3-HYDROXYTERPHENYLLIN, A NEW METABOLITE OF *ASPERGILLUS CANDIDUS*

STRUCTURE ELUCIDATION BY ^{1}H AND ^{13}C NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY*

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A new compound isolated from cultures of *Aspergillus candidus* LINK is shown to be 3-hydroxyterphenyllin. The structure was assigned by comparing the ^{1}H and ^{13}C nmr spectra of the metabolite and its acetate derivative with those of terphenyllin and terphenyllin triacetate.

p-Terphenyl derivatives are rare in microfungi. Volucrisporin,[1] produced by cultures of the Hyphomycete *Volucrispora aurantiaca* HASKINS, was the first to be isolated. *De novo* synthesis of this metabolite in the culture was demonstrated,[2] and evidence was presented for a biosynthetic pathway from phenylalanine.[3] Subsequently, terphenyllin (**1**) was isolated from cultures of *Aspergillus candidus*.[4] Its biosynthetic pathway is not known in detail but radioactivity is efficiently incorporated from ^{14}C-labelled phenylalanine.[5] TAKAHASHI *et al.* independently isolated **1** and several other metabolites including 4″-deoxyterphenyllin (**2**), and reported on the cytotoxicity of **1** and **2** to cultured HeLa cells.[6,7]

We report here the isolation of a new terphenyl metabolite, 3-hydroxyterphenyllin (**3**) from *A. candidus*, and the elucidation of its structure by ^{1}H and ^{13}C nmr studies.

Materials and Methods

Organism

Aspergillus candidus LINK strain CMI 16046 was obtained from the Commonwealth Mycological Institute, Kew Gardens, England.

Culture media

The inoculum medium contained (per liter) D-glucose, 50 g; corn steep liquor, 50 ml; and calcium carbonate, 2 g. The fermentation medium contained (per liter) D-glucose, 54.5 g; L-leucine, 6.56 g; KH_2PO_4, 0.45 g; $MgSO_4 \cdot 7H_2O$, 0.45 g; salt solution, 4.5 ml; trace mineral solution, 4.5 ml; and ferrous sulfate solution, 4.5 ml containing 0.2% (w/v) of $FeSO_4 \cdot 7H_2O$. The salt solution consisted of 1% (w/v) each of NaCl and $CaCl_2$; the trace mineral solution contained $CuSO_4 \cdot 5H_2O$, 39.3 mg; H_3BO_3, 5.7 mg; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 3.7 mg; $MnSO_4 \cdot 4H_2O$, 6.1 mg; and $ZnSO_4 \cdot 7H_2O$, 880 mg per liter.

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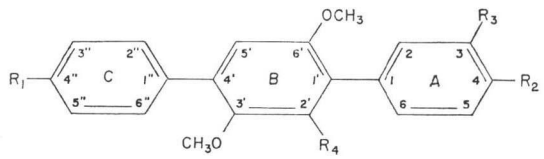
Cultivation

The mycelium was transferred from potato-dextrose-agar slants to 50 ml of inoculum medium in 250-ml Erlenmeyer flasks and incubated for 4 days at 25°C with shaking (220 rpm). This primary inoculum was homogenized for 15 seconds in a Waring blender and 2-ml portions were transferred to 50 ml of fresh inoculum medium. A secondary inoculum was grown under the same conditions as used for the primary inoculum. For the fermentation a 2-ml portion of the 2-day old secondary inoculum was transferred to each 50-ml of medium in a 250-ml Erlenmeyer flask. Cultures were grown for 12 days at 25°C with shaking (220 rpm).



1) $R_3 = H$; $R_1 = R_2 = R_4 = OH$
2) $R_1 = R_3 = H$; $R_2 = R_4 = OH$
3) $R_1 = R_2 = R_3 = R_4 = OH$
4) $R_3 = H$; $R_1 = R_2 = R_4 = OCOCH_3$
5) $R_1 = R_2 = R_3 = R_4 = OCOCH_3$

Isolation

Mycelium from 1 liter of culture was harvested by filtration and leached with hot acetone. The acetone was removed by evaporation *in vacuo*, and the residue was washed first with petroleum ether (b.p. 30~60°C) to remove lipids and then with chloroform to remove chlorflavonin and dechlorflavonin. The remainder of the residue was dissolved in hot acetone and filtered; the material which crystallized was purified by column chromatography on silicic acid using benzene - ethyl acetate mixtures as eluants. A 3: 1 mixture gave **1** (814 mg), m.p. 240~241°C (colourless prisms from acetone); M^+ at *m/e* 338.1134 (calcd for $C_{20}H_{18}O_5$: 338.11543); λ_{max} (95% EtOH) 225 and 275 nm (log ϵ 4.42 and 4.41); ν_{max} (KBr) 3380 cm^{-1} (s). The physical constants and spectral features are indistinguishable from those previously reported for **1**.[4] Decreasing the benzene-ethyl acetate ratio to 3: 2 gave **3** (91 mg), m.p. 221~222°C (colourless prisms from acetone); M^+ at *m/e* 354.1099 (calcd for $C_{20}H_{18}O_6$: 354.11034); λ_{max} (95% ethanol) 225 and 275 nm (log ϵ 4.43 and 4.31); ν_{max} (KBr) 3370 cm^{-1} (s).

Acetylation of 1 and 3

Acetic anhydride was added to pyridine solutions of **1** and **3**. After 20 hours at room temperature the reaction mixtures were evaporated to dryness *in vacuo*, the residues were dissolved in chloroform, and hexane was added to promote crystallization. **1** gave terphenyllin triacetate (**4**) as colourless needles, m.p. 156~157°C; M^+ at *m/e* 464; λ_{max} (95% ethanol) 228, 260 and 300 nm (log ϵ 4.24, 4.34 and 3.98); ν_{max} (KBr) 1700 cm^{-1} (s). **3** gave 3-hydroxyterphenyllin tetraacetate (**5**) as colourless needles, m.p. 153~154°C; M^+ at *m/e* 552; λ_{max} (95% ethanol) 228, 260 and 300 nm (log ϵ 4.33, 4.25 and 3.89); ν_{max} (KBr)1750 cm^{-1} (s).

Analytical Instruments

Accurate mass measurements were obtained with a Dupont/CEC model 21-110 B high resolution mass spectrometer. A Dupont/CEC model 21-491 mass spectrometer was used to determine the fragmentation of **4** and **5**. UV spectra were measured in 95% ethanol with a Unicam model SP 8000 recording spectrophotometer. Infrared spectra were obtained with a Perkin-Elmer model 521 grating infrared spectrometer using samples compressed in KBr pellets. ^1H nmr spectra were

recorded with a Varian model HA-100D NMR spectrometer equipped with a 12″ magnet, internal lock and multiple resonance capabilities; ^{13}C nmr spectra were obtained with a Varian model XL-100-15 high resolution NMR spectrometer.

Discussion

A comparison of the ultraviolet and infrared spectra of **1** and **3** (and their acetates **4** and **5**) indicated that their structures were not markedly different; accurate mass measurements established that **3** possessed a molecular formula of $C_{20}H_{18}O_6$, and thus had one oxygen atom more than **1**. Comparison of the ^1H nmr spectra of the compounds (Table 1) revealed that the penta substituted ring and one of the disubstituted rings of **1** were retained in **3**, and that one aromatic hydrogen in the second disubstituted ring of **1** was replaced by a hydroxyl group in **3**. Thus an AA′BB′ sub-spectrum arising from the hydrogens of a symmetrical 1,4-disubstituted ring in **1** was replaced by a typical ABC multiplet for three hydrogens in the spectrum of **3**. Spin-spin coupling between these hydrogens and chemical shift substituent effects[8] placed the additional hydroxyl group in **3** at the 3- or 3″-position. A choice between the alternatives was made from ^{13}C nmr evidence.

The ^{13}C nmr spectra (Tables 2 and 3) contained resonances for the numbers and types of carbons in structures **1**, **3**, **4** and **5**. Those due to aromatic carbons bonded to oxygen were, as expected,[10] at lowest field, and characteristic one-bond ^{13}C-H spin-spin coupling[10] in the high

Table 1. ^1H nuclear magnetic resonance data (100 MHz) for terphenyllin (**1**), 3-hydroxyterphenyllin (**3**), and the tri- and tetraacetate derivatives (**4**, **5**).*

Compound	H-2	H-6	H-3	H-5	H-5′	3′-OCH$_3$	6′-OCH$_3$
1	7.12(m)		6.77(m)		6.40(s)	3.32(s)	3.64(s)†
3	6.71(d)	6.57(dd)		6.73(d)	6.40(s)	3.33(s)	3.65(s)
4	7.33(m)		7.13(m)		6.82(s)	3.41(s)	3.72(s)
5					6.81(s)	3.40(s)	3.74(s)

	H-2″	H-6″	H-3″	H-5″	3-OH	4-OH	4″-OH
1	7.44(m)		6.85(m)			9.27(s)	9.48(s)
3	7.44(m)		6.85(m)		8.72(bs, 2H)		9.42(bs)
4	7.64(m)		7.17(m)				
5	7.63(m)		7.17(m)				

	2′-OH	3-O(CO)CH$_3$	4-O(CO)CH$_3$	4″-O(CO)CH$_3$	2′-O(CO)CH$_3$
1	8.46(s)				
3	8.41(bs)				
4			2.28(s), 2.30(s)		2.04(s)
5		2.28(s)	2.29(s)	2.32(s)	2.11(s)

* Spectra for compounds **1** and **3** were recorded (Varian HA-100D spectrometer) in dimethylsulfoxide-d_6, and for **4** and **5** in chloroform-d using tetramethylsilane as an internal standard. H-2″, H-6″, H-3″ and H-5″ formed an AA′BB′ system typical for a para-disubstituted benzene ring in all compounds. $N=J_{AB}+J_{AB'}=8.4\sim8.6$ Hz, with $J_{AB'}$, probably <0.5 Hz[9]. H-2, H-6, H-3 and H-5 formed a similar system in **1** and **4**. H-5, H-6, and H-2 formed an ABC system in compounds **3** and **5**. Analysis of this system for **3**, with the Varian spin simulation program on a 620L computer, gave J_{AB} 8.3, J_{BC} 1.8, J_{AC} <0.5. This system in **5** gave a much narrower complex multiplet ($\sim$14 Hz base, centered at δ 7.19) and was not analyzed.

† A small increase in peak height ($J<0.4$ Hz) was observed for this signal on irradiation of the signal for H-5′.

Table 2. ^{13}C nuclear magnetic resonance chemical shift data (δ_c TMS) for terphenyllin (**1**), 3-hydroxyterphenyllin (**3**), and the tri- and tetraacetate derivatives (**4**, **5**)*.

	C-1	C-2	C-3	C-4	C-5	C-6	C-1′	C-2′	C-3′
1	124.58	131.89	114.36	155.93	114.36	131.89	116.98	148.13	139.35
3	125.04	118.44	144.22	143.87	114.86	121.97	117.24	148.10	139.25
4	130.43	131.20	121.00	150.09	121.00	131.20	124.03	142.84	143.60
5	131.30	128.62	141.59	141.38	122.70	125.29	123.00	142.78	143.53

	C-4′	C-5′	C-6′	C-1″	C-2″	C-3″	C-4″	C-5″	C-6″
1	132.38	103.02	153.10	128.81	129.71	115.20	156.77	115.20	129.71
3	132.24	102.98	153.11	128.77	129.67	115.17	156.64	115.17	129.67
4	134.05	110.57	153.13	135.32	130.03	121.55	150.31	121.55	130.03
5	134.34	110.48	152.96	135.18	130.01	121.56	150.28	121.56	130.01

	3′, 6′-OCH_3	3,4,2′,4″-$CH_3\underline{C}O_2$	3,4,2′,4″-$\underline{C}H_3CO_2$
1	55.52, 60.00		
3	55.53, 60.00		
4	56.09, 60.88	169.04, 169.12, 169.27	20.28, 21.07(2C)
5	56.03, 60.85	169.09, 169.30(2C), 169.31	20.26, 20.55(2C), 21.01

* Pulse Fourier transform spectra recorded at 30°C in dimethyl sulfoxide-d_6 (**1**, **3**) and chloroform-d (**4**, **5**); with aquisition time 0.8~1.6 s, spectral width 5120 Hz, flip angle 35°C, ^1H-decoupling field, $\gamma H_2/2\pi \sim 3800$ Hz, internal ^2H pulse lock. Broadband ^1H-decoupling from ^{13}C was obtained by phase modulation of the decoupling field from 0°~180°C at 150 Hz.

resolution spectra identified signals arising from carbons bearing hydrogen. The remaining resonances in the aromatic region arose from the quaternary carbons C-1, C-1′, C-4′, and C-1″ and can be assigned as follows. In the spectra of **1** and **4** the C-1 signal is a triplet due to three-bond coupling to the chemically equivalent hydrogens H-3 and H-5; the corresponding resonance for C-1″ is a triplet of doublets due to similar coupling to H-3″ and H-5″ and an additional smaller three-bond coupling to H-5′. C-1′ should be shielded to the greatest extent as both adjacent carbons bear oxygen substituents.[10] It would be expected to appear as a doublet of triplets due to coupling with H-5′ and to a lesser extent with H-2 and H-6. This is observed in the spectrum of **4** but the individual components are unresolved in the spectrum of **1**. The remaining quaternary carbon, C-4′, is a triplet due to a small coupling with H-2″ and H-6″. It follows that a change in multiplicity of either the C-1 or C-1″ resonance on going from **1** to **3** or **4** to **5** provides the means to locate the additional substituent unequivocally in **3** and **5**. From Table 3 it can be seen that the C-1 resonance in **3** and **5** has changed from a triplet to a doublet whereas the signals for C-1′, C-4′ and C-1″ have remained unchanged; C-3 is a doublet because it is coupled to H-5 only. This evidence places the additional hydroxyl group on C-3 and establishes that the new metabolite from *A. candidus* is 3-hydroxyterphenyllin.

Assignment of the remaining resonances in the ^{13}C spectrum of **3** (**5**) was trivial but necessary for the identification of signals arising from the 1,4-disubstituted rings of **1**(**4**). Prior assignment of the ^1H nmr for **3**(**5**) was also necessary before complete interpretation of the corresponding spectrum of **1**(**4**) was possible.

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Table 3. ^{13}C-^{1}H coupling constants ($^{n}J_{CH}$, Hz) for terphenyllin (**1**), 3-hydroxyterphenyllin (**3**), and their acetate derivatives (**4, 5**).*

	C-1	C-2	C-3	C-4	C-5	C-6	C-1′	C-2′	C-3′
1	t ^{3}J 7.6	dd ^{1}J 159.3 ^{3}J 7.5	dd ^{1}J 158.8 ^{3}J 3.8	tt ^{2}J 2.3 ^{3}J 9.2	dd ^{1}J 158.8 ^{3}J 3.8	dd ^{1}J 159.3 ^{3}J 7.5	m	s	dq $^{3}J_{d}\sim 8$ $^{3}J_{q}$ 4.3
3	d ^{3}J 8.2	dd ^{1}J 160.0 ^{3}J 7.0	m	m	ds ^{1}J 156.4	dd ^{1}J 157.0 ^{3}J 6.3	m	s	dq $^{3}J_{d}$ 8.4 $^{3}J_{q}$ 4.1
4	t ^{3}J 7.6	dd ^{1}J 162.3 ^{3}J 7.9	dd ^{1}J 163.3 ^{3}J 4.6	m	dd ^{1}J 163.3 ^{3}J 4.6	dd ^{1}J 162.3 ^{3}J 7.9	dt $^{3}J_{d}$ 6.3 $^{3}J_{t}$ 3.3	s	dq $^{3}J_{d}$ 8.6 $^{3}J_{q}$ 3.8
5	d ^{3}J 8.6	dd ^{1}J 164.5 ^{3}J 7.8	m	m	d ^{1}J 164.4	dd ^{1}J 164.4 ^{3}J 4.9	m	s	dq $^{3}J_{d}$ 8.3 $^{3}J_{q}$ 3.7

	C-4′	C-5′	C-6′	C-1″	C-2″	C-3″	C-4″	C-5″	C-6″
1	t ^{3}J 3.1	d ^{1}J 159.3	dq $^{2}J_{d}\sim 3$ $^{3}J_{q}\sim 4$	dt $^{3}J_{d}$ 4.3 $^{3}J_{t}$ 7.5	dd ^{1}J 159.4 ^{3}J 7.5	dd ^{1}J 159.9 ^{3}J 3.8	tt ^{2}J 2.4 ^{3}J 9.3	dd ^{1}J 159.9 ^{3}J 3.8	dd ^{1}J 159.4 ^{3}J 7.5
3	t ^{3}J 3.3	d ^{1}J 158.8	dq $^{2}J_{d}$ 2.9 $^{3}J_{q}$ 4.1	dt $^{3}J_{d}$ 4.2 $^{3}J_{t}$ 7.6	dd ^{1}J 159.7 ^{3}J 6.6	dd ^{1}J 159.4 ^{3}J 3.8	tt ^{2}J 2.5 ^{3}J 9.3	dd ^{1}J 159.4 ^{3}J 3.8	dd ^{1}J 159.7 ^{3}J 6.6
4	m	d ^{1}J 159.0	dq $^{2}J_{d}$ 3.1 $^{3}J_{q}$ 3.9	dt $^{3}J_{d}$ 4.5 $^{3}J_{t}$ 7.8	dd ^{1}J 162.3 ^{3}J 7.5	dd ^{1}J 163.4 ^{3}J 5.0	tt $^{2}J\sim 3.5$ $^{3}J\sim 10$	dd ^{1}J 163.4 ^{3}J 5.0	dd ^{1}J 162.3 ^{3}J 7.5
5	t $^{3}J\sim 3$	d ^{1}J 159.3	dq $^{2}J_{d}$ 3.1 $^{3}J_{q}$ 3.8	dt $^{3}J_{d}$ 4.6 $^{3}J_{t}$ 7.8	dd ^{1}J 162.5 ^{3}J 7.4	dd ^{1}J 163.6 ^{3}J 4.8	tt ^{2}J 3.8 ^{3}J 10.1	dd ^{1}J 163.6 ^{3}J 4.8	dd ^{1}J 162.5 ^{3}J 7.4

* Characteristic coupling constants were observed for the carbons of the methoxyl groups (^{1}J 143.8～144.7), and the acetoxyl groups (CH_3, ^{1}J 129.9～130.3; CO, ^{2}J 6.9～7.1). Signals designated as multiplets (m) were either obscured by overlap, or not resolved sufficiently to allow coupling constants to be estimated.

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