Arthrichitin. A New Cell Wall Active Metabolite from *Arthrinium phaeospermum*

E. K. S. Vijayakumar,* Kirity Roy, Sugata Chatterjee, S. K. Deshmukh, and B. N. Ganguli

Research Centre, Hoechst Marion Roussel Limited, Mulund (W), Bombay, India 400 080

H.-W. Fehlhaber and H. Kogler

Central Pharma Research, G 838, Hoechst AG, D-65926, Frankfurt am Main, Germany

Received April 26, 1996[⊗]

Arthrichitin (**1**), $C_{33}H_{46}N_4O_9$, is a new cell wall active depsipeptide isolated from the fermentation broth of *Arthrinium phaeospermum* (HIL Y-903022). Its structure was elucidated on the basis of spectroscopic and chemical degradation studies. Arthrichitin consists of serine, β-keto tryptophan, glutamic acid, and 2,4-dimethyl-3-hydroxydodecanoic acid units.

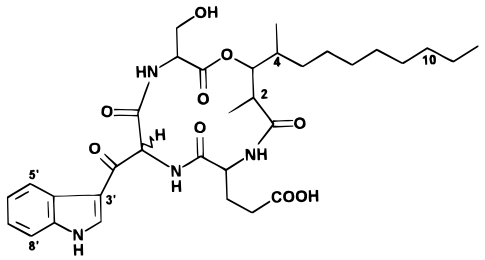
Introduction

A major potential world crop harvest worth several billion dollars is lost every year due to plant diseases caused by fungi and insects. Chitin, which is a polymer of β-1,4-linked *N*-acetylglucosamine, is one of the main components of fungal cell wall and also the main component of the cuticle of insects. The synthesis of chitin in the cell wall is regulated mainly by two enzymes: chitin synthetase for its synthesis and chitinase for its degradation. Because of this fundamental role, it is believed that inhibitors of these enzymes should provide good leads for designing either fungicidal or insecticidal compounds. Whereas nikkomycins and polyoxins,[1] the peptidyl nucleoside antibiotics, are the known fungal chitin synthesis inhibitors isolated from *Streptomyces*, allosamidin,[2] also isolated from a *Streptomyces* species, is a potent insect chitinase inhibitor. Similar attempts were made to isolate such inhibitors from fungi. Asperfuran[3] is the only weakly active antifungal metabolite isolated from a fungal culture of *Aspergillus oryzae*. These facts led us to an intensive search for more effective and safer non-nucleoside chitin synthesis inhibitors.

Results and Discussion

During our screening program for the discovery of chitin synthesis inhibitors, we have isolated a new depsipeptide, arthrichitin, from the fermentation broth of *Arthrinium phaeospermum* (Corda) Ellis (culture HIL Y-903022).[4,5] The crude arthrichitin was obtained from the culture filtrate by chromatography over Diaion HP-20 (aqueous MeOH eluant) and from the mycelium by acetone extraction followed by chromatography over Diaion HP-20 (aqueous MeOH eluant). The combined crude material was purified by medium-pressure liquid chromatography (MPLC) over reverse-phase silica gel (RP-18) (30−60 μm, aqueous phosphate buffer (pH 7.0)−CH_3CN mixtures as eluants), followed by desalting of the active fractions over Diaion HP-20 (aqueous MeOH eluant). The isolation was monitored both by HPLC and a bioassay model using *Neurospora crassa*, a strain specifically designed to identify cell wall acting compounds or chitin synthesis inhibitors.[6]

Arthrichitin (**1**) is a depsipeptide having a molecular formula of $C_{33}H_{46}N_4O_9$ determined by HRFABMS (matrix MeOH/NBA, internal reference PEG 600) [$(M + H)^+$: found *m*/*z* 643.3341; calcd for $C_{33}H_{47}N_4O_9$ *m*/*z* 643.3345].



(**1**)

The IR frequencies at 3515, 3360 and 3300, 1720, 1680, and 1660 cm^{-1} indicated the presence of hydroxyls, carbonyls, and amide carbonyls. Acid hydrolysis (6 N HCl, 115 °C, 20 h) of **1** yielded glutamic acid, serine, and a hydroxy C_{14} acid (GCMS of *N*-TFA/*O*-Me derivatives).

Arthrichitin (**1**) gave well-resolved ^1H and ^{13}C NMR spectra in DMSO-d_6. Table 1 summarizes the ^1H and ^{13}C NMR spectra of arthrichitin (**1**). The carbon multiplicities were determined by DEPT-135 spectrum. The assignments of all the protons were done by the analysis of phase-sensitive double quantum filtered HH COSY. The protonated carbons were assigned by the analysis of HMQC spectrum, while the quaternary carbons were identified by the interpretation of HMBC spectrum optimized for $^nJ_{CH}$ values of 6 and 12 Hz. In DMSO-d_6 as the solvent, arthrichitin revealed the presence of six D_2O exchangeable protons at δ 12.26 (br s), 12.20 (br s), 8.93 (d, 6.4 Hz), 8.54 (d, 7.3 Hz), 6.98 (d, 5.7 Hz), and 4.79 (t, 5.6 Hz) corresponding to one COOH, one indole NH, three amides, and one hydroxyl, respectively. Besides the expected spin systems for serine and glutamic acid, four additional spin systems *viz*. an aromatic system consisting of the four neighboring protons **A** [δ 8.17 (dm),

[⊗] Abstract published in *Advance ACS Abstracts*, August 15, 1996.

(1) Cohen, E.; Cashida, J. E. *Pestic. Biochem. Physiol.* **1982**, *17*, 301.
(2) Sakuda, S.; Isogai, A.; Matsumoto, S.; Suzuki, A. *J. Antibiot.* **1987**, *40*, 296.
(3) Pfefferle, W.; Anke, H.; Bross, M.; Steffan, B.; Vianden, R.; Steglich, W. *J. Antibiot.* **1990**, *43*, 648.
(4) The producing organism has been deposited at the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany with the accession number DSM 6245.
(5) Kirity Roy, Vijayakumar, E. K. S.; Deshmukh, S. K.; Chatterjee, S.; Ganguli, B. N.; Kogler, H.; Fehlhaber, H.-W. Eur. Pat. Appl. 96100859.6, Jan, 23, 1996.
(6) Kirsch, D. R.; Lai, M. H. *J. Antibiot.* **1986**, *39*, 1620.

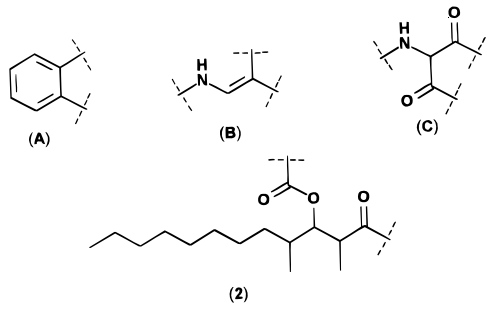
S0022-3263(96)00769-4 CCC: $12.00

Table 1. ^{13}C and ^{1}H NMR Data of Arthrichitin (1) in DMSO-d_6

	$^{13}C^a$	$^{1}H^b$
serine		
CO	169.12 (s)	
α	55.01 (d)	3.93 (ddd, 8.9, 5.3, 4.1 Hz)
β	58.94 (t)	3.41 (m)
NH		6.98 (d, 5.7 Hz)
OH		4.79 (t, 5.6 Hz)
β-keto tryptophan		
CO	167.54 (s)	
α	62.15 (d)	5.64 (d, 6.4 Hz)
β	184.95 (s)	
NH		8.93 (d, 6.4 Hz)
1′ (NH)		12.26 (d, 1.0 Hz)
2′	136.98 (d)	8.48 (d, 1.0 Hz)
3′	125.59 (s)	
4′	113.52 (s)	
5′	121.17 (d)	8.17 (d, 7.4 Hz)
6′	122.46 (d)	7.25 (m)
7′	123.36 (d)	7.27 (m)
8′	112.52 (d)	7.52 (d, 7.8 Hz)
9′	136.66 (s)	
glutamic acid		
CO	171.99 (s)	
α	51.99 (d)	4.45 (q, 7.6 Hz)
β	25.04 (t)	1.89 (m)
γ	30.09 (t)	2.29 (m)
δ (CO)	173.77 (s)	
NH		8.54 (d, 7.3 Hz)
COOH		12.20 (broad s)
2,4-dimethyl-3-hydroxy-dodecanoic acid		
1	179.06 (s)	
2	41.29 (d)	2.81 (dq, 9.7, 6.5 Hz)
3	78.36 (d)	4.99 (dd, 9.7, 1.8 Hz)
4	33.23 (d)	1.74 (m)
5	33.47 (t)	1.27 (m)
6	26.78 (t)	1.32 (m)
7	29.24 (t)^c	1.25 (m)
8	29.07 (t)^c	1.25 (m)
9	28.76 (t)^c	1.25 (m)
10	31.33 (t)	1.25 (m)
11	22.14 (t)	1.25 (m)
12	13.98 (q)	0.86 (t, 6.7 Hz)
C2-Me	16.15 (q)	1.07 (d, 6.7 Hz)
C4-Me	13.49 (q)	0.84 (d, 6.7 Hz)

^{a} 125 MHz, 15 mg/0.7 mL concentration ^{b} 500 MHz, 15 mg/0.7 mL concentration. ^{c} Values interchangeable.

7.52 (dm), 7.27 (m), and 7.25 (m)], the two isolated two-proton systems **B** [an aromatic proton at *δ* 8.48 (d, 1 Hz) having a weak correlation with a D_2O exchangeable NH at *δ* 12.26 (d, 1 Hz)] and **C** [a methine proton at *δ* 5.64 (d, 6.4 Hz) having a correlation with a D_2O exchangeable NH at *δ*8.93 (d, 6.4 Hz)], and a branched C_{14} aliphatic chain **2** could be extracted from the analysis of COSY spectrum. In **2**, the relatively downfield shift of the



terminal methine (δ_H 2.81) suggested that it was adjacent to a carbonyl group. Furthermore, the chemical shift (δ_H 4.99) of the oxymethine indicated acyloxy nature. The presence of the spin systems for serine, glutamic acid,

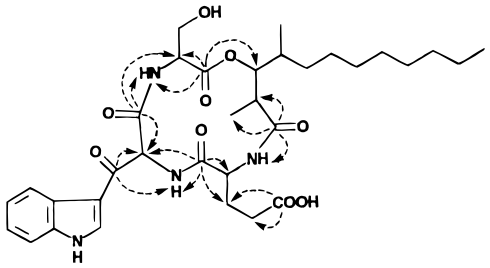
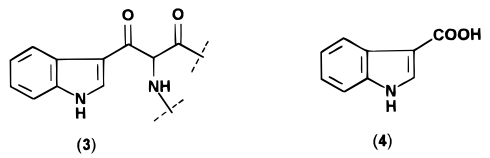


Figure 1. HMBC network establishing the subunit connectivities (- - > $^{13}C-^{1}H$).

and **2** were also in agreement with the results obtained from the GCMS analysis of the acid hydrolysate of **1**.

$^3J_{CH}$ correlations were observed between **A**, **B**, and **C** in the HMBC spectrum of arthrichitin (**1**). Thus, the proton at δ_H 8.17 of the spin system **A** showed a correlation with the carbon signal at δ_C 125.59 of **B** and the NH at δ_H 12.26 of the spin system **B** had a correlation with the carbon signal at δ_C 112.52 of **A**. Furthermore, the proton at δ_H 8.48 of **B** showed a correlation with the carbonyl at δ_C 184.95 of **C**. The spin systems **A**, **B**, and **C** could, thus, be connected leading to the subunit *β*-keto tryptophan **3**, the presence of which was further confirmed by alkaline hydrolysis (2 N KOH, rt, 16 h) of **1** followed by purification on preparative TLC. The product, thus obtained, was identified as indole-3-carboxylic acid (**4**) by comparison of its ^1H and ^{13}C NMR data with the reported values.[7] However, attempts to isolate *β*-keto tryptophan **3** using various conditions of acid and alkaline hydrolysis were not successful. This could probably be due to the instability of the 1,3-diketo system.



Finally, the connectivities of all the four subunits *viz.* glutamic acid, serine, 2,4-dimethyl-3-hydroxydodecanoic acid **2**, and *β*-keto tryptophan **3** were established by the analysis of HMBC spectra. Figure 1 illustrates the relevant long-range $^{13}C-^{1}H$ correlations between the four subunits extracted from the analysis of the HMBC spectrum of arthrichitin (**1**).

The sequencing of the subunits established by HMBC was confirmed by the analysis of the electrospray ionization (ESI) MS of the hydrolyzed product **5**. Thus, a mild alkaline hydrolysis (0.2 N KOH, 30 min, rt) of **1** followed by purification on Diaion HP-20 (elution with aqueous MeOH) and Sephadex G-10 (elution with water) afforded product **5**. Figure 2 illustrates the sequential fragmentations observed in the ESIMS of **5** induced by raising the cone voltage.

The structure of arthrichitin was, thus, established to be represented as **1**.

In polar solvents like MeOH and DMSO, arthrichitin (**1**) was found to undergo conformational changes, and the HPLC analysis indicated that after 1 h arthrichitin existed as an equilibrium mixture of epimers in a 5:1 ratio. This equilibrium could also be explicitly seen in the ^1H NMR spectrum of arthrichitin (**1**), recorded after

(7) *The Aldrich Library of ^{13}C and ^{1}H FT NMR Spectra*; Pouchert, C. J., Behnke, J., Eds.; Aldrich Chemical Co.: Milwaukee, WI, 1993;Vol. 3.

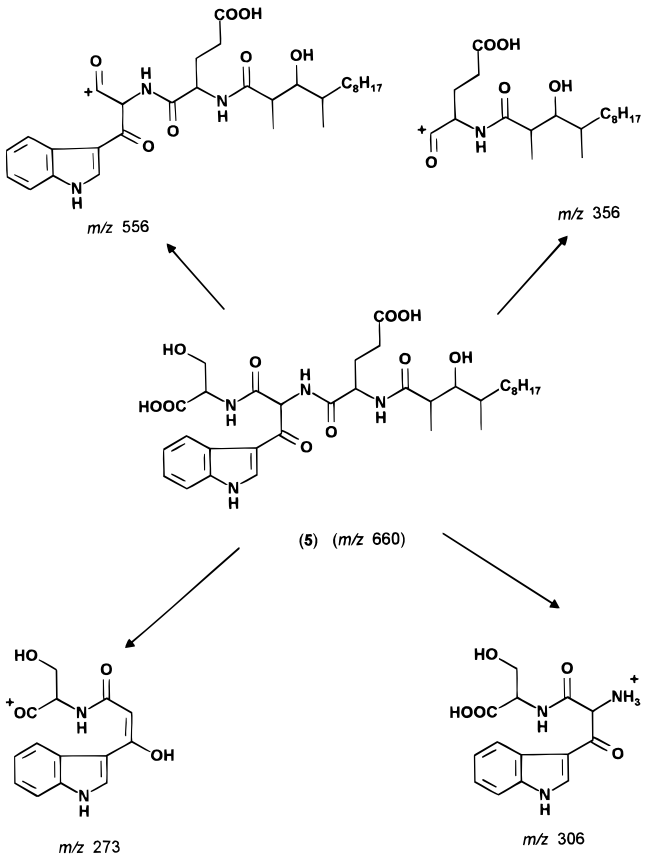


Figure 2. ESIMS fragmentations of **5**.

2 h in solution, by the presence of a second set of identical signals with different chemical shifts, for most of the protons. The most likely interpretation for this equilibrium could be the slow epimerization at the C^{α} position of the β-keto tryptophan **3** wherein the C^{α}-H is flanked by two carbonyls and one NH. This assumption of epimerization at C^{α} position was confirmed by the disappearance of C^{α} H of **3** (δ 5.64) in the ^{1}H NMR spectrum of arthrichitin (**1**) recorded in DMSO-d_6 + D_2O after 1 week.

Arthrichitin displayed morphological abnormalities for *in vitro Botrytis cinerea* and showed fungicidal efficiency of 75 and 85% against *Pyricularia oryzae* infection of rice and *B. cineria* infection of cucumber at 5000 ppm under greenhouse conditions, respectively.

Experimental Section

Melting points are uncorrected. HPLC analysis was carried out on a 10 μm ODS−Hypersil [4 mm × (30 + 100) mm] column using 35% CH_3CN in 0.02 M aqueous sodium phosphate buffer (pH 7.0) as the eluant with a flow rate of 1 mL/min and detection at 220 nm. Coupling constants are reported in hertz following the multiplicity.

Isolation. Approximately 14 L of the culture broth was harvested and centrifuged to separate the mycelium (3 kg). The culture filtrate was passed through a column of Diaion HP-20 (1 L) and successively washed with demineralized water (10 L), 1 M aqueous NaCl (5 L), and demineralized water (10 L). The column was then eluted with 50% MeOH in water (7 L) and MeOH (12 L). The eluates were collected as 1 L fractions and monitored by their activity against *N. crassa*. The active fractions, eluted in MeOH, were concentrated under reduced pressure and lyophilized to afford crude arthrichitin (1.2 g). The mycelium was extracted with acetone (4 × 5 L). The combined extracts were concentrated under reduced pressure to approximately 2 L and passed through a column of Diaion HP-20 (500 mL). The column was washed with demineralized water (10 L) and eluted with 50% MeOH in water (2.5 L) and MeOH (5 L). The fractions were of 500 mL size and monitored by activity against *N. crassa*. The active fractions were concentrated under reduced pressure and lyophilized to obtain crude arthrichitin (2.1 g).

The combined crude material (3.3 g) was subjected to medium-pressure liquid chromatography (MPLC) over RP-18 (30−60 μm, 1.2 L) and eluted with a 5% stepwise gradient of CH_3CN in 0.02 M aqueous sodium phosphate buffer (pH 7.0) with a flow rate of 20 mL/min. The eluates were collected in sizes of 150 mL, and the presence of arthrichitin was monitored by UV detection at 220 nm as well as by activity against *N. crassa*. Arthrichitin was eluted with 30% CH_3CN in phosphate buffer. The combined active eluates were concentrated under reduced pressure and diluted with water to 4.5 L. Desalting of this material was done by passing it through Diaion HP-20 (800 mL). The column was washed with demineralized water (4 L) and eluted with MeOH (3.5 L). The active eluates were concentrated under reduced pressure and lyophilized to obtain pure arthrichitin as white solid: yield = 1.3 g (93 mg/L); mp 245 °C; $[\alpha]_D$ +24.12° (*c* 0.475, MeOH); HPLC t_R = 5.4 min; UV (MeOH) 247, 266, and 310 nm; IR (KBr) 3515, 3360, 3300, 2920, 2850, 1720, 1680, 1660, 1640, 1520, 1420, 1310, 1190, 1055, 975, 750 cm^{-1}. The ^{1}H and ^{13}C NMR data are given in Table 1.

Alkaline Hydrolysis of 1. Ten milliliters of 2 N KOH was added to **1** (100 mg, 0.15 mmol) and stirred at rt for 16 h. The reaction mixture was then neutralized with 2 N HCl and extracted with CH_2Cl_2 (3 × 15 mL). The combined CH_2Cl_2 extracts were concentrated under reduced pressure to obtain 32 mg of crude product. The crude product was purified by preparative TLC on SiO_2 gel plates (article no. 13794, E. Merck) using 5:3:1 EtOAc−2-propanol−water for developing the plates and MeOH for elution to afford pure indole-3-carboxylic acid (**4**) (11 mg, 0.06 mmol): EIMS *m*/*z* 117 (M − CO_2)$^+$; ^{1}H NMR (DMSO-d_6 + DCl) δ 11.85 (s, 1H), 7.96 (s, 1H), 7.95 (d, 7.3 Hz, 1H), 7.43 (d, 7.4 Hz, 1H), 7.17 (m, 1H), 7.14 (m, 1H); ^{13}C NMR (DMSO-d_6 + DCl) δ 166.00, 136.37, 132.25, 126.03, 122.31, 121.16, 120 66, 112.33, 107.28.

Mild Alkaline Hydrolysis of 1. Ten milliliters of 0.2 N KOH was added to **1** (50 mg, 0.08 mmol) and strirred at rt for 30 min. The reaction mixture was then neutralized with 0.5 N HCl to pH 6, and the resulting solution was desalted by being passed through a column of Diaion HP-20. The column was washed with demineralized water and eluted with 75% MeOH in water. The eluates were concentrated under reduced pressure and lyophilized to obtain the crude product (48 mg) which was finally purified by Sephadex G-10 in water. Compound **5** was obtained as a white powder (46 mg, 0.07 mmol): mp 240 °C (dec); $[\alpha]_D$ +5.53° (*c* 0.47, MeOH); HPLC t_R = 2.6 min; UV (MeOH) 245, 263, 305 nm; IR (KBr) 3520, 3340, 2925, 2860, 1650, 1570, 1520, 1450, 1420, 1320, 1245, 1130, 960, 750 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.14 (br s, 1H, NH), 8.59 (d, 6.3 Hz, NH), 8.52 (br s, =CH), 8.27 (d, 7.3 Hz, NH), 8.17 (dd, 6.1, 2.4 Hz, =CH), 7.79 (d, 6.1 Hz, NH), 7.64 (br s, OH), 7.48 (d, 7.3 Hz, =CH), 7.21 (m, 2 × =CH), 5.79 (d, 6.3 Hz, CH), 5.58 (br s, OH), 4.21 (q, 7.6 Hz, CH), 4.07 (m, CH), 3.79 (ddd, 8.8, 5.2, 4.0 Hz, CH), 3.48 (m, CH_2), 2.56 (m, CH), 2.41 (m, CH_2), 2.09 (m, CH_2), 1.79 (m, CH), 1.45−1.20 (m, 7 × CH_2), 0.89 (d, 6.5 Hz, CH_3), 0.86 (t, 6.6 Hz, CH_3), 0.80 (d, 6.5 Hz, CH_3); ESIMS *m*/*z* 661 (M + H)$^+$. The mass spectral fragmentations are shown in Figure 2.

Acknowledgment. We thank Mr. Jitesh Bhambani and Mr. M. S. Phansalkar for technical assistance. We are grateful to Dr. B. Sachse, AgrEvo, Frankfurt, Germany, for providing the *in vivo* data. We acknowledge the support of Dr. P. K. Inamdar and Dr. G. Subbaiah for elemental analysis, mass spectra, and GCMS. The helpful discussions with Dr. J. M. Kenia are sincerely acknowledged.

JO960769N