Hypomycetin – an Antifungal, Tetracyclic Metabolite from *Hypomyces aurantius*: Production, Structure and Biosynthesis

Jens Breinholt,[a,*] Georg W. Jensen,[a] Anders Kjær,[b] Carl Erik Olsen[c] and Connie N. Rosendahl[a]

[a]Novo Nordisk A/S, DK-2880, Bagsværd, Denmark, [b]Department of Organic Chemistry, The Technical University of Denmark, DK-2800 Lyngby, Denmark and [c]Chemistry Department, Royal Veterinary and Agricultural University, DK-1871 Frederiksberg C, Denmark

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As part of a screening programme a new antifungal substance, hypomycetin, has been isolated from the mycophilic fungus *Hypomyces aurantius*. Its tetracyclic structure, including the absolute configuration, has been established by spectroscopic methods and CD measurements. The biosynthetic pathway to hypomycetin has been unveiled by feeding experiments with ^{13}C-labelled precursors, followed by extensive NMR analyses. The extent of its relationship to viridicatumtoxin, a known, structurally similar fungal metabolite, and to the tetracyclic *Streptomyces* antibiotics, such as tetracycline and various congeners, is discussed in terms of biosynthetic origins. A polyketide folding mode, different from that involved in the biosynthesis of the tetracyclines, has been documented as the starting point on the path to hypomycetin.

During a search for antifungal metabolites with growth inhibitory activity against phytopathogenic fungi our attention was drawn to *Hypomyces aurantius*, a commonly encountered species of the mycophilic *Hypomyces* genus. Solvent extracts of mycelia and culture filtrates from laboratory scale fermentations of *H. aurantius* (deposited at the Centraalbureau voor Schimmelculturen (CBS), Holland, under the designation CBS 654.93) were purified by chromatographic fractionation to yield a crystalline, yellow metabolite, named *hypomycetin*, inhibiting the growth of various phytopathogens. We report the isolation, structure elucidation, absolute stereochemistry, and biosynthetic derivation of hypomycetin and discuss its relationship to viridicatumtoxin and the tetracyclines, all structurally related, antibiotic compounds.

Isolation, properties and structure elucidation. After six days of fermentation (see Experimental) the cultures were filtered. The combined ethyl acetate extracts of mycelia and acidified filtrates were dried, the solvent was removed, and a methanol solution of the residue was subjected to purification by reversed-phase MPLC, monitored by UV-absorbtion at 225 nm, to give hypomycetin

(about 40 mg per litre of culture broth) as yellow crystals possessing the elementary composition $C_{26}H_{26}O_{10}$, established by elemental analysis and HR-MS (EI).

According to ^{13}C NMR spectroscopy, including multiplicity analysis (DEPT), 16 of the 26 individual carbon signals were attributable to quaternary carbon atoms. The ^{1}H NMR spectrum revealed the presence in hypomycetin of four three-proton signals supplemented by fourteen individual one-proton singlets, or multiplets, six of which were exchangeable, i.e., derived from oxygen-bound protons. Other directly recognizable structural elements, confirmed by ^{1}H–^{1}H COSY, were a dimethylallyl grouping, two *meta*-positioned aromatic methines and one carbinol–methine, a geminally coupled methylene, a methoxy group, and a methyl group attached to an α,β-unsaturated keto group, diagnosed by the lack of absorption at frequencies higher than $1680\ \text{cm}^{-1}$ within the double bond region of the IR-spectrum of hypomycetin. The observed ^{13}C and ^{1}H signals were supplemented by direct (HMQC) and long-range (HMBC) ^{1}H-detected heteronuclear shift correlation spectroscopy in order to assign the proton-bearing carbon atoms and establish a series of long-range ^{1}H–^{13}C correlations, respectively. The combined spectroscopic data provided satisfactory evidence for hypomycetin possessing the tetracyclic structure **1**.
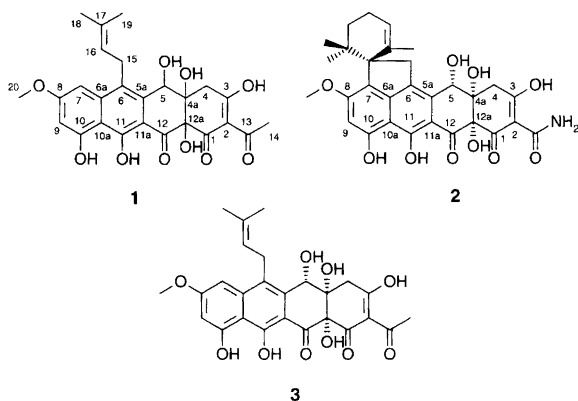
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*To whom correspondence should be addressed.

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Fig. 1. Selected ^{1}H,^{13}C long-range correlations observed in the HMBC-spectrum ($J_{CH} = 6$ Hz) of hypomycetin.

The assigned ^{13}C and ^{1}H NMR signals are listed in Table 1 and the established long-range ^{1}H,^{13}C correlations portrayed in Fig. 1. Structurewise, hypomycetin (1) has several features in common with viridicatumtoxin, a bright-yellow mycotoxin isolated from *Penicillium viridicatum*[1] and possessing the structure and absolute configuration 2, both deduced by single crystal X-ray

Table 1. ^{1}H and ^{13}C NMR data (CDCl$_3$) of hypomycetin (3). For comparison, relevant ^{13}C NMR data of viridicatumtoxin (2) are listed in the column Vtox. Numbering as indicated in formulae 1 and 2.

Carbon No.	^{1}H	^{13}C	Vtox[a]
1		189.8	190.5
2		110.4	99.6
3		194.3	192.9
4	2.76 (1 H, d, J = 19 Hz)	40.5	40.4
	2.88 (1 H, d, J = 19 Hz)		
4a		70.9	71.5
5	4.73 (1 H, s(br))	71.0	71.7
5a		130.7	123.9
6		130.3	137.1
6a		140.6	147.2
7	6.86 (1 H, d, J = 2.3 Hz)	99.8	122.7
8		164.5	160.8
9	6.62 (1 H, d, J = 2.3 Hz)	102.0	99.9
10		160.7	158.0
10a		108.5	105.5
11		166.7	166.0
11a		104.1	105.1
12		195.8	195.2
12a		79.8	80.2
13		202.3	
14	2.73 (3 H, s)	27.7	
15	3.87 (1 H, dd, J = 7/16 Hz)	27.2	
	3.60 (1 H, dd, J = 5/16 Hz)		
16	5.04 (1 H, m)	122.1	
17		133.2	
18	1.74 (3 H, d, J = 1 Hz)	18.3	
19	1.90 (3 H, brs)	25.6	
20	3.92 (3 H, s)	55.6	
OH's	18.1 (1 H, s), 15.7 (1 H, s),		
	9.94 (1 H, s), 5.46 (1 H, s)		
	4.09 (1 H, brs), 3.29 (1 H, brs)		

[a]All signals listed in this column are identical with those reported.[4]

analysis.[2,3] A crystalline metabolite, isolated, quite fortuitously, in our laboratory from an unidentified (sterile) fungal strain was unequivocally identified as viridicatumtoxin (2). Its ^{13}C signals, identical with those previously reported,[4] are listed in Table 1 and underscore the overall similarity of hypomycetin and viridicatumtoxin, with marked and predictable deviations notable solely in the ^{13}C NMR signals arising from carbon atoms differing in their substitution patterns in the two metabolites.

Attention was thereafter directed towards determination of the stereochemistry of hypomycetin.

Stereochemistry. Our access to viridicatumtoxin (2) enabled us to compare its CD curve with that of hypomycetin (1). The UV spectra (in acetonitrile) of the two metabolites were strikingly similar as were their CD curves, recorded in the same solvent within the wavelength range 220–600 nm, as regards both wavelength positions, signs, and relative rotational strength of the CD extremes: slightly negative at 415–430 nm, very strongly positive at about 275 nm, and strongly negative at ca. 240 nm. The pronounced CD curve resemblance is accepted as evidence that formula 3 represents the structure of hypomycetin including its absolute configuration.

Biosynthesis. The partially hydrogenated naphthacene structures of viridicatumtoxin (2) and hypomycetin (3), produced by species of the fungal genera *Penicillium* and *Hypomyces*, respectively, suggest a less restricted biological distribution of such structural types than was generally assumed. Various species of the *Streptomyces* genus were long regarded as typical and exclusive sources of such tetracyclic antibiotics as tetracycline and closely related congeners, the biosyntheses of which, from polyketide precursors, have been studied in great detail. Surprisingly, the *in vivo* pathway to viridicatumtoxin (2) in *P. expansum* (*syn. P. viridicatum*), though unsettled with regard to a single carbon atom, clearly implies a folding of the polyketide chain different from that prevalent in the *Streptomyces* species on the path to tetracycline and its congeners.[4] This fact prompted us to subject the biosynthetic derivation of hypomycetin in *H. aurantius* to closer scrutiny.

Shake-flask cultures of the fungus were grown in the presence of variously labelled precursors as outlined in Table 2. The hypomycetin specimens were isolated after six days of growth, purified by HPLC, and subjected to ^{13}C NMR analysis. The results of the feeding experiments are presented in Table 3.

Table 2. Feeding experiments with *H. aurantius,* conducted within a total fermentation time of 144 h. The pH-values at harvest time varied between 7.3 and 8.0.

Expt. No.	Precursor	Amount[a]	Yield[b]
1	Sodium [1-¹³C]acetate	150	1.5
2	Sodium [1-¹³C]acetate	250	2.4
3	Sodium [2-¹³C]acetate	150	1.0
4[c]	Sodium [2-¹³C]acetate	250	—
5	Sodium [1,2-¹³C₂]acetate	150	2.3
6	Sodium [1,2-¹³C₂]acetate	250	4.3
7	(2S)-[Me-¹³C]methionine	150	0.9
8[c]	(2S)-[Me-¹³C]methionine	250	—
9	Sodium acetate	150	3.5
10	—	—	6.5

[a]Total number of milligrams; in experiments Nos. 1,3,5,7, and 9: added in one portion after 66 h; in experiments Nos. 2,4,6, and 8: added in three equal portions after 24, 47 and 74 h. [b]Amount (in milligrams) of hypomycetin (3) isolated after HPLC. [c]Discarded because of bacterial infection.

Based on the assignments listed in Table 1 the enhancement patterns presented in Table 3 provided convincing evidence that all carbon atoms of hypomycetin (3), save for C-20, are derived from acetate units. Feeding (2S)-[Me-¹³C]methionine to the *H. aurantius* culture resulted in an almost thirtyfold enhancement of the C-20 signal as estimated by comparing the intensities of the center

line with the ¹³C-satellite signals of the methoxy group in the ¹H NMR spectrum of hypomycetin.

The 2D-¹³C,¹³C-INADEQUATE-spectrum (J_{CC} = 55 Hz) of the [1,2-¹³C₂]acetate-derived hypomycetin (from the combined experiments Nos. 5 and 6, Table 2) unequivocally demonstrated the incorporation of twelve intact C₂-units into hypomycetin (Table 3). Because of the high enrichment some of the doublets were accompanied by low intensity satellite peaks arising from less frequent incorporation of adjacent [1,2-¹³C₂]acetate units.

The established labelling and coupling patterns discussed above are intelligible only in terms of a biosynthetic derivation of hypomycetin from a decaketide chain with a folding mode as shown in Scheme 1.

Introduction of an acetate-derived dimethylallyl-grouping into an activated methylene chain link, as well as selective reduction of a single oxo-group to hydroxy, are unexceptional biosynthetic reactions, most likely occurring prior to stabilization of the folded chain. Subsequent steps on the path to hypomycetin (3) are few and simple, *viz.,* straightforward oxidations and a standard methionine-mediated anisole formation (Scheme 1).

Formally, hypomycetin (3) resembles the tetracyclic antibiotics, produced by *Streptomyces* species and known generically as tetracyclines. Prominent members of the

Table 3. ¹³C NMR data for hypomycetin (3) derived from sodium [1-¹³C]- and [2-¹³C]-acetate.

| Carbon No.[a]	δ¹³C	Enrichment factor[b] [1-¹³C]acetate[d]	Enrichment factor[b] [2-¹³C]acetate[e]	J_{CC}/Hz[c] [1,2-¹³C₂]acetate
1	189.8	4/9		42
2	110.4		3	59
3	194.3	6/13		59
4	40.5		5	41
4a	70.9	7/14		nd
5	71.0		7	nd
5a	130.3	6/12		nd
6	130.7		7	nd
6a	140.6	8/12		55
7	99.8		6	69
8	164.5	4/8		69
9	102.0		6	74
10	160.7	3/6		74
10a	108.5		3	64
11	166.7	7/11		66
11a	104.1		3	58
12	195.8	5/12		58
12a	79.8		2	nd
13	202.3	6/12		43
14	27.7		5	43
15	27.2	10/16		nd
16	122.1		4	74
17	133.2	12/16		42
18	18.3		5	42
19	25.6		9	—
20	55.6			—

[a]Numbering according to formula 1. [b]Relative to natural abundance spectrum normalized to the signal for C-20 (55.6 ppm). [c]nd: not determined. [d]Experiments Nos.1/2 (Table 2). [e] Experiment No. 3 (Table 2).
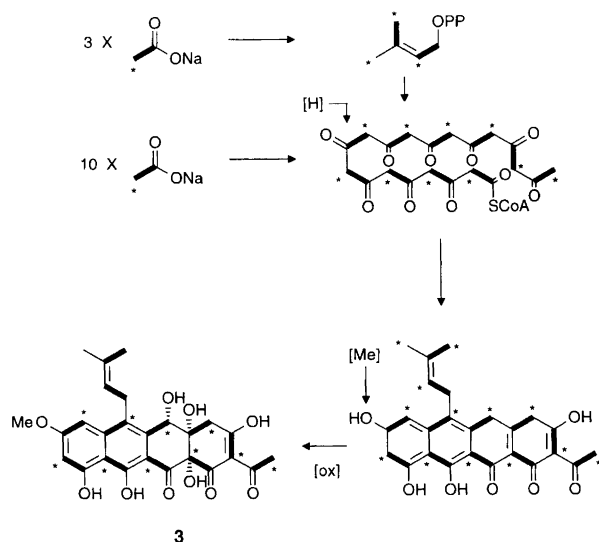
Scheme. 1. Proposed biosynthetic pathway from acetate to hypomycetin (3). Solid bars signify intact acetate units, asterisks carbon atoms deriving from acetate C-2 carbons, and Me a methyl group donated from methionine.

class are tetracycline (4), 7-chlorotetracycline (5), and oxytetracycline (6), all sharing with viridicatumtoxin (2) the presence of a carboxamido group at the 2-position of the tetracyclic ring systems. In contrast, an acetyl group is found at C-2 in hypomycetin (3), a feature recurring in the 2-acetyl-2-decarboxamido analogues of tetracycline (7),[5] 7-chlorotetracycline (8),[5] and oxytetracycline (9),[6] all recognized as minor congeners of the major carboxamido products and seemingly produced via a biosynthetic pathway corresponding to that of the latter products.[7]

The remarkable experimental observation that hypomycetin (3) (Scheme 1) and 2-acetyl-2-decarboxamido-oxytetracycline (9)[7] (Scheme 2) are derived biosynthetically from identical, but differently folded decaketide chains, may well reflect a more general biological divergence between the *Streptomyces* and *Hypomyces* genera, in accordance with their taxonomical remoteness. Though still unsettled,[4,7] the biosynthetic derivation
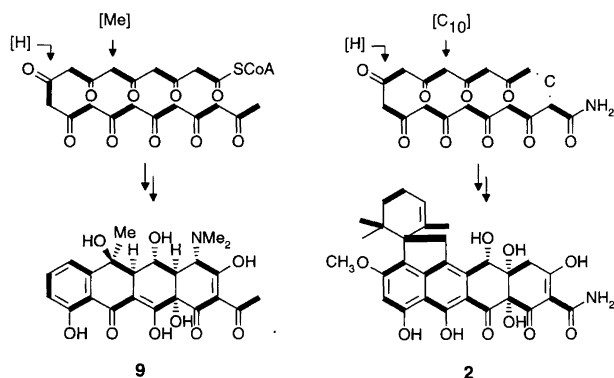
Scheme. 2. Proposed biosynthetic pathways from acetate to 2-acetyl-2-decarboxamidooxytetracycline (9) and viridicatum-toxin (2). Solid bars signify intact acetate units.

of viridicatumtoxin (2) in *Penicillium expansum* (*P. viridicatum*) (Scheme 2) implies that the folding mode of the polyketide chain is certainly different from that prevalent in the *Streptomyces*-produced tetracyclic antibiotics. Resemblance to hypomycetin folding (Scheme 1), however, is evident.
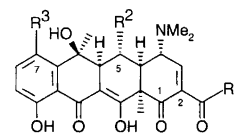
4: R¹=NH₂; R²=R³=H

5: R¹=NH₂; R²=H; R³=Cl

6: R¹=NH₂; R²=OH; R³=H

7: R¹=Me; R²=R³=H

8: R¹=Me; R²=H; R³=Cl

9: R¹=Me; R²=OH; R³=H

Discussion

When tested *in vitro* hypomycetin (3) displayed growth inhibitory activity towards a wide range of phytopathogenic fungi, including *Aphanomyces euteiches*, *Phytophthora cryptogea*, *Pythium ultimum*, *Pythium* Type F (Oomycetes), *Colletotrichum graminicola*, *Gaumannomyces graminis*, *Pyricularia oryzae*, *Alternaria alternata* (Ascomycetes) and *Sclerotium rolfsii* (Basidiomycetes). The potency of hypomycetin against the two *Pythium*-species *P. ultimum* and *P.* Type F (MIC 1.3 and 7.0 ppm, respectively) was similar to that of the commercial fungicide Captan (MIC 1.7 and 7.0 ppm, respectively). Compared with Captan, the growth inhibitory activities of hypomycetin against *Phytophthora cryptogea* and *Aphanomyces euteiches* were eight- and sixty-times weaker, respectively.

Literature reports on the chemistry of metabolites of species of the widely distributed, mycoparasitic *Hypomyces* genus are scant and inconclusive. Siderova *et al.*[8] reported the production of unidentified, antifungal substances in various *Hypomyces* species, including *H. aurantius*. UV data, the sole reported characteristics, preclude identification as hypomycetin of any of these metabolites. Later, Briggs *et al.*[9] described the isolation from *H. aurantius* of mannitol, ergosterol, and a small amount (0.01%) of a red, unidentified substance possessing physical data (m.p., UV, IR) quite different from those of hypomycetin. Another species, *H. rosellus*, produced aurofusarin, a well known quinone, again structurally unrelated to hypomycetin. Finally, attempts by Kellock and Dix[10] at identifying a diffusable, non-specific toxin, produced by *H. aurantius*, remain inconclusive and non-supportive as regards its identification as hypomycetin.

Experimental

CD curves were recorded on a JASCO 720 spectropolarimeter. NMR data (for samples in $CDCl_3$) were acquired on a Bruker AC300P spectrometer; chemical shifts (δ-values) were measured relative to residual solvent peaks at 7.27 ppm (^1H) and 77.0 ppm (^{13}C). The high-resolution mass spectrum was obtained at 70 eV ionization potential on a JEOL AX505W instrument and is presented as m/z (% rel. int.).

Antifungal activity. Antifungal activity was assessed by means of agar diffusion assays as described previously.[11] Minimum inhibitory concentration (MIC) values for hypomycetin and the commercial fungicide Captan against oomycetous fungi were estimated by plotting areas (mm^2) of inhibition zones versus concentration (ppm) of test compound and recording the intercept between the regression lines and the x-axis.

Fermentation. Hypomyces aurantius (CBS 654.93) was cultivated on agar slants (Bacto™ (Difco) 12 ml/slant) at 25 °C for seven days, at the end of which profuse growth and sporulation were noticeable. Each slope was washed with sterile, distilled water (10 ml) containing 0.1% of Tween 20. The resulting spore-mycelium suspension (1 ml) was used to inoculate an Erlenenmeyer flask, with 2 baffles, containing PCS-medium (100 ml) prepared as follows. Pharmamedia (10 g), Rofec (5 g), glucose (30 g), and KH_2PO_4 (3 g) were mixed and supplemented with distilled water to a total volume of 1 l. The pH-value was adjusted to 7.0 and the medium was autoclaved (121 °C, 40 min). The fermentation took place with shaking (150 rpm) at 25 °C for six days.

A profile of the metabolite concentration as function of time was constructed in the following way. Acidified (1 M HCl, pH 3) whole-broth was stirred with one volume of EtOAc for 1 h. After phase separation (centrifugation) the EtOAc extract (1 ml) was evaporated, the residue was dissolved in MeOH (100 µl), and an aliquot (2 µl) was analyzed by HPLC as follows. SpheriSorb ODS 1 (5 µm), 60 × 4 mm, flowrate 2.5 ml min⁻¹, gradient elution: 25% MeCN (0.15% TFA), increasing to 100% MeCN (0.15% TFA) in the course of 5 min, detection by UV absorption at 280 and 420 nm; t_R(hypomycetin) 3.5 min. Hypomycetin production was found to set in between days 3 and 4, and levelled out after day 6.

In the feeding experiments (Table 2), the ^{13}C-labelled precursors were dissolved in water (1–2 ml) and administered to the shake-flask cultures via syringes and sterile filters. The fermentations were harvested 144 h after inoculation and processed as outlined above.

Isolation and purification of hypomycetin. In the experiments involving ^{13}C-labelled precursors, the contents of a single shake-flask were filtered (glass fiber) and the mycelium extracted by vigorous stirring for 1 h with two consecutive portions (100 ml) of EtOAc. The cleared

broth, following acidification (1 M HCl, pH 3), was extracted three times with 100-ml portions of EtOAc. The combined organic phases were freed of water (by freezing and filtration) and evaporated. The crude extract from each experiment was dissolved in MeOH (850 µl), centrifuged, and applied to a 20 × 250 mm Dupont ODS (10 µm) column. Elution, at a flow rate of 15 ml min⁻¹, was performed with a linear gradient of 25% MeCN (0.15% TFA), increasing to 100% MeCN (0.15% TFA) in the course of 45 min. Collection of fractions (0.5 min per fraction) was begun 20 min after injection, and high-purity hypomycetin was collected in fractions Nos. 16 and 17 (t_R 28 min). The combined fractions were evaporated *in vacuo* and the residue was dissolved in $CDCl_3$ and subjected to ^{13}C NMR analysis, the results of which are presented in Table 3.

Larger quantities of non-labelled hypomycetin were procured by subjecting an EtOAc-extract, produced as described above, to reversed-phase MPLC on a LiChroprep RP18 column (26 × 230 mm, 15–25 µm), that was eluted at a flow rate of 15 ml min⁻¹ with a linear gradient of aqueous MeOH, ranging from 60% to 100% MeOH (0.05% TFA), within 60 min. The hypomycetin-containing fractions (t_R 50 min), identified by UV detection at 225 nm, were combined and evaporated to dryness, leaving yellow crystals in a yield of about 40 mg per litre of culture broth. Recrystallization from EtOAc afforded homogeneous hypomycetin, m.p. 212–216 °C (decomp.), $[\alpha]_D^{21} + 116°$ (c 0.2, MeOH). Anal. $C_{26}H_{26}O_{10}$: C,H. EI-MS: 498 (80, M^+), 480 (10, $M - H_2O$), 424 (25), 396 (16), 379 (13), 340 (21), 327 (11), 316 (48), 314 (21), 312 (19), 311 (17), 296 (100), 281 (36), 254 (10), 185 (16), 169 (10), and 129 (10). HR-EIMS: M^+: found 498.1560, calc. for $C_{26}H_{26}O_{10}$ 498.1526. IR (KBr): 2975, 2925, 2850, 1682, 1630, 1578, 1522, 1400, 1170, and 995 cm⁻¹. UV (MeCN) (log ε): 235 (4.50), 279 (4.61), 327 (3.78), 340sh, 417 (4.11).

^1H and ^{13}C NMR data: see Table 1.

References

1. Hutchinson, R. D., Steyn, P. S. and van Rensburg, S. J. *Toxicol. Appl. Pharmacol. 24* (1973) 507.
2. Kabuto, C., Silverton, J. V., Akiyama, T., Sankawa, U., Hutchinson, R. D., Steyn, P. S. and Vleggaar, R. *J. Chem. Soc., Chem. Commun.* (1976) 728.
3. Silverton, J. V., Kabuto, C. and Akiyama, T. *Acta Crystallogr., Sect. B 38* (1982) 3032.
4. de Jesus, A. E., Hull, W. E., Steyn, P. S., van Heerden, F. R. and Vleggaar, R. *J. Chem. Soc., Chem. Commun.* (1982) 902.

5. Miller, M. W. and Hochstein, F. A. *J. Org. Chem. 27* (1962) 2525.
6. Hochstein, F. A., von Wittenau, M. S., Tanner, F. W. Jr. and Murai, K. *J. Am. Chem. Soc. 82* (1960) 5934.
7. Thomas, R. and Williams, D. J. *J. Chem. Soc., Chem. Commun.* (1983) 128.
8. Sidorova, I. I., Lerner, L. E. and Makarova, M. O. *Mikologiya y Fitopathologiya 11* (1977) 315.
9. Briggs, L. H., Cambie, R. C., Dean, I. C., Dromgoole, S. H., Fergus, B. J., Ingram, W. B., Lewis, K. G., Small,

C. W., Thomas, R. and Walker, D. A. *N. Z. J. Sci. 18* (1975) 565.
10. Kellock, L. and Dix, N. J. *Trans. Br. Mycol. Soc. 82* (1984) 327.
11. Berova, N., Breinholt, J., Jensen, G. W., Kjær, A., Lo, L.-C., Nakanishi, K., Nielsen, R. I., Olsen, C. E., Pedersen, C. and Stidsen, C. E. *Acta Chem. Scand. 48* (1994) 240.