl protons (14-H_3) on irradiation of the methine proton (2-H) suggested that the methyl group (C-14) was pseudo-equatorial on the supposition that the 2-H proton was pseudo-axial.

Neovasipyridone B (**4b**) had the same molecular formula, $C_{21}H_{35}NO_3$ (HRFABMS and ^{13}C NMR data), as neovasipyridone A (**4a**), and their UV spectra were very similar. Their 16 ^{13}C resonances due to C-2 to C-17 (Table 3.1) were nearly superimposable, indicative that **4b** has the same structure, except for the *N*-alkyl group, as **4a**. The ^1H NMR (Table 3.2) and H,H-COSY data indicated the attachment of 3-methylbutyl to the nitrogen atom on the 2,3-dihydro-4-pyridinone ring. Neovasipyridone C (**4c**) had a molecular formula of $C_{20}H_{33}NO_3$ (HREIMS and ^{13}C NMR data), which is equal to the loss of CH_2 from **4a** and **4b**. The 16 ^{13}C resonances due to C-2 to C-17 (Table 3.1) were nearly superimposable on those of **4a** and **4b**. The ^1H NMR (Table 3.2) and H,H-COSY data indicated that the 2-methylpropyl is an *N*-alkyl instead of the 2-methylbutyl in **4a** or the 3-methylbutyl in **4b**.

The structures of neovasipyridones D (**4d**, $C_{24}H_{33}NO_3$), E (**4e**, $C_{18}H_{29}NO_3$), and F (**4f**, $C_{20}H_{32}N_2O_4$) were determined by the same method as used for **4b** and **4c**. The EI mass spectra of **4d**, **4e**, and **4f** had ion peaks at *m/z* $[M-43]^+$, $[M-57]^+$, $[M-153]^+$, and 125, indicative of neovasipyridones. The ^1H NMR (Table 3.2) and H,H-COSY data for **4d** and **4e** indicated that the phenylethyl is an *N*-alkyl in **4d** and that the ethyl in **4e**. The

carbamoyl group in **4f** was confirmed by the IR absorption band at 1671 cm^{-1} [11] and the D_2O exchangeable resonance at δ_H 5.48.

Neovasipyridones A–F (**4a–f**) have different *N*-alkyl groups: 2-methylbutyl in **4a**, 3-methylbutyl in **4b**, 2-methylpropyl in **4c**, phenylethyl in **4d**, ethyl in **4e**, and 3-carbamoylpropyl in **4f**. These compounds and neovasinin (**1**) have the same side chain. The stereochemistries of the asymmetric carbons in neovasipyridones A–F (**4a–f**) have yet to be resolved, but the absolute stereochemistries of neovasinin (**1**) [2], neovasipyrones (**2**), and neovasifuranones (**3**) suggest that the stereochemistries of C-2, C-3, and C-9 in neovasipyridones (**4**) are (*R*,*S*,*S*).

Preliminary leaf spot bioassay indicated that neovasipyridones (**4**) as well as neovasipyrones (**2**) were not phytotoxic to soybean plant, although neovasinin (**1**) and neovasifuranones (**3**) are phytotoxic.

Experimental Section

General procedure.

NMR spectra were recorded in $CDCl_3$ on a JEOL JNM GX-270 or GX-400 FT NMR spectrometer. NMR chemical shifts were referenced to $CDCl_3$ (δ_H 7.26, δ_C 77.0). MS were obtained with a JEOL DX-300 or AX-505 spectrometer. Glycerol was the matrix used for FABMS. In EIMS, the ionization voltage was 70 eV. 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 measured with a Horiba SEPA-200 high sensitive polarimeter. Daisogel IR-60 was the silica gel used for column chromatography. Preparative TLC was performed on a Merck Kieselgel 60 HF_{254} glass plate (20 x 20 x 0.05 cm).

Fungal material.

Strain NHL2298 of *Neocosmospora vasinfecta* E. F. Smith var. *africana* (von Arx) Cannon et Hawksworth used in this study was a gift from Dr. Shun-ichi Udagawa (National Institute of Hygienic Sciences, Tokyo) in 1983 and was maintained on potato

dextrose agar.

Fermentation and extraction.

The fungus was grown in a 500-mL conical flask containing liquid medium (200 mL x 200) composed of sucrose (50 g/L), peptone (3 g/L), the extract from 100 g/L on malt, L-methionine (0.3 g/L), and water, without shaking at 24°C for 21 days in the dark. The mycelial mats obtained after filtering the culture broth were rinsed with H_2O and dried under a hood. The dried mats (620 g) were soaked in Me_2CO overnight, after which the Me_2CO extract was obtained by filtration. This treatment was repeated three times. The combined Me_2CO extracts were evaporated to dryness. The residue was dissolved in 1 M $NaHCO_3$ (500 mL) and treated with EtOAc (500 mL). The EtOAc solution obtained was placed over Na_2SO_4 and evaporated to dryness. The residue was partitioned with 90% aq. MeOH (250 mL) and *n*-hexane (250 mL). The *n*-hexane layer obtained was extracted with 90% aq. MeOH (2 x 250 mL). The combined MeOH solution was evaporated to dryness.

Isolation of neovasipyridones A–F (4a–f).

The residue (13.5 g) from MeOH solution was subjected to silica gel column chromatography (340 g, 4.6 i.d. x 32 cm). The column was developed successively with 2000 mL (5 x 400 mL) each of 0, 2, 5, 10, 20, 30, 40, and 50% Me_2CO in *n*-hexane. Fraction 1 (455 mg), eluted with 10% Me_2CO in *n*-hexane, was purified by Sephadex LH-20 column chromatography (2.7 i.d. x 80 cm) with MeOH as the solvent. Each 7 mL of eluate constituted one fraction, and fractions 41–44 were combined and evaporated to dryness. The residue (30 mg) further was purified by preparative TLC [Me_2CO–$CHCl_3$ (1 : 19)] to afford neovasipyridone A (**4a**, 15.5 mg). Fractions 2 and 3, eluted with 10% Me_2CO in *n*-hexane, were combined and evaporated to dryness. The residue (1.29 g) was subjected to Sephadex LH-20 column chromatography (3.2 i.d. x 122 cm) with MeOH as the solvent. Each 10 mL of eluate constituted one fraction, and fractions 56–61 were combined and evaporated to dryness. The residue (73 mg) contained neovasipyridones A (**4a**), B (**4b**), and C (**4c**). Removal of **4a** from the mixture was achieved by preparative

TLC [EtOAc–C_6H_6 (7 : 93), quadruple development], giving **4a** (6.7 mg) as a constituent of a higher R_f band on TLC. The lower R_f band contained **4b** and **4c**. Compound **4b** was separated from **4c** by repeated HPLC [DAISOPAK SP-120-5-ODS-A (10 i.d. x 250 mm), 90% aq. MeOH, 1.0 mL/min], giving **4b** (14.0 mg, R_t 28.0 min) and **4c** (11.1 mg, R_t 33.0 min). Fraction 4 (349 mg), eluted with 10% Me_2CO in *n*-hexane, was purified on a Sephadex LH-20 column chromatography (2.7 i.d. x 80 cm) with MeOH as the solvent. Each 7 mL of eluate constituted one fraction, and fractions 44–47 were combined and evaporated to dryness. The residue (168 mg) was purified by preparative TLC [Me_2CO–$CHCl_3$ (1 : 9)], giving neovasipyridone D (**4d**, 13.8 mg). Fraction 5 (203 mg), eluted with 10% Me_2CO in *n*-hexane, was purified on a silica gel column chromatography (30 g, 2.2 i.d. x 20 cm) with Me_2CO–$CHCl_3$ (1 : 9) as the solvent. Each 10 mL of eluate constituted one fraction, and fractions 7 and 8 were combined and evaporated to dryness. The residue (13 mg) contained neovasipyridones D (**4d**) and E (**4e**), which were separated by preparative TLC [Me_2CO–$CHCl_3$ (1 : 9)], giving **4d** (7.9 mg, higher R_f band) and **4e** (4.9 mg, lower R_f band). Fractions 4 and 5, eluted with 40% Me_2CO in *n*-hexane, and fraction 1, eluted with 50% Me_2CO in *n*-hexane, were combined (195 mg) and subjected to column chromatography on Sephadex LH-20 (2.3 i.d. x 100 cm) with MeOH as the solvent. Each 6 mL of eluate constituted one fraction, and fractions 42–47 were combined and evaporated to dryness. The residue (23 mg) was purified by preparative TLC [Me_2CO–$CHCl_3$ (3 : 7)], giving neovasipyridone F (**4f**, 2.7 mg).

Neovasipyridone A (4a).

Oil. $[\alpha]_D^{20}$ +366° (*c* 1.0, EtOH). UV (EtOH) λ_{max} nm (log ε): 202 (3.79), 262 (4.10), 322 (4.14). IR (film) ν_{max} cm^{-1}: 3398, 2964, 2930, 2874, 2858, 1647, 1580, 1462, 1375, 1350, 1315, 1267, 1241, 1172, 1114. ^1H and ^{13}C NMR spectral data: Tables 3.1 and 3.2. FABMS *m/z* (rel. int.): 350 $[M+H]^+$ (100), 332 (10), 306 (14), 236 (30), 196 (12); exact mass calcd for $C_{21}H_{36}NO_3$ 350.2695, found 350.2655.

Neovasipyridone B (4b).

Oil. $[\alpha]_D^{20}$ +392° (*c* 1.0, EtOH). UV (EtOH) λ_{max} nm (log ε): 200 (3.71), 263

(4.15), 322 (4.19). IR (film) ν_{max} cm^{-1}: 3362, 2960, 2874, 1638, 1574, 1460, 1392, 1377, 1334, 1292, 1257, 1201, 1156, 1116, 1083, 1029. ^{1}H and ^{13}C NMR spectral data: Tables 3.1 and 3.2. FABMS *m/z* (rel. int.): 350 $[M+H]^+$ (100), 306 (15), 196 (10), 154 (39), 149 (27), 136 (33), 107 (15); exact mass calcd for $C_{21}H_{36}NO_3$ 350.2695, found 350.2666.

Neovasipyridone C (4c).

Oil. $[\alpha]_D^{20}$ +383° (*c* 1.0, EtOH). UV (EtOH) λ_{max} nm (log ε): 203 (3.98), 262 (4.11), 323 (4.14). IR (film) ν_{max} cm^{-1}: 3406, 2967, 2926, 2874, 1639, 1579, 1460, 1369, 1315, 1261, 1157, 1113. ^{1}H and ^{13}C NMR spectral data: Tables 3.1 and 3.2. EIMS *m/z* (rel. int.): 335 $[M]^+$ (55), 292 (100), 278 (23), 222 (15), 182 (41), 125 (19); exact mass calcd for $C_{20}H_{33}NO_3$ 335.2461, found 335.2488.

Neovasipyridone D (4d).

Oil. $[\alpha]_D^{20}$ +188° (*c* 1.0, EtOH). UV (EtOH) λ_{max} nm (log ε): 202 (3.91), 262 (3.88), 323 (3.93). IR (film) ν_{max} cm^{-1}: 3434, 2966, 2928, 2876, 1638, 1576, 1458, 1375, 1350, 1309, 1261, 1151, 1116. ^{1}H and ^{13}C NMR spectral data: Tables 3.1 and 3.2. EIMS *m/z* (rel. int.): 383 $[M]^+$ (34), 340 (100), 326 (20), 267 (26), 230 (36), 212 (20), 162 (26), 125 (16), 105 (22); exact mass calcd for $C_{24}H_{33}NO_3$ 383.2460, found 383.2436.

Neovasipyridone E (4e).

Oil. $[\alpha]_D^{20}$ +178° (*c* 0.5, EtOH). UV (EtOH) λ_{max} nm (log ε): 201 (3.82), 262 (3.72), 322 (3.70). IR (film) ν_{max} cm^{-1}: 3416, 2966, 2930, 1638, 1580, 1462, 1352, 1311, 1265, 1151, 1118. ^{1}H and ^{13}C NMR spectral data: Tables 3.1 and 3.2. EIMS *m/z* (rel. int.): 307 $[M]^+$ (50), 278 (16), 264 (100), 250 (18), 154 (89), 125 (40); exact mass calcd for $C_{18}H_{29}NO_3$ 307.2148, found 307.2157.

Neovasipyridone F (4f).

Oil. $[\alpha]_D^{20}$ +260° (*c* 0.2, EtOH). UV (EtOH) λ_{max} nm (log ε): 202 (3.61), 262 (3.91), 322 (3.90). IR (film) ν_{max} cm^{-1}: 3326, 2964, 2930, 2860, 1671, 1638, 1576, 1460, 1375, 1309, 1263, 1151, 1116. ^{1}H NMR spectral data: Table 3.2. EIMS *m/z* (rel. int.):

364 $[M]^+$ (28), 335 (17), 321 (100), 307 (12), 248 (10), 211 (88), 154 (17), 125 (45); exact mass calcd for $C_{20}H_{32}N_2O_4$ 364.2362, found 364.2352.

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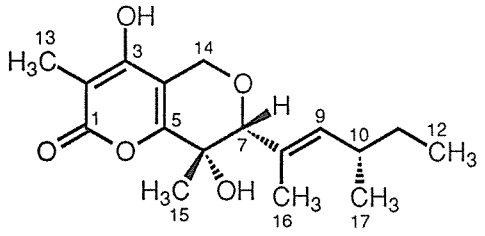
CHAPTER 4

Incorporation of ^{13}C- and ^{2}H-Labeled Precursors into Neovasinin, Neovasipyrones, and Neovasifuranones and the Conversion of Neovasifuranone A Aldehyde to Neovasifuranone A

Introduction

Neovasinin (**1**), produced by *Neocosmospora vasinfecta* NHL2298, has a unique bicyclic unit, 2*H*,5*H*-pyrano[4,3-*b*]pyran-2-one [1, 2]. The fungus simultaneously produces metabolites biogenetically related to neovasinin (**1**): neovasinone (**11**) [3], neovasipyrones (**2**), neovasifuranones (**3**), vasinfectins (**10**), and neovasipyridones (**5**). These metabolites have the following structural features: The carbon skeletons and stereochemistries of asymmetric carbons (C-6 and C-10) in neovasipyrones (**2**) are identical to those in neovasinin (**1**). Neovasipyrones (**2**), neovasifuranones (**3**), and vasinfectins (**10**) have the same side chain. Except for *N*-alkyl groups, C_{16} carbon networks of the neovasipyridones (**5**) are in agreement with the networks of neovasifuranones (**3**). Moreover, the variety of *N*-alkyl groups suggests that a Schiff base would be formed with alkyl amines and an aldehyde group in the biosynthetic pathway of the neovasipyridones (**5**) [4, 5]. These features strongly suggest that neovasinin (**1**), neovasipyrones (**2**), neovasifuranones (**3**), and neovasipyridones (**5**) have the same origin, probably biosynthesis from a hexaketide plus five C_1 units, the common intermediate being an aldehyde compound. To explore this hypothesis, I did incorporation experiments using ^{13}C-labeled acetates and $^{13}CH_3$- and $^{13}C^{2}H_3$-labeled methionine. I also did replacement culture and enzymatic conversion of the probable aldehyde compound. The incorporation patterns of labeled precursors into neovasinin (**1**), neovasipyrones (**2**), and neovasifuranones (**3**), and the *in vivo* and *in vitro* conversion of neovasifuranones A aldehyde (**4a**) to neovasifuranone A (**3a**) are described in this chapter.



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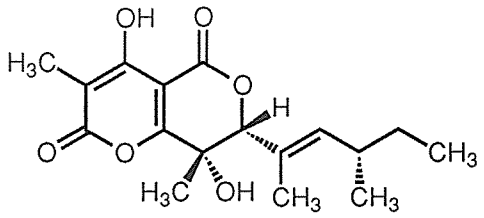

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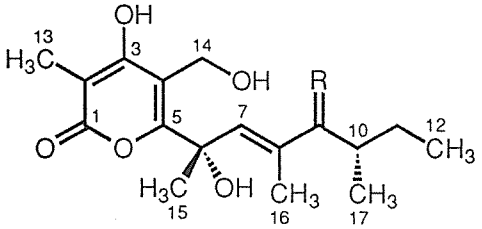
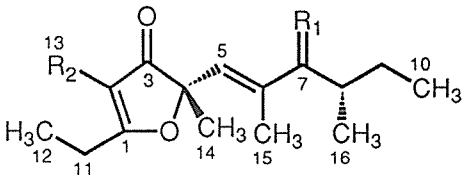
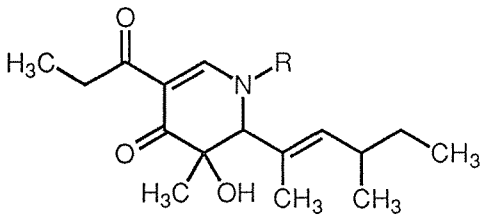


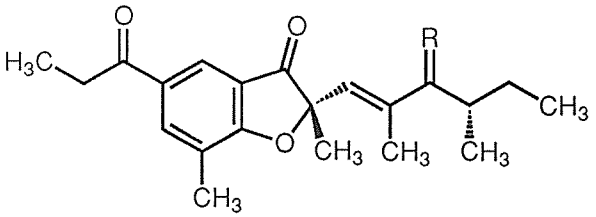
2a R = α-OH, β-H
2b R = α-H, β-OH



3a R_1 = α-OH, β-H, R_2 = CH_2OH
3b R_1 = α-H, β-OH, R_2 = CH_2OH
4a R_1 = α-OH, β-H, R_2 = CHO
4b R_1 = α-H, β-OH, R_2 = CHO



5a R = $CH_2CH(Me)CH_2Me$
5b R = $CH_2CH_2CH(Me)_2$
5c R = $CH_2CH(Me)_2$
5d R = $CH_2CH_2C_6H_5$
5e R = CH_2Me
5f R = $CH_2CH_2CH_2CONH_2$



10a R = α-OH, β-H
10b R = α-H, β-OH

Results and Discussion

Preliminary feeding experiments done to clarify the biosynthetic origin of neovasinin (**1**) from this fungus grown on malt extract medium supplemented with peptone and methionine [6] were unsuccessful because of the low incorporation of ^{13}C-labeled acetate into **1**. This probably was caused by dilution of the fed labeled acetate with the endogenous unlabeled acetate of the fungus. To solve this problem, various media and carbon sources were tested. As a result, incorporation experiments with ^{13}C-labeled

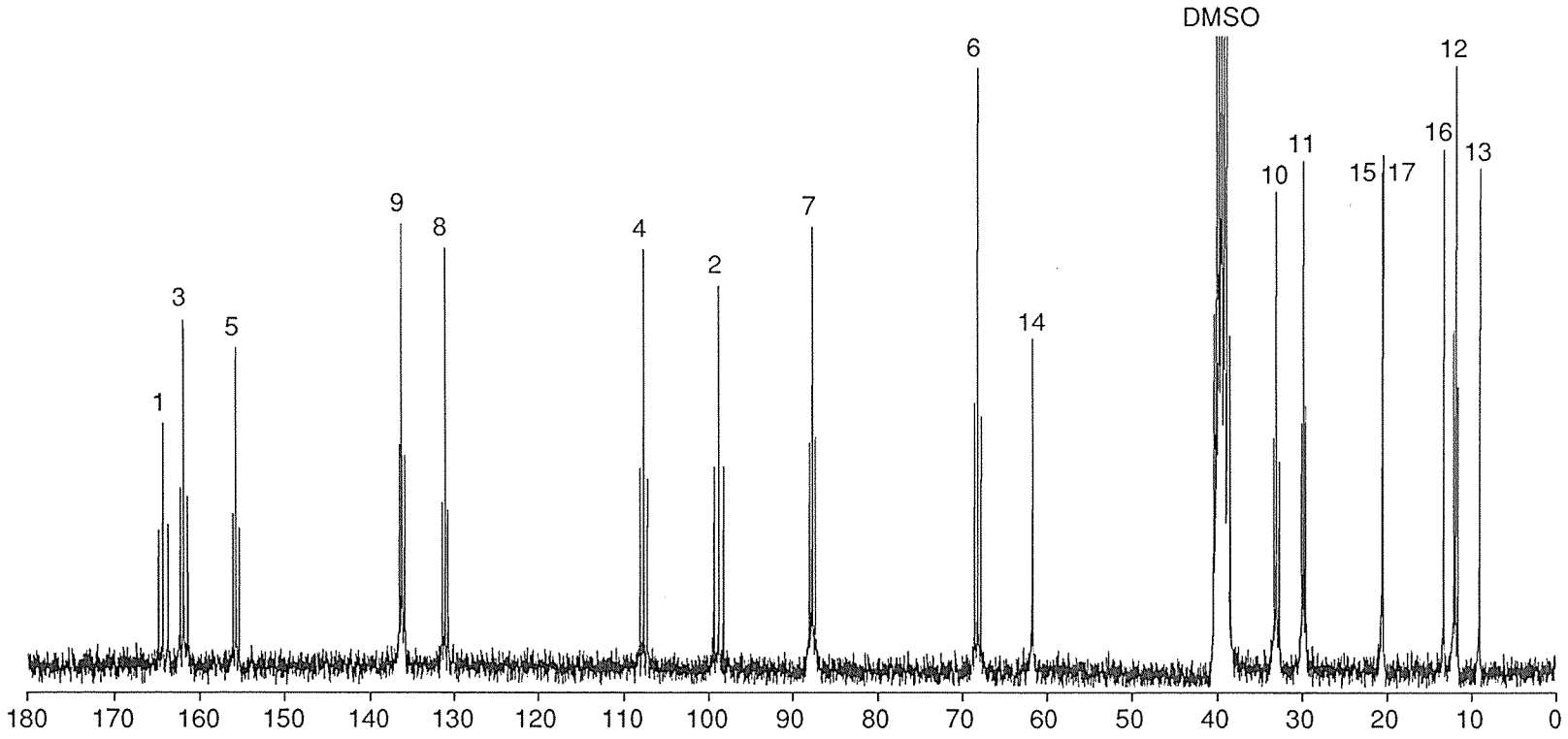


Fig. 4.1. ^{13}C NMR spectrum (67.8 MHz) of neovasinin (**1**) enriched with sodium [1,2-$^{13}C_2$]acetate in DMSO-d_6.

acetates were done with glycerol-based malt medium to control the carbon economy of the cell [7, 8]. In contrast to the studies with labeled acetates, incorporation studies done with labeled methionines and conventional malt–peptone–sucrose medium were successful. The timing for the supply of precursor to this fungus was determined from growth curves obtained by detecting metabolites after various growth periods (data not shown).

The ^{13}C NMR spectrum of neovasinin (**1**) enriched with sodium [1,2-$^{13}C_2$]acetate is shown in Fig. 4.1. Results of the feeding experiments with ^{13}C-labeled precursors are given in Table 4.1 and Fig. 4.2. In the ^{13}C NMR spectrum of **1** derived from sodium [1,2-$^{13}C_2$]acetate, the signals for C-1 to C-12 were flanked by doublets due to ^{13}C–^{13}C coupling [9]. The coupling constants could be unambiguously paired, indicative of the existence of six intact acetate units. Six carbons (C-1, C-3, C-5, C-7, C-9, and C-11) were enriched by sodium [1-^{13}C]acetate, and the other six carbons (C-2, C-4, C-6, C-8, C-10, and C-12) were enriched by sodium [2-^{13}C]acetate. The incorporation of [*S*-$^{13}CH_3$]-L-methionine indicated that the remaining five carbons (C-13 to C-17) were derived from the C_1 unit. These results show that neovasinin (**1**) is biosynthesized from a linear hexaketide chain and five C_1 units.

In the incorporation experiments with the labeled acetates, neither neovasipyrones A

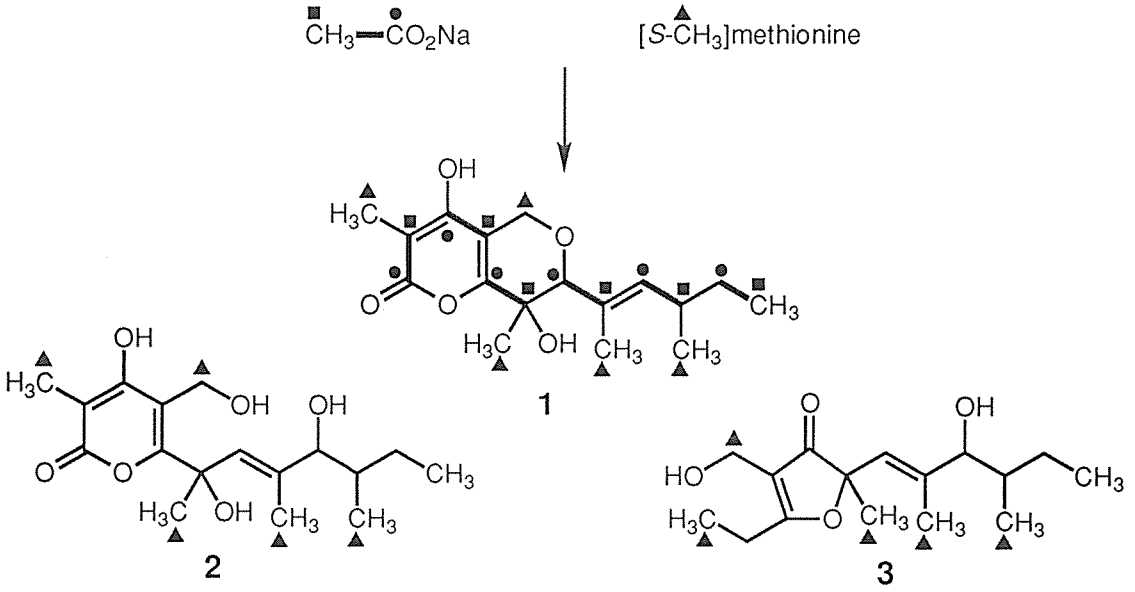


Fig. 4.2. Incorporation patterns of labeled precursors into neovasinin (**1**), neovasipyrones (**2**), and neovasifuranones (**3**).

Table 4.1. ^{13}C NMR data (67.8 MHz) for neovasinin (**1**) enriched with labeled precursors in DMSO-d_6.

			relative enrichment			
carbon	δ_C	*J* cc (Hz)	[1-^{13}C]acetate	[2-^{13}C]acetate	[$^{13}CH_3$]methionine	α-shift
1	164.3	76.3	2.25	0.99	0.78	
2	98.9	76.3	0.83	1.74	1.00*	
3	161.8	60.6	2.65	1.36	0.91	
4	107.6	60.2	0.92	2.25	1.01	
5	155.7	53.3	1.78	1.18	0.99	
6	68.2	53.8	0.82	2.21	1.05	
7	87.7	50.4	2.05	0.97	1.57	
8	131.1	50.4	0.81	2.02	0.98	
9	136.2	43.0	2.00	0.94	1.18	
10	33.2	43.0	1.05	2.64	0.96	
11	29.9	35.2	2.12	0.91	1.26	
12	11.9	35.2	1.02	2.22	1.12	
13	9.1		1.23	1.23	19.36	
14	61.7		1.00*	1.00*	17.95	0.30
15	20.6		1.02	1.06	23.41	
16	13.3		0.97	1.30	16.08	
17	20.5		1.11	1.20	23.70	

* Enrichments were normalized to these signals.

(**2a**) and B (**2b**) nor neovasifuranones A (**3a**) and B (**3b**) were detected in the culture filtrate. In the incorporation study done with ^{13}C-labeled methionine, however, these metabolites were obtained. In the ^{13}C NMR spectra of the neovasipyrones (**2**) and neovasifuranones (**3**) enriched with ^{13}C-labeled methionine, five of the signals had enhanced intensities greater than the natural abundance (Tables 4.2 and 4.3). The labeling patterns of neovasinin (**1**), neovasipyrones (**2**), and neovasifuranones (**3**) with C_1 units (Fig. 4.2) suggested that **2** and **3** are derived from a hexaketide plus five C_1 units, the same biosynthetic origin as **1**, and that **3** lose one carbon from the hexaketide chain.

Table 4.2. ^{13}C NMR data (67.8 MHz) for neovasipyrones A (**2a**) and B (**2b**) enriched with labeled methionine in acetone-d_6.

	2a			**2b**		
carbon	δ_C	rel. enrichment	α-shift	δ_C	rel. enrichment	α-shift
1	164.9	1.25		164.7	0.69	
2	100.6	1.00*		100.7	1.00*	
3	167.7	1.48		167.5	1.46	
4	110.4	1.28		110.7	0.85	
5	163.5	2.70		163.3	1.42	
6	75.5	1.51		75.2	1.29	
7	132.7	2.02		134.7	1.21	
8	140.3	2.12		140.0	1.16	
9	80.8	1.77		83.2	2.17	
10	38.9	2.52		38.9	1.30	
11	27.6	1.38		26.1	1.05	
12	12.4	1.43		11.9	1.13	
13	9.1	35.96		9.1	13.54	
14	58.8	15.11	0.32	58.7	11.94	0.32
15	28.7	37.33		28.1	10.39	
16	13.8	26.54		12.4	13.19	
17	14.7	23.31		16.4	16.23	

* Enrichments were normalized to these signals.

The methylene carbon, C-14, of neovasinin (**1**) that is involved in the formation of the pyran ring in the bicyclic unit is derived from C_1 unit. In general, *C*-methylation occurs by direct nucleophilic displacement on the *S*-methyl group of *S*-adenosyl-L-methionine before release of the completed polyketide from the synthase complex occurs [10–13]. To clarify the level of oxidation at C-14 in **1** and neovasipyrones (**2**) and at C-13 in neovasifuranones (**3**) during their biosyntheses, [*S*-$^{13}C^2H_3$]-L-methionine was used as the precursor [14]. To determine the number of deuterium atoms retained, α-shifted signals in the ^{13}C NMR spectra of the derived metabolites were observed [9, 15]. In the ^{13}C NMR spectrum of **1**, deuterium-induced α-isotope shifts occurred for C-13 to C-17 (Fig. 4.3).

Table 4.3. ^{13}C NMR data (67.8 MHz) for neovasifuranones A (**3a**, in CD_3OD) and B (**3b**, in $CDCl_3$) enriched with labeled methionine.

	3a			**3b**		
carbon	δ_C	rel. enrichment	α-shift	δ_C	rel. enrichment	α-shift
1	194.0	1.42		189.5	1.00	
2	114.1	0.48		111.9	1.31	
3	208.2	1.16		205.6	0.88	
4	91.0	0.42		88.5	0.44	
5	124.4	0.60		123.7	0.80	
6	146.0	0.67		143.5	0.73	
7	82.3	0.78		82.1	0.65	
8	39.5	0.75		37.5	0.67	
9	28.1	0.72		24.4	1.02	
10	12.7	1.00*		11.1	1.00*	
11	24.2	0.42		22.5	1.40	
12	11.6	24.85		10.6	15.64	
13	53.2	16.54	0.30	53.4	14.80	0.32
14	25.2	14.71		23.9	15.96	
15	14.8	18.86		12.8	11.32	
16	15.2	28.85		15.6	16.58	

* Enrichments were normalized to these signals.

The intensity of the signals for C-13 to C-17 being lower than that of the expected normal signals is consistent with the retention of deuterium atoms at these positions. In particular, a triplet (J = 22 Hz) centered 0.30 ppm upfield of the main signal (C-14) is consistent with the retention of one deuterium atom (Table 4.1 and Fig. 4.3). In the ^{13}C NMR spectra of neovasipyrones A (**2a**) and B (**2b**) derived from this precursor, the natural signals of C-14 showed triplets centered respectively 0.32 and 0.32 ppm upfield of the C-14 signal (Table 4.2). In the ^{13}C NMR spectra of neovasifuranones A (**3a**) and B (**3b**), the natural signals of C-13 also showed respective triplets centered 0.30 and 0.32 ppm upfield of the C-13 signal (Table 4.3). The multiplicity and overlapping of the α-shifted signals of the other

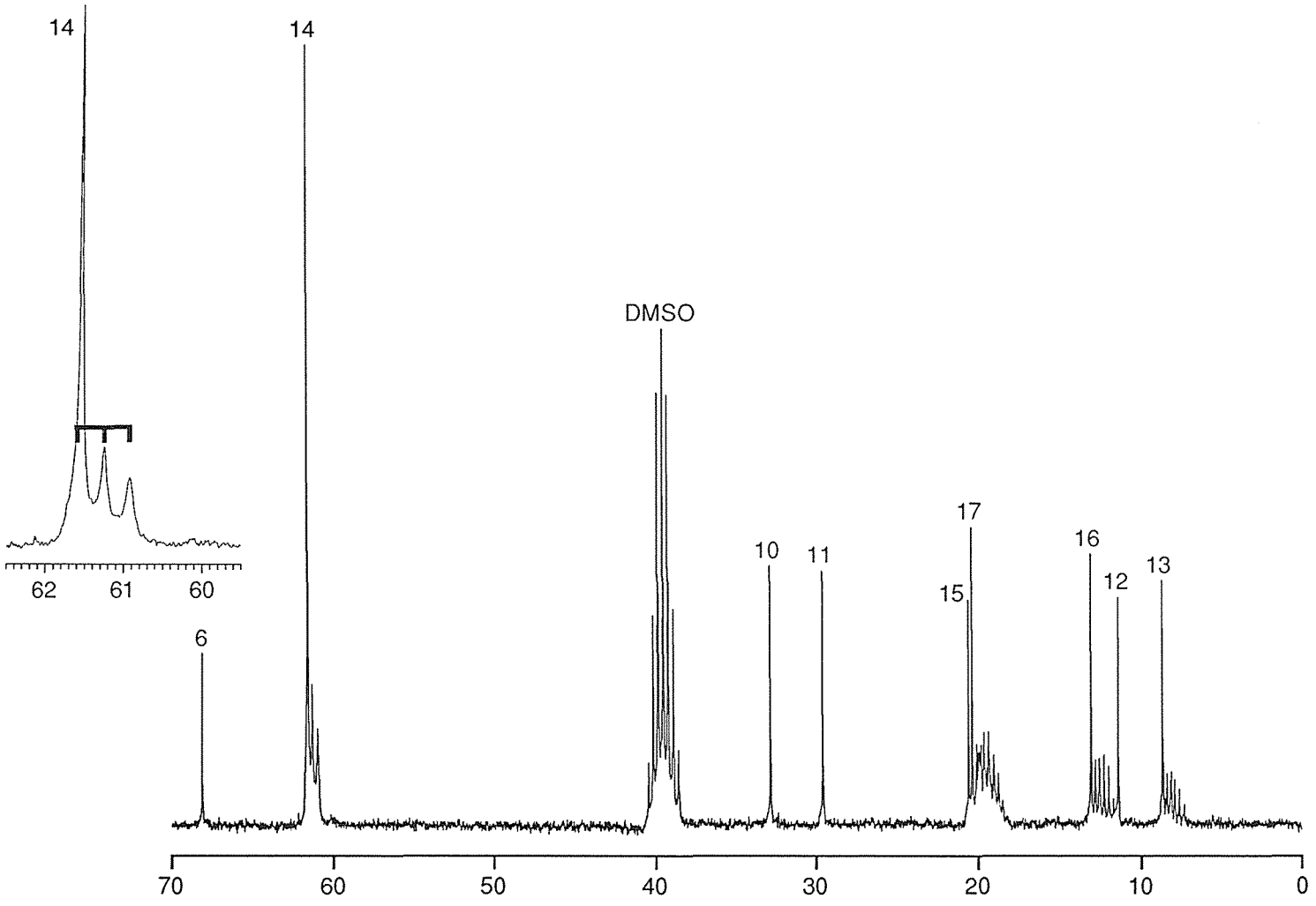


Fig. 4.3. ^{13}C NMR spectrum (67.8 MHz) of neovasinin (**1**) enriched with [*S*-$^{13}C^{2}H_3$]methionine in DMSO-d_6.

C_1 unit carbons (C-13, C-15, C-16, and C-17 in **1** and **2**; C-12, C-14, C-15, and C-16 in **3**) hampered further examination, but their carbons mainly seem to retain three deuterium atoms; the actual number of deuterium atoms retained at these positions would be determined by obtaining simultaneous proton- and deuterium-decoupled ^{13}C NMR spectra [9, 15, 16]. These findings indicate that the key intermediate in **1**, **2**, and **3** is an aldehyde rather than a hydroxymethyl compound, and that it is formed by the oxidation of the introduced C_1 unit, C-14 in **1** and **2** and of C-13 in **3**.

To investigate this possibility, synthesis and isolation of the aldehyde intermediates were attempted. Unfortunately, they were unsuccessful for neovasinin (**1**) and the neovasipyrones (**2**). A small amount of neovasifuranone A aldehyde (**4a**) and neovasifuranone B aldehyde (**4b**), however, were isolated from the culture filtrate (each 0.05 mg/L of medium, data not shown). Moreover, neovasifuranone A aldehyde (**4a**) was synthesized from neovasifuranone A (**3a**) by oxidation with manganese dioxide (MnO_2) [17, 18]. In the replacement culture experiment done with this fungus, compound **4a** was converted to **3a**; but, **3a** was not converted to any compound. As expected, neovasinone (**11**) and neovasipyrones A (**2a**) and B (**2b**) were not converted to neovasinin (**1**), nor was **1** converted to **11**. Enzymatic conversion was done with the cytosol fraction from this fungus [19, 20]. When this fraction was incubated with **4a** in the presence of NADPH, only **3a** was formed (Fig. 4.4). When, however, this fraction was incubated with **4a** in the presence of NADH, or in the absence of the coenzyme, **4a** was not converted to **3a**. No reverse reaction (from **3a** to **4a**) occurred under various conditions (pH, cofactor, etc.). These results indicate that the enzyme(s) catalyzes reduction of the aldehyde group in **4a** to the hydroxymethyl group and specifically requires NADPH as the primary electron donor.

From the results described above, I propose the biosynthetic pathway shown in scheme 1. Neovasinin (**1**), neovasipyrones (**2**), neovasifuranones (**3**), and neovasipyridones (**5**) are derived from a hexaketide chain plus five C_1 units. Neovasinin (**1**) and the neovasipyrones (**2**) are biosynthesized from hypothetical common intermediates (**6**, two diastereomers with respect to the hydroxymethine carbon) that are produced by the oxidation of one C_1 unit in the hexaketide chain. Compound **1** is formed from **6** via neovasipyrone aldehydes (**7**). The dehydration/cyclization of **6** into **7** and the

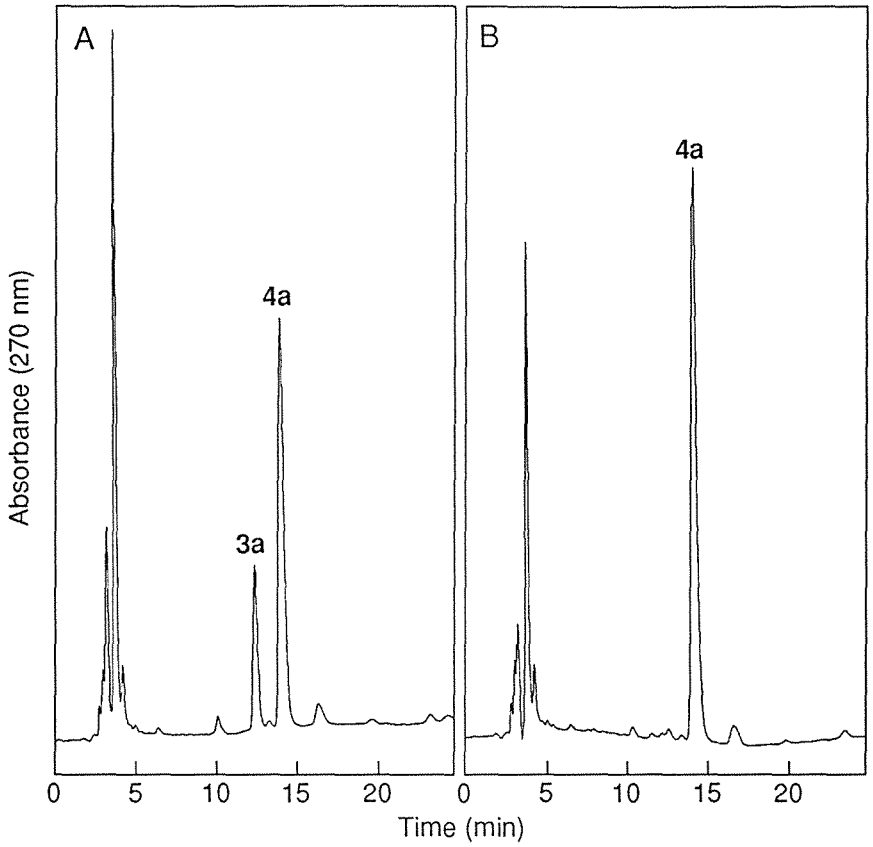
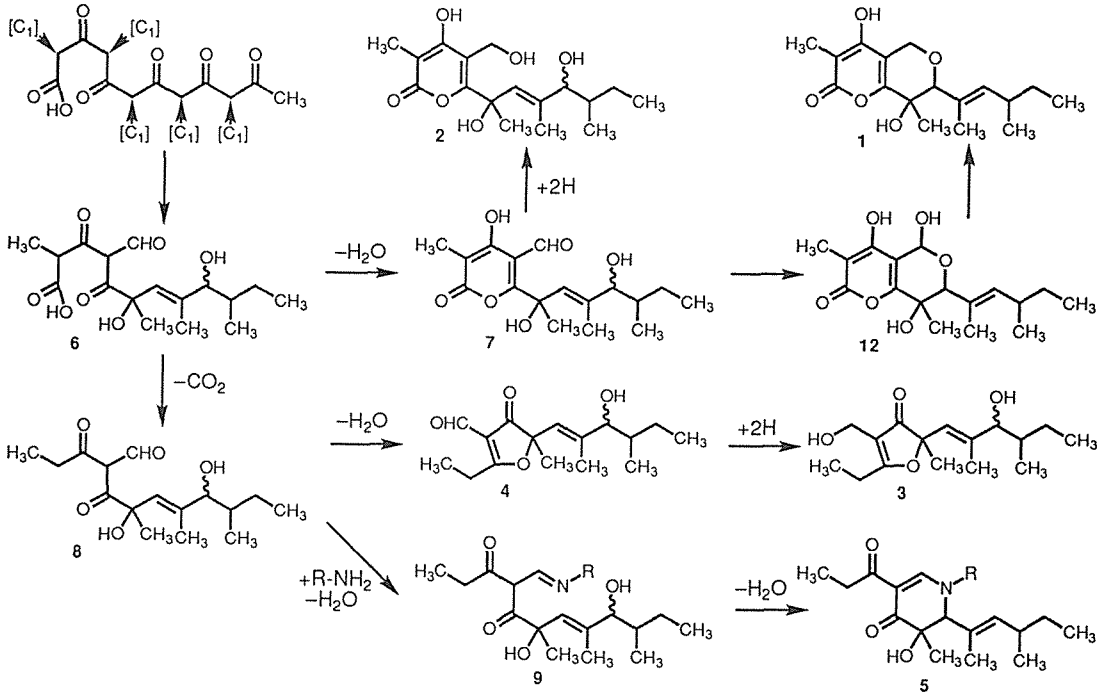


Fig. 4.4. Enzymatic conversion of neovasifuranone A aldehyde (**4a**) to neovasifuranone A (**3a**) in the presence of NADPH (A) or in the absence of the coenzyme (B).

subsequent reduction of **7** give neovasipyrones A (**2a**) and B (**2b**). Neovasifuranones (**3**) and neovasipyridones (**5**) are biosynthesized from hypothetical intermediates (**8**) that are produced by decarboxylation of carboxy group in the intermediates (**6**). Neovasifuranones A (**3a**) and B (**3b**) are formed through the dehydration/cyclization of **8** into the neovasifuranone aldehydes (**4**) and the subsequent reduction of **4**. Neovasipyridones (**5**) are formed by the Schiff base formation of **8** with alkyl amines which seem to come from amino acids (**5a**: isoleucine, **5b**: leucine, **5c**: valine, **5d**: phenylalanine, **5e**: alanine, **5f**: glutamine) and the subsequent dehydration/cyclization of **9**. Neovasinone (**11**) would be biosynthesized by the dehydrogenation of hemiacetal (**12**) or through oxidation of neovasipyrone aldehydes (**7**) to their carboxylic acids. Vasinfectins (**10**) could be formed from intermediates **6** plus an additional C_4 unit or from intermediates **8** plus a C_5 unit. Metabolites containing the bicyclic unit, as in neovasinin (**1**), have been reported in

Fusarium chlamydosporum and *F. tricinctum* (chlamydosporol, isochlamydosporol, and *O*-methylisochlamydosporol) [21, 22] and in *Gelasinospora multiforis* (multiforisin C) [23]. Multiforisin C, in particular, seems to be an artifact derived from an aldehyde compound (multiforisin B) such as neovasipyrone aldehydes (**7**). An acetal compound such as multiforisin C and O-methylisochlamydosporol also was isolated from *N. vasinfecta* NHL2298 (data not shown), but isolation of its aldehyde was unsuccessful, probably owing to unstableness and easy conversion. Although the biosynthesis of chlamydosporol and isochlamydosporol have yet to be investigated, they probably are formed via similar aldehyde intermediates (**7**) as in the biosynthesis of neovasinin (**1**).



Scheme 1. Proposed biosynthetic pathway of neovasinin (**1**) and the related metabolites.

Experimental Section

General procedure.

NMR spectra were recorded on a JEOL JNM GX-270 FT NMR spectrometer. All NMR chemical shifts were referenced against the deuterated solvent used ($CDCl_3$, δ_H 7.26, δ_C 77.0; Me_2CO-d_6, δ_C 30.3; CD_3OD, δ_C 49.8; DMSO-d_6, δ_C 39.5). Mass spectrum was obtained with a JEOL AX-505 spectrometer. IR spectrum was measured with a JASCO FT/IR-7000 spectrometer, and UV spectrum with a Shimadzu UV-vis recording spectrophotometer. Optical rotation was determined with a Horiba SEPA-200 high sensitive polarimeter. Daisogel IR-60 was the silica gel used for the column chromatography. Preparative TLC was done on a Merck Kieselgel 60 HF_{254} glass plate (20 x 20 x 0.05 cm).

Fungal material.

Strain NHL2298 of *Neocosmospora vasinfecta* E. F. Smith var. *africana* (von Arx) Cannon et Hawksworth used in this study was a gift from Dr. Shun-ichi Udagawa (National Institute of Hygienic Sciences, Tokyo) in 1983 and was maintained on potato dextrose agar.

Incorporation of labeled acetates.

The fungus was grown in a 500-mL conical flask containing liquid medium (100 mL x 20) composed of glycerol (10 g/L), peptone (3 g/L), the extract from 50 g/L on malt, and water, without shaking at 24°C. Sodium [1,2-$^{13}C_2$]acetate (99 atom % ^{13}C, 200 mg/day), sodium [1-^{13}C]acetate (99 atom % ^{13}C, 200 mg/day), or sodium [2-^{13}C]acetate (99 atom % ^{13}C, 200 mg/day) and unlabeled L-methionine (100 mg/day) dissolved in sterilized water (20 mL) were supplied to 5-day-old culture, every 24 hr from day 5 to day 9. After further 3 days, the culture filtrate obtained after filtering the culture broth was acidified to pH 2.0 with HCl and extracted with EtOAc (4 x 500 mL). The EtOAc extracts were dried over Na_2SO_4, concentrated, and extracted with 1 M $NaHCO_3$ (2 x 0.5 vol.). The $NaHCO_3$ solution was acidified to pH 2.0 with HCl and extracted with EtOAc (4 x 1 vol.) to afford

the acidic EtOAc-soluble (AE) fraction. AE fraction was subjected to silica gel partition column chromatography (20 g, impregnated with 12 mL of 0.1 M HCO_2H, 2.1 i.d. x 13 cm). The column was developed successively with each 100 mL of 10, 20, 30, 40, and 50% EtOAc in *n*-hexane saturated with 0.1 M HCO_2H. The 20 and 30% fractions were combined and concentrated. The residue was purified by preparative TLC [Me_2CO–$CHCl_3$–AcOH (10 : 90 : 1), quadruple development] to afford neovasinin (ca. 14 mg).

Incorporation of labeled methionines.

The fungus was grown in a 500-mL conical flask containing liquid medium (100 mL x 10) composed of sucrose (50 g/L), peptone (3 g/L), the extract from 100 g/L on malt, and water, without shaking at 24°C. [*S*-$^{13}CH_3$]-L-methionine (99 atom % ^{13}C, 100 mg/day) or [*S*-$^{13}C^2H_3$]-L-methionine (99 atom % ^{13}C, 99 atom % ^2H, 50 mg/day) dissolved in sterilized water (10 mL) was supplied to 8-day-old culture, every 24 hr from day 8 to day 12. After further 9 (^{13}C-labeled methionine) or 7 days ($^{13}C^2H_3$-labeled methionine), the culture filtrate obtained after filtering the culture broth was acidified to pH 2.0 with HCl and extracted with EtOAc (4 x 500 mL). The EtOAc extracts were dried over Na_2SO_4, concentrated, and washed with 1 M $NaHCO_3$ (2 x 0.5 vol.) to afford the neutral EtOAc-soluble (NE) fraction. The $NaHCO_3$ washings were acidified to pH 2.0 with HCl and extracted with EtOAc (4 x 1 vol.) to afford the acidic EtOAc-soluble (AE) fraction. AE fraction was subjected to silica gel partition column chromatography (20 g, impregnated with 12 mL of 0.1 M HCO_2H, 2.1 i.d. x 13 cm). The column was developed successively with each 100 mL of 10, 20, 30, 40, and 50% EtOAc in *n*-hexane saturated with 0.1 M HCO_2H. The 20 and 30% fractions were combined and concentrated. The residue was purified by preparative TLC [Me_2CO–$CHCl_3$–AcOH (10 : 90 : 1), quadruple development] to afford neovasinin (^{13}C-labeled methionine: 63 mg, $^{13}C^2H_3$-labeled methionine: 91 mg). The 40 and 50% fractions were purified by preparative TLC [Me_2CO–$CHCl_3$–AcOH (25 : 75 : 1), quadruple development] to afford neovasipyrones A (^{13}C: 47 mg, $^{13}C^2H_3$: 61 mg) and B (^{13}C: 18 mg, $^{13}C^2H_3$: 22 mg). NE fraction was purified by preparative TLC [Me_2CO–$CHCl_3$ (1 : 3), triple development] to afford neovasifuranones A (^{13}C: 11 mg,

$^{13}C^{2}H_3$: 10 mg) and B (^{13}C: 6 mg, $^{13}C^{2}H_3$: 7 mg).

Neovasifuranone A aldehyde (4a).

A solution of **4a** (10 mg) and MnO_2 (200 mg) in dry C_6H_6 (3 mL) was stirred for 8 hr at 28°C. The reaction mixture was diluted with water-saturated EtOAc and filtered through silica gel (Kieselgel 60 HF_{254}), and the filtrate was concentrated. The residue was purified by preparative TLC [Me_2CO–*n*-hexane (3 : 7), double development] to afford **4a** (2.7 mg) as an oil. $[\alpha]_D^{22}$ –165° (*c* 0.1, EtOH). UV (EtOH) λ_{max} nm (log ε): 202 (3.90), 234 (4.05), 271 (4.03). IR (film) ν_{max} cm^{-1}: 3439, 2965, 1717, 1680, 1570, 1462, 1420, 1366, 1262, 1047. ^1H NMR (270 MHz, $CDCl_3$): δ 0.84 (3H, *d*, *J* = 6.8 Hz, H-16), 0.91 (3H, *t*, *J* = 7.3 Hz, H-10), 1.14 (1H, *m*, H-9), 1.28 (3H, *t*, *J* = 7.6 Hz, H-12), 1.33 (1H, *m*, H-9), 1.52 (1H, *m*, H-8), 1.59 (3H, *s*, H-14), 1.72 (3H, *d*, *J* = 1.2 Hz, H-15), 3.03 (1H, *dq*, *J* = 15.2, 7.6 Hz, H-11), 3.10 (1H, *dq*, *J* = 15.2, 7.6 Hz, H-11), 3.82 (1H, *br. d*, *J* = 5.8 Hz, H-7), 5.53 (1H, *dq*, *J* = 1.2, 1.2 Hz, H-5), 9.80 (1H, *s*, H-13). EIMS *m/z* (rel. int.): 280 $[M]^+$ (8), 262 (21), 223 (23), 205 (10), 195 (14), 167 (21), 149 (15), 127 (40), 97 (100), 71 (14), 57 (36).

Replacement culture.

The fungus was grown in a test tube (10 i.d. x 75 mm) containing liquid medium (0.5 mL x 5) composed of sucrose (50 g/L), peptone (3 g/L), malt extract (DIFCO, 20 g/L), and water, without shaking at 24°C. After 8 days incubation the medium was replaced with 0.1 M potassium phosphate buffer (pH 7.0), and the fungus was cultured for 2 days. Then the buffer was replaced with fresh phosphate buffer with or without the test materials (10 μg/test tube), and the fungus was cultured for 4 days. The converted products were extracted from the culture filtrate (with or without acidifying) using EtOAc. The products were detected by HPLC analysis [COSMOSIL $5C_{18}$-AR (4.6 i.d. x 150 mm), MeOH–H_2O–CH_3CN–AcOH (50 : 40 : 10 : 0.5), 0.5 mL/min, 270 nm].

Preparation of cytosol fraction.

The fungus was grown in a 500-mL conical flask containing liquid medium (100 mL

x 10) composed of sucrose (50 g/L), peptone (3 g/L), the extract from 100 g/L on malt, L-methionine (0.3 g/L), and water, without shaking at 24°C for 8 days. Subsequent all 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 used as the buffer, unless otherwise stated. The mycelial mats obtained after filtering the culture broth were washed with water (x 3) and the buffer (x 1). The washed mycelia (69 g, wet weight) were ground with sea sand (24 g) and the buffer (140 mL) in a mortar and pestle. The homogenate was centrifuged at 800 x *g* for 10 min and then at 12,000 x *g* for 15 min to give the cell-free extract. The extract was centrifuged at 105,000 x *g* for 90 min, and the resulting supernatant was obtained as the cytosol fraction. The cytosol fraction, to which glycerol (final concentration, 25%) was added, was stored at –80°C until use. Protein content (1.1 mg/mL) was estimated by a method of Bradford [24] with bovine serum albumin as the standard.

Enzyme assay.

The reaction mixture (total 50 μL) in a microtube (1.5 mL) consisted of the buffer (33 μL), 1.5 mM test material (2 μL in MeOH), 20 mM coenzyme (5 μL in the buffer), and the cytosol fraction (10 μL). NADH, NADPH, NAD^+, or $NADP^+$ was used as the coenzyme. The reaction mixture was incubated at 30°C for 30 min with shaking (100 rpm), and the products were extracted with water-saturated EtOAc (100 μL x 3). The reaction products were detected by HPLC analysis [COSMOSIL $5C_{18}$-AR (4.6 i.d. x 150 mm), MeOH–H_2O–CH_3CN–AcOH (50 : 40 : 10 : 0.5), 0.5 mL/min, 270 nm].

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CHAPTER 5

Conclusion

Neocosmospora vasinfecta E. F. Smith is a pathogen which causes root- and fruit-rot and seedling damping-off in the Malvaceae, Leguminosae, Piperaceae, Cucurbitaceae, etc., species that include pepper, groundnuts, soybean, beans, coconuts, *Albizzia*, *Crotalaria* spp. Neovasinin has been isolated from the culture filtrate of a strain (NHL2298) of this fungus and is a new type of fungal metabolite with a unique bicyclic unit, 2*H*,5*H*-pyrano[4,3-*b*]pyran-2-one. The purpose of this study was to determine the biosynthetic pathway of neovasinin, in particular the bicyclic unit formation mechanism.

First, the isolation of metabolites biogenetically related to neovasinin was attempted. Neovasipyrones A and B, neovasifuranones A and B, and vasinfectins A and B were isolated from the culture filtrate of this fungus. Their structures were clarified by spectroscopic methods, X-ray analysis, and chemical reactions. The neovasipyrones and neovasinin have the same carbon skeleton. Neovasipyrones, neovasifuranones, and vasinfectins have the same side chain with the same stereochemistry of asymmetric carbons. The stereochemistries of the asymmetric carbons in these metabolites are consistent with the stereochemistry of neovasinin. These findings suggest that neovasinin and these isolated metabolites are biosynthesized from a common intermediate. Neovasipyridones A–F were isolated from mycelial mats of *N. vasinfecta* NHL2298, and their structures were determined by spectroscopic methods. The neovasipyridones and neovasinin have the same side chain, the neovasipyridones each having a different *N*-alkyl group. These findings suggest that neovasinin and these metabolites are formed from a common intermediate; an aldehyde.

To confirm the polyketide origin of neovasinin, incorporation experiments with ^{13}C-labeled acetate and ^{13}C-labeled methionine of neovasinin next were done. The findings indicate that neovasinin is biosynthesized from a linear hexaketide plus five C_1 units. The data from the incorporation experiments done with the ^{13}C-labeled methionine of the neovasipyrones and neovasifuranones suggest that they also are derived from a linear

hexaketide plus five C_1 units and that in the case of neovasifuranones the hexaketide is subject to further decarboxylation.

Finally, incorporation experiment using [*S*-$^{13}C^{2}H_3$]-L-methionine of neovasinin, neovasipyrones, and neovasifuranones was done to examine the oxidation level of the intermediate. The C_1 unit carbons of these metabolites that correspond to the C-14 in neovasinin retained only one deuterium atom through the biosynthetic pathway. These findings suggest that the common intermediate of these metabolites is an aldehyde.

This possibility was examined using the replacement culture method and enzymatic conversion. In those experiments, the neovasifuranone A aldehyde specifically was converted to neovasifuranone A, but the reverse reaction did not occur. These results are a strong indication of the existence of a common aldehyde intermediate. The intermediate that is converted directly to neovasinin, however, has yet to be obtained.

Investigations that require for a deeper understanding of neovasinin biosynthesis are
(i) Determination of the stereochemistries of all the asymmetric carbons in the neovasifuranones and neovasipyridones.
(ii) Obtaining the hypothetical intermediates, such as the neovasifuranone A aldehyde, by synthesis, isolation, and enzyme inhibition.
(iii) Conversion of the hypothetical intermediates *in vivo* and *in vitro*.

The studies reported here contribute to our understanding of the biosynthesis of neovasinin-related metabolites and various secondary metabolites. Furthermore, *N. vasinfecta* can be used to study the regulation of secondary metabolism as related to pathogenesis.

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Summary

Neocosmospora vasinfecta E. F. Smith is a pathogen which causes root- and fruit-rot and seedling damping-off in the Malvaceae, Leguminosae, Piperaceae, Cucurbitaceae, etc. Neovasinin has been isolated from a strain (NHL2298) of *N. vasinfecta* and is phytotoxic to soybean, a host plant of this fungus. Its structure have been deduced by spectroscopic methods, X-ray analysis, and degradation reaction. Neovasinin has a unique bicyclic unit, 2*H*,5*H*-pyrano[4,3-*b*]pyran-2-one and probably is biosynthesized from a hexaketide plus five C_1 units. The purpose of this study was to determine the biosynthetic pathway of neovasinin, in particular the bicyclic unit formation mechanism.

First, the isolation of metabolites biogenetically related to neovasinin was attempted. Neovasipyrones A and B, neovasifuranones A and B, and vasinfectins A and B were isolated from the culture filtrate of this fungus. Their structures were clarified by spectroscopic methods. The stereochemistry of the neovasipyrones were determined by X-ray analysis and oxidation and degradation reaction. This results indicated that neovasipyrones are diastereomer of each other. The stereochemistries of neovasifuranones and vasinfectins were determined by oxidation and degradation reaction, and these compounds also were diastereomer of each other.

Neovasipyridones A–F were isolated from the mycelial mats of this fungus. Their structures were determined by spectroscopic methods. Neovasipyridones have each different *N*-alkyl group.

Neovasinin and neovasipyrones have the same carbon skeleton, and the stereochemistries of asymmetric carbons in neovasipyrones are consistent with the stereochemistry of neovasinin. Neovasipyrones and neovasifuranones have different ring system, but they have the same side chain. Except for the *N*-alkyl groupsC_{16} carbon networks of neovasipyridones is agreement with the networks of neovasifuranones. Moreover, the variety of *N*-alkyl groups suggests that a Schiff base would be formed with

alkyl amines and an aldehyde group in the biosynthetic pathway of the neovasipyridones. These features suggest that these metabolites have the same biosynthetic origin, probably biosynthesis from the common aldehyde intermediate. To explore this hypothesis, incorporation experiments with isotope-labeled acetate and methionine were done. The incorporation pattern of labeled precursors into neovasinin indicated that neovasinin is biosynthesized from a linear hexaketide plus five C_1 units. In the incorporation experiments of neovasipyrones and neovasifuranones with ^{13}C-labeled methionine, five of C_1 unit were introduced. The labeling patterns of these metabolites suggested that neovasipyrones and neovasifuranones are derived from a hexaketide and that neovasifuranones lose one carbon from the hexaketide chain. In the incorporation experiment of neovasinin with $^{13}C^{2}H_3$-labeled methionine, the C_1 unit carbon, C-14, in neovasinin retained only one deuterium atom. The same results were observed with neovasipyrones and neovasifuranones. These findings indicate that the C1 unit carbons are oxidized to aldehyde in the step of biosynthesis. This possibility was examined using the replacement culture method and enzymatic conversion. In these experiments, neovasifuranone A aldehyde specifically was converted to neovasifuranone A, but the reverse reaction did not occur. These results are a strong indication of the existence of a common aldehyde intermediate.

摘　要

Neocosmospora vasinfecta E. F. Smith はネムノキの苗立ち枯れ病，ダイズの株枯れ病などを起こす多犯性の植物病原菌である．*N. vasinfecta* NHL2298 株からは宿主植物であるダイズに対して植物毒性を示す neovasinin が単離され，これまでに各種機器分析，X 線結晶構造解析および分解反応により，不斉炭素の立体配置を含めた全構造が明らかにされている．Neovasinin は独特の二環式構造（2*H*,5*H*-pyrano[4,3-*b*]pyran-2-one）を有しており，その構造からポリケチド由来の代謝産物であることが示唆された．本研究は NHL2298 株における neovasinin の生合成経路，特に二環式構造の形成様式の解明を目的として行われた．

まず，NHL2298 株の生産する代謝産物中に neovasinin 生合成関連化合物の検索を行い，培養濾液から新規化合物である neovasipyrones A，B，neovasifuranones A，B，vasinfectins A, B を単離した．各種機器分析により構造解析を行った結果，これら化合物は環構造は異なっているが同一の側鎖構造を有していることが示された．さらに，neovasipyrone B の X 線結晶構造解析，ならびに neovasipyrone 類の酸化反応や分解反応を行い，neovasipyrone 類の全ての不斉炭素の立体配置を決定した．これらの結果から，neovasipyrone 類は互いにジアステレオマーの関係にあることが明らかとなった．また，neovasifuranone 類の酸化反応により，これらも互いにジアステレオマーの関係にあることが示された．Neovasipyrone 類の分解反応生成物との比較により，neovasifuranone 類の側鎖部分の立体構造も決定された．Vasinfectin 類についても同様の方法により，立体構造が決定された．

次に，菌体から新規化合物である neovasipyridones A–F を単離し，各種機器分析によりそれらの構造解析を行った．その結果，これら化合物は *N*–アルキル基のみが異なる化合物であることが示された．

Neovasinin と neovasipyrone 類は同一の炭素骨格を持ち，対応する不斉炭素の立体配置も同一である．また，neovasipyrone 類と neovasifuranone 類は環構造は異なっている

が同一の側鎖構造を有している．さらに，neovasipyridone 類の *N*-アルキル基以外の構造は neovasifuranone 類と同一の C_{16} 単位を持ち，またそれぞれ異なる *N*-アルキル基の存在から，生成段階においてアルキルアミンとアルデヒド基との間にシッフ塩基の形成が起こっていることが予想された．これらの事から，neovasinin，neovasipyrone 類，neovasifuranone 類および neovasipyridone 類は同一の生合成起源を有し，共通の生合成中間体としてアルデヒド化合物の存在が示唆された．この仮説を証明するために，安定同位体標識酢酸ナトリウムとメチオニンを用いた投与実験を行った．得られた標識パターンから，neovasinin は直鎖のヘキサケチド鎖と５つの C_1 ユニットから生合成されるポリケチドであることが確認された．また，neovasipyrone 類と neovasifuranone 類にはそれぞれ５つの C_1 ユニットが取り込まれ，それらの位置は neovasinin での位置と対応していた．この結果より，これら化合物もヘキサケチドであり，neovasifuranone 類の生成段階には脱炭酸反応が存在することが示唆された．さらに，$^{13}C^{2}H_3$ 標識メチオニンの投与実験を行い，neovasinin の二環式構造の形成にとって重要と考えられる C_1 ユニット由来のメチレン炭素には１つの重水素しか保持されていないことを明らかにした．同様に，neovasipyrone 類と neovasifuranone 類においても対応するメチレン炭素には１つの重水素しか保持されていないことがわかった．従って，これら化合物の生合成経路のいずれかの段階でその炭素はアルデヒドまで酸化されることが示された．次に，neovasifuranone A から対応するアルデヒド化合物を合成し，置換培養と粗酵素による変換実験を行った．その結果，そのアルデヒド化合物は選択的に neovasifuranone A に変換されたが，その逆反応は起こらなかった．以上の結果から，neovasinin をはじめとするこれら代謝産物は共通の生合成中間体から生合成され，その中間体はアルデヒド化合物であることが示された．

List of Publications

Chapter 2 is originally described in

Furumoto, T., Fukuyama, K., Hamasaki, T. and Nakajima, H. (1995) Neovasipyrones and neovasifuranones: four new metabolites related to neovasinin, a phytotoxin of the fungus, *Neocosmospora vasinfecta*. *Phytochemistry* **40**, 745-751.

Furumoto, T., Hamasaki, T. and Nakajima, H. (1997) Vasinfectins A and B: new phytotoxins from *Neocosmospora vasinfecta*. *Tetrahedron Lett.* **38**, 5523-5524.

Chapter 3 is originally described in

Nakajima, H., Shimomura, K., Furumoto, T. and Hamasaki, T. (1995) Neovasipyridones A, B and C: metabolites related to neovasinin, a phytotoxin of the fungus, *Neocosmospora vasinfecta*. *Phytochemistry* **40**, 1643-1647.

Nakajima, H., Shimomura, K., Furumoto, T. and Hamasaki, T. (1996) Neovasipyridones related to neovasinin, a phytotoxin of the fungus *Neocosmospora*. *Phytochemistry* **43**, 1015-1017.