Bioactive Substances from Insect Pathogenic Fungi

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ABSTRACT

Insect pathogenic fungi have opened up a relatively untapped area of natural product research which, unfortunately, has not received much attention to date. Found in wild abundance in wet tropical Thailand, the insect fungi are shown to contribute not only as controllers of insect populations but also as rich sources of structurally novel biologically active substances.

The 80 000 known fungi represent about 5% of those estimated to exist.[1] Of these, only a fraction are utilized as food or medicine, both modern and traditional,[2] and as biological control agents.[3]

Insect fungi exist as commensals deriving nutrition from gut contents without causing harm to the host, as ectoparasites getting nutrition from cuticular waxes, or as true insect pathogenic fungi obtaining nutrients from within the insect.[4,5] Insect pathogenic fungi are unique in being able to infect across the insect cuticle, the first barrier to infection. Germinating conidia produce extracellular lipases, chitinases, and proteases to initiate cuticle invasion. Once inside the host, the fungus develops as a yeast-like form, producing metabolites that inhibit the insect's immune system, modify the insect's behavior, or act as post-mortem antibiotics against competing microorganisms.[6,7] After death, the fungus reverts to a filamentous form and typically digests the remaining internal organs, leaving only the chitin/protein exoskeleton.[4,5]

The most well-known insect pathogenic fungi are members of the highly host-specific megagenus *Cordyceps*, with 300+ species.[8] For example *Cordyceps nutans* infects only Hemiptera (stink bugs).[9] Within Hymenoptera (ants, wasps, and bees) *Cordyceps myrmecophila* and *Cordyceps irangiensis* infect formicine ants, which inhabit leaf litter,[10] *Cordyceps unilateralis* and *Cordyceps pseudolloydii* infect ants on the underside of leaves,[11] while the related *Cordyceps sphecocephala* infects wasps.[12] *Cordyceps* spp. usually have restricted geographical ranges. *Cordyceps militaris* is known from Lepidoptera pupae in northern temperate regions (Europe, Northern Asia, and North America).[13] *Cordyceps stylophthora* is known from North America and Japan;[13] for example, *Cordyceps nutans* has an East Asian range from Japan, Korea, China, and Thailand.[9] Although restricted in host range and geography, the asexual states of some *Cordyceps* spp. (e.g., *Metarhizium* and *Beauveria*) have migrated to agricultural ecosystems, increased their host range, and become panglobal.[14]

Cordyceps sinensis has been known and used in Chinese traditional medicine for about 2000 years.[15,16] Interest in *Cordyceps sinensis* has increased in the last 15−20 years as demand for alternative medicines has spread into Western culture. In the early 1990s, several relatively unknown Chinese distance runners broke world records, and suspicion initially fell on performance enhancing drugs.[2] Eventually, a cocktail of natural Chinese herbal medicines was implicated, with *Cordyceps sinensis* being the major ingredient. However, the medicinal value of *Cordyceps sinensis* remains to be scientifically evaluated.[15,16]

In contrast to most *Cordyceps* found at lower elevations and in forested areas, *Cordyceps sinensis* grows in Himalayan alpine grasslands above 4300 m, where it infects larvae of *Hepialus* (Lepidoptera, ghost moths).[17] It is called the "Winter Worm Summer Grass" in Chinese, because in winter months it is seen as a "worm" (larva), while in the summer the infected larva dies and the fungus fruiting body grows above the soil as a "grass" (Figures 1−4).

A country rich in biological resources, Thailand has proven to be a rich source of insect pathogenic fungi. Mycologists at the National Center for Genetic Engineering and Biotechnology (BIOTEC) have been surveying these fungi for several years and have yielded numerous species.[8] Isolates, after identification, are deposited in the BIOTEC Culture Collection (BCC) and are available for other laboratories. Most of the work described in this Account is derived from collaborative research focusing

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Table 1

	biological activities (μg/mL)						
	anti-*P. falciparum* K1[a]	anti-*M. tuberculosis*	anticancer (IC_{50})		cytotoxicity[b]	additional biological activities[d]	
compound	IC_{50}	MIC[c]	BC	KB	Vero (IC_{50})		ref
1	4.0	*e*	9.7	23	15		27
2	7.5	*e*	6.0	12.4	30		27
3	10.1	*e*	5.0	24	10		27
4	7.0	*e*	4.2	7.2	7.5		27
5	8.5	*e*	10	20	10		27
6	2.5	*e*	>50	>50	>50		27
7	0.066	*e*	3.9	15.7	6.3		29
8	0.037	*e*	3.7	8.4	5.3		29
9	>20	*e*	*e*	*e*	*e*		29
10	7.8	*e*	>20	>20	>20		29
14	>100	*e*	>100	>100	>100		32, 33
15	>100	*e*	>100	>100	>100		32, 33
16	4.7	*e*	63	30	36		33
17	8.1	*e*	>100	>100	>100		33
18	2.2	*e*	>100	>100	>100		33
19	3.7	*e*	>100	>100	44		33
20	5.2	*e*	>100	>100	>100		33
21	8.4	*e*	>100	6.3	37		33
22	1.1	*e*	14	28	20		33
23	5.9	*e*	18	17	33		33
25	7.1	*e*	47	>100	>100		33
26	18	*e*	>100	>100	>100		33
28	64	*e*	63	>100	64		33
29	2.2	*e*	2.2	17	11		40
42	1.3	1.6	15	>20	10		51
43	1.8	1.6	14	13	9.1		51
44	2.3	1.6	9.0	10	9.1		51
46	2.0	0.8	15	>20	5.9		51
47	2.4	0.8	4.4	>20	4.4		51
48	1.6	0.8	3.3	14	5.2		51
49	0.27	3.12	18	16	17		52
50	0.20	3.12	12	11	18		52
51	0.46	6.25	>20	>20	45		52
52	1.1	6.25	>20	>20	>50		52
53	1.9	6.25	5.5	>20	38		52
54	0.24	6.25	18	>20	38		52
55	0.22	1.56	8.1	11	1.4		52
56a	3.2 (mixture of **56a**, **56b**, **56c**)	3.13 (mixture of **56a**, **56b**, **56c**)	1.4 (mixture of **56a**, **56b**, **56c**)	2.4 (mixture of **56a**, **56b**, **56c**)	*e*	anti-NCI-H187 (IC_{50}, 0.78) (mixture of **56a**, **56b**, **56c**)	54
56b					*e*		54
56c					*e*		54
57	3.3	12.5	3.8	3.6	6.4	anti-NCI-H187 (IC_{50}, 2.1)	55
58a	3.4 (1:1 mixture of **58a** and **58b**)	6.25 (1:1 mixture of **58a** and **58b**)	2.0 (1.1 mixture of **58a** and **58b**)	3.1 (1.1 mixture of **58a** and **58b**)	3.7 (1:1 mixture of **58a** and **58b**)	anti-NCI-H187 (IC_{50}, 2.2) (1:1 mixture of **58a** and **58b**)	55
58b							55
59	3.4	6.25	0.78	4.0	4.7	anti-NCI-H187 (IC_{50}, 1.2)	55
60	>20	100	>20	>20	>20	anti-NCI-H187 (IC_{50}, >50)	56
61	2.5	*e*	3.9	15	8.9		56
62	*e*	25	>20	>20	49.9	anti-NCI-H187 (IC_{50}, 6.6)	57
63	*e*	12.5	8.3	>20	4.9	anti-NCI-H187 (IC_{50}, 4.4)	57
64	*e*	12.5	16.8	>20	9.7	anti-NCI-H187 (IC_{50}, 3.5)	57
66	*e*	*e*	8.4	19	6.9	anti-NCI-H187 (IC_{50}, 7.9) inactive against HSV-1	58
67	*e*	*e*	>20	>20	38	anti-NCI-H187 (IC_{50}, >20) inactive against HSV-1	58

Table 1 (Continued)

	biological activities (μg/mL)						
	anti-*P. falciparum* K1[a]	anti-*M. tuberculosis*	anticancer (IC_{50})		cytotoxicity[b]	additional biological activities[d]	
compound	IC_{50}	MIC[c]	BC	KB	Vero (IC_{50})		ref
68	*e*	*e*	1.4	2.7	3.4	anti-NCI-H187 (IC_{50}, 2.2) inactive against HSV-1	58
69	*e*	0.000 15	*e*	*e*	*e*		*f*
70	2.8	6.0−12	*e*	*e*	>50		59
71	>20	0.78	>20	>20	>50	anti-NCI-H187 (IC_{50}, >20) inactive against HSV-1	60
72	>20	0.78	>20	>20	>50	anti-NCI-H187 (IC_{50}, 6.0) inactive against HSV-1	60
73	>20	0.78	3.2	4.6	12	anti-NCI-H187 (IC_{50}, 8.3) inactive against HSV-1	60
74	*e*	3.13	>20	>20	*e*	anti-NCI-H187 (IC_{50}, 7.3)	60
76	*e*	12.5	*e*	*e*	>50		63
77	*e*	>50	*e*	*e*	>50		63
78	*e*	12.5	*e*	*e*	>50		63
79	*e*	>50	*e*	*e*	33.8		63

[a] Anti-*P. falciparum* K1 (multidrug-resistant strain) assay was performed according to the methods of Desjardins.[66] Inhibitory concentration 50 (IC_{50}) represents the concentration that causes 50% reduction in growth. IC_{50} values of chloroquine diphosphate and dihydroartemisinin were 0.16 and 0.0012 μg/mL, respectively. [b] Cytotoxicity was determined using a colorimetric method previously described by Skehan et al.[67] [c] Anti *M. tuberculosis* assay was performed against the H37Ra strain using the Microplate Alamar Blue Assay (MABA) as described by Collins and Franzblau.[68] Minimum inhibitory concentration (MIC) values of isoniazid and kanamycin were 0.04−0.09 and 2.5−5.0 μg/mL, respectively. [d] Anti-HSV1 (herpes simplex type 1, ATCC VR-260) assay was determined colorimetrically as modified from Skehan et al.[67] IC_{50} values of acyclovir were 2−5 μg/mL. Inactive indicates less than 25% inhibition at concentrations noncytotoxic to the host cells (Vero). BC, human breast-cancer cells. IC_{50} values of ellipticine were 0.3−1.5 μg/mL. KB, human oral epidermoid carcinoma (ATCC CCL-17). IC_{50} values of ellipticine were 0.3−1.3 μg/mL. NCI-H187, human small-cell lung-cancer cells (ATCC CRL-5804). The IC_{50} value of ellipticine was 0.39 μg/mL. Vero, African green monkey kidney fibroblasts (ATCC CCL-81). IC_{50} values of ellipticine were 0.4−1.0 μg/mL. [e] Not tested. [f] Unpublished results.

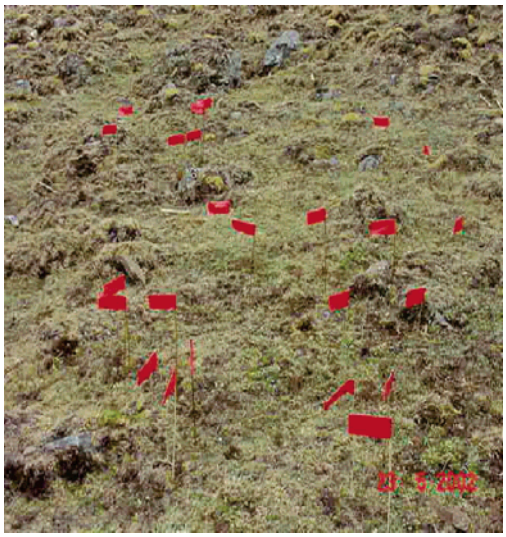


FIGURE 1. Red flags marking the positions of *Cordyceps sinensis* fruiting bodies in Himalayan (Bhutan) alpine grassland at 4700 meters.

FIGURE 2. Stroma of *Cordyceps sinensis* emerging from the grassland.

on the search for novel bioactive compounds against *Plasmodium falciparum* and *Mycobacterium tuberculosis* from insect pathogenic fungi deposited at the BCC. In addition, growth inhibition of different human and primate cell lines was also determined to indicate cytotoxicity. In certain cases, biological activities against Herpes simplex virus type 1 were also tested (Table 1).

Cordyceps are rich sources of novel biologically active substances with diverse structural architecture. For example, extracts of *C. sinensis* exhibited antioxidation,[18] immunomodulatory,[19] hypoglycemic,[20] hypotensive and vasorelaxant,[21] and antitumor activities.[22,23] Chemical investigation of *C. sinensis* led to the identification of polysaccharides and sterols.[23,24] The panglobal species *Cordyceps unilateralis* BCC 1869, collected from Khao Luang National Park and specific to ants (Figure 5) produces both known[25,26] and novel[27] naphthoquinone

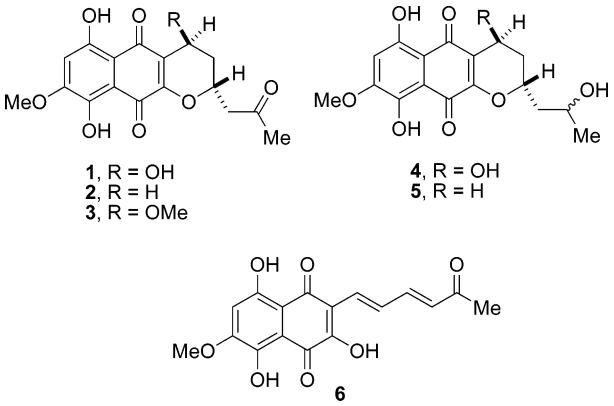
FIGURE 3. Excavation revealing the larval host of *Cordyceps sinensis*.

FIGURE 4. *Cordyceps sinensis* stroma on Lepidoptera larvae.

FIGURE 5. *Cordyceps unilateralis* on an ant.

derivatives, **1**−**6**. Interestingly, these naphthoquinones exhibited antimalarial activity with IC_{50} values of 2.5−10.1 μg/mL (Table 1). The above naphthoquinones show a deep red color under acidic conditions but intense purple in basic environments; such color characteristics are attractive to the pigment industry. Production of naphthoquinones by *C. unilateralis*, after optimization of fermentation conditions, can attain yields up to 3 g/L of culture broth.[28]

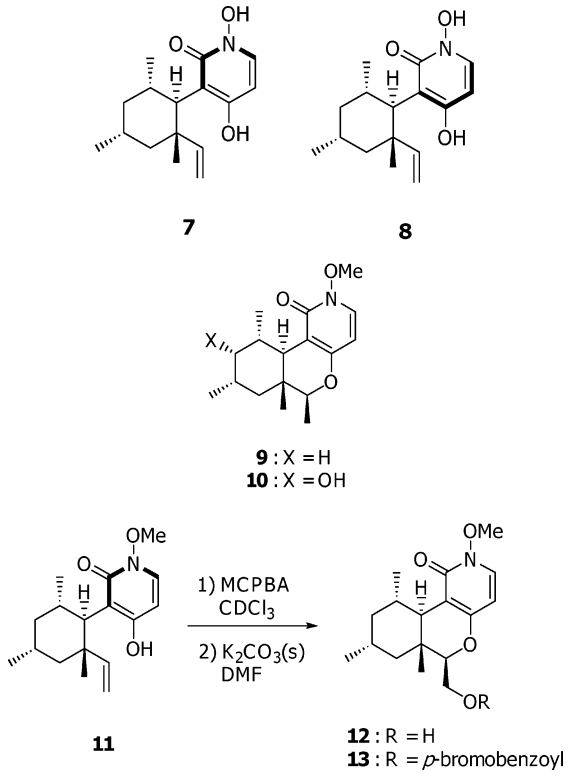
FIGURE 6. *Cordyceps nipponica* on ant lions.



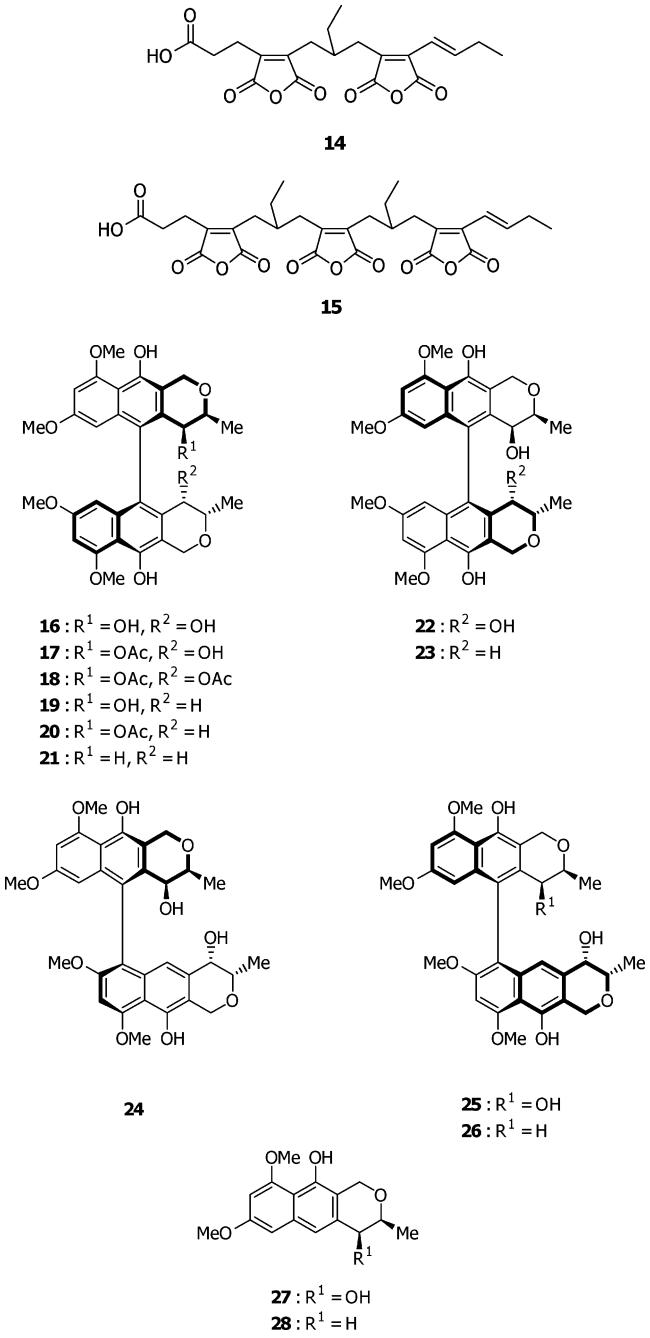
Cordyceps nipponica was originally described from cicadas in Japan and is found infecting both cicadas and ant lions (*Neuroptera*) in Thailand. Two *N*-hydroxy-2-pyridones, cordypyridones A (**7**) and B (**8**), and two tricyclic *N*-methoxy-2-pyridones, cordypyridones C (**9**) and D (**10**), were isolated from *Cordyceps nipponica* BCC 1389 (collected from Khao Yai National Park, central Thailand, Figure 6).[29] Cordypyridone A (**7**) is identical to 8-methyl-pyridoxatin, previously isolated from an unidentified fungus OS-F61800,[30] while its atropisomer, cordypyridone B (**8**), was shown to be a metabolite of BCC 1389. A careful study indicated that interconversion between compounds **7** and **8** occurred upon heating the solution, and the absolute configuration of cordypyridone **7** (and hence its atropisomer, **8**) was later determined using chemical means. Epoxidation of compound **11** (1-*O*-methyl derivative of **7**) and subsequent cyclization gave the major product **12**, which is the 14-hydroxy derivative of cordypyridone C (**9**). X-ray analysis of **13**, the *p*-bromobenzolate derivative of **12**, revealed the proposed absolute configuration. Cordypyridones A (**7**) and B (**8**) exhibited potent antimalarial activity with respective IC_{50}

FIGURE 7. *Cordyceps pseudomilitaris* on a Lepidoptera larva.

values of 0.066 and 0.037 μg/mL but showed weak cytotoxicity (Vero cells; IC_{50} values of 6.3 and 5.3 μg/mL, respectively).



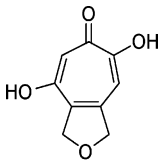
Cordyceps pseudomilitaris is known only from Thailand and, to date, only from Sam Lan National Park, where it infects Lepidoptera larvae from August to October.[31] Cordyanhydrides A (**14**) and B (**15**),[32] two unique anhydrides, were isolated from *Cordyceps pseudomilitaris* BCC 1620 (Figure 7). Most importantly, cordyanhydride B (**15**) is the first naturally occurring nonadride containing three C-9 units. However, anhydrides **14** and **15** showed negative results in the antimalarial, antituberculous, and cytotoxicity assays. Fermentation studies focused on secondary metabolites revealed that these anhydrides were effectively produced when *C. pseudomilitaris* was incubated in potato dextrose broth (PDB) on rotary shakers, while another class of metabolites, bioxanthracenes **16**−**26** and the corresponding monomers, **27** and **28**, were isolated from cultures incubated statically in yeast extract sucrose (YES) medium.[33,34] Compounds **16**−**21** and **26** (ES-242-4, -3, -2, -5, -1, -6, and -8, respectively), were previously isolated from *Verticillium* sp. as *N*-methyl-D-aspartate receptor antagonists.[35,36] Synthetically known compounds, **22** and **23** [atropisomers of ES-242-4 (**16**) and ES-242-5 (**19**), respectively],[37−39] have now been shown to be naturally occurring bioxanthracenes produced by *C. pseudomilitaris* BCC 1620.



Cordytropolone (**29**), a new tropolone isolated as the predominant constituent of *Cordyceps* sp. BCC 1681 (collected from Khao Soi Dao Wildlife Sanctuary, Chant-

FIGURE 8. *Cordyceps* sp. on Coleoptera (elaterid larva).

aburi, Figure 8) exhibited moderate antimalarial and cytotoxic activities.[40] BCC 1681 is an as yet unnamed new species infecting elaterid larvae. Recent unpublished molecular work places this species close to *Cordyceps sinensis*.



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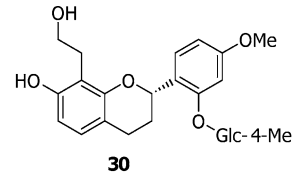
A related insect pathogenic fungus used as an analgesic and anticonvulsant in north Asian traditional medical practice is the dried silkworm larva infected by *Beauveria bassiana*. Recent chemical investigation of the crude drug led to the identification of compounds **30**−**33**, all bearing a 4-*O*-methylglucose unit.[41] While several other chemical entities were obtained from cultivated *Beauveria* species,[42] the isolation of beauveriolides from the culture broth of *Beauveria* species FO-6979 might prove to be pharmaceutically useful because the cyclodepsipeptides beauveriolides I (**34**) and III (**35**) show promising antiatherogenic activity.[43]

Apart from *Cordyceps*, extracts of mycelia and fruiting bodies of entomopathogenic fungi of the genus *Paecilomyces* have also yielded novel biologically active secondary metabolites. New trichothecanes, paecilomycines A (**36**), B (**37**), and C (**38**), were isolated from *Paecilomyces tenuipes* (the asexual state of *Cordyceps takaomontana*), a well-known panglobal fungus commonly used as health food in north Asian countries (China, Korea, and Japan). From cultured fruiting bodies of this same fungus, three



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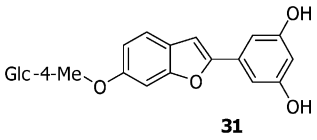

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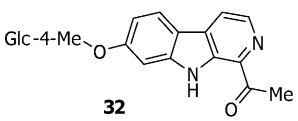

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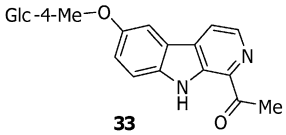

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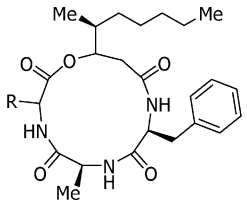

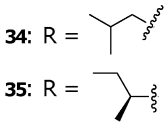

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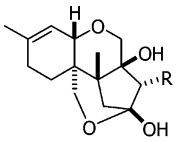
additional trichothecanes, tenuipesine A (**39**)[44] and spirotenuipesines A (**40**) and B (**41**),[45] were also identified.



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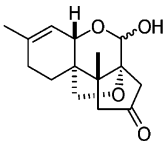

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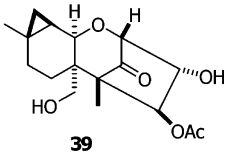

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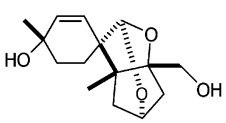

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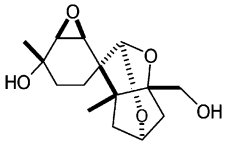

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Biological screening of fungal fermentation products deposited at the BCC indicated that beauvericin (**42**) and beauvericin A (**43**) were constituents responsible for antimalarial and antituberculous activities of *Paecilomyces tenuipes* BCC 1614 (collected from Khlong Nakha Wildlife Sanctuary, Ranong, southern Thailand, on Lepidoptera pupa, Figure 9).[46]

Beauvericin (**42**)[47] is an ionophoric cyclodepsipeptide exhibiting insecticidal and antibiotic activities. It consists of three residues each of L-*N*-methylphenylalanine and D-2-hydroxyisovaleric acid (Hiv) linked alternately to furnish an 18-membered cyclohexadepsipeptide structure. Although beauvericin (**42**) was first isolated over 40 years ago and has been detected as metabolites of various fungi, particularly *Beauveria*, *Fusarium*, and *Paecilomyces*, it was in 1995 that the related minor analogues, beauvericin A (**43**) and B (**44**), were isolated from *B. bassiana*.[48]

Biosynthetic studies by Zocher and co-workers revealed that L-phenylalanine and D-Hiv (derived from L-valine) are biosynthetic precursors.[49,50] A study on the precursor-directed biosynthesis of beauvericin by *P. tenuipes* BCC 1614 involving the feeding of four isomers of isoleucine independently as precursors for 2-hydroxy-3-methylpentanoic acid (Hmp) residues was undertaken.[51] Although precursor-directed biosynthesis is frequently employed as

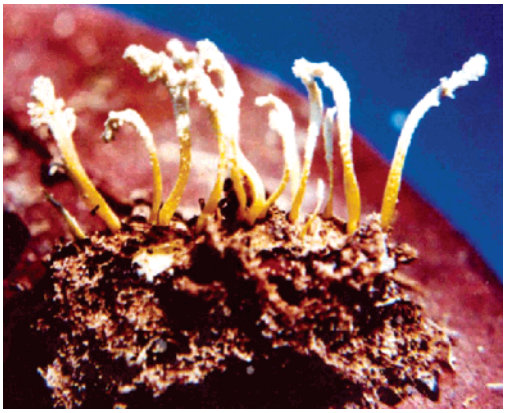
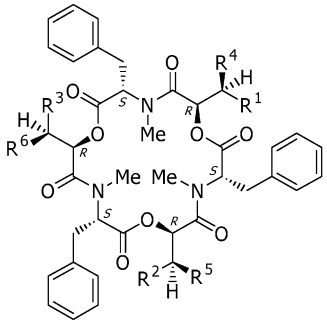


FIGURE 9. *Paecilomyces tenuipes* on a Lepidoptera pupa.

a common tool for enhanced production of certain metabolites, the symmetrical structural feature of beauvericin template was of interest because it is capable of accepting several precursors that would lead to the biosynthesis of many "unnatural natural products" in a single fermentation.

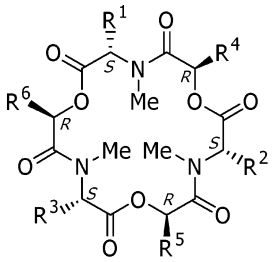
Feeding of L-(2*S*,3*S*)-isoleucine or D-(2*R*,3*S*)-alloisoleucine (50 mM each) in the culture liquid medium resulted in enhanced production of beauvericin A (**43**) and the appearance of beauvericin B (**44**) and a third isomer, named beauvericin C (**45**). Hence, the 3*S* configuration of the Hmp residues was established on the basis of the absolute configuration of the precursor. When (3*R*,3*R*)-D-isoleucine or (3*S*,3*R*)-L-alloisoleucine was fed, diastereoisomers of beauvericins A, B, and C, namely, allobeauvericins A (**46**), B (**47**), and C (**48**), were isolated and characterized.[51]



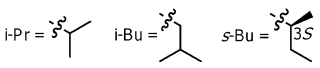
	R^1	R^2	R^3	R^4	R^5	R^6
beauvericin (**42**)	Me	Me	Me	Me	Me	Me
beauvericin A (**43**)	Et	Me	Me	Me	Me	Me
beauvericin B (**44**)	Et	Et	Me	Me	Me	Me
beauvericin C (**45**)	Et	Et	Et	Me	Me	Me
allobeauvericin A (**46**)	Me	Me	Me	Et	Me	Me
allobeauvericin B (**47**)	Me	Me	Me	Et	Et	Me
allobeauvericin C (**48**)	Me	Me	Me	Et	Et	Et

Verticillium hemipterigenum is the asexual state of *Torrubiella hemipterigena* infecting only leafhoppers in the Indian Ocean region (including Thailand). BCC 1449 (collected from Khlong Nakha Wildlife Sanctuary, Ranong, southern Thailand, Figure 10) produced enniatins, which are also well-known cyclohexadepsipeptides. During the investigation of the antimalarial constituents of BCC 1449, two new analogues, enniatins H (**53**) and I (**54**), bearing respectively one and two Hmp residues, instead of Hiv, were isolated and identified together with the known enniatins B and B_4.[52] To confirm the presence of Hmp residues in **53** and **54** and to determine their stereochemistries, precursor-directed biosynthesis was also applied in this case. Interestingly, unique substrate selectivity was observed. Feeding L-leucine (20 mM in PDB) resulted in selective uptake of this precursor as L-*N*-methylamino acid units in the enniatin molecule, thus enhancing production of enniatins B_4 (**50**), G (**51**), and C (**52**). In contrast, feeding with (2*S*,3*S*)-L-isoleucine (20 mM in PDB) resulted in enhanced production of enniatins H (**53**), I (**54**), and MK1688 (**55**), indicating that the precursor was used in the biosynthesis selectively as Hmp residues. This latter experiment demonstrated the 3*S* configuration of the Hmp residues in **53** and **54**, as well as that in MK1688,[53] whose stereochemistry had not previously been addressed.

A drawback observed in this study was the low efficiency of enniatin production by BCC 1449, resulting in the failure to isolate/characterize the minor enniatin analogues because of their small quantities. Subsequent study indicated that YES was the most suitable medium for enniatin production, and this medium also promoted rapid mycelial growth. Enniatins were found mostly in mycelial extracts, and the enniatin composition was similar to that observed in the PDB fermentation. Large-scale fermentation in YES medium revealed the presence of three minor analogues, enniatins O_1 (**56a**), O_2 (**56b**), and O_3 (**56c**), which were obtained and characterized as an inseparable mixture (ca. 1:1:1).[54] Importantly, enniatin C (**52**), previously reported as a synthetically known compound, has now been isolated from the same fermentation as a bona fide natural product.



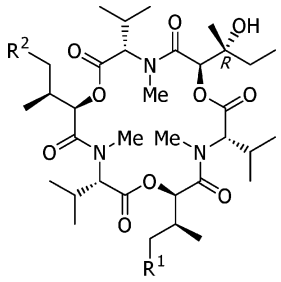
	R^1	R^2	R^3	R^4	R^5	R^6
enniatin B (**49**)	i-Pr	i-Pr	i-Pr	i-Pr	i-Pr	i-Pr
enniatin B_4 (**50**)	i-Bu	i-Pr	i-Pr	i-Pr	i-Pr	i-Pr
enniatin G (**51**)	i-Bu	i-Bu	i-Pr	i-Pr	i-Pr	i-Pr
enniatin C (**52**)	i-Bu	i-Bu	i-Bu	i-Pr	i-Pr	i-Pr
enniatin H (**53**)	i-Pr	i-Pr	i-Pr	*s*-Bu	i-Pr	i-Pr
enniatin I (**54**)	i-Pr	i-Pr	i-Pr	*s*-Bu	*s*-Bu	i-Pr
MK1688 (**55**)	i-Pr	i-Pr	i-Pr	*s*-Bu	*s*-Bu	*s*-Bu
enniatin O_1 (**56a**)	i-Bu	i-Pr	i-Pr	*s*-Bu	i-Pr	i-Pr
enniatin O_2 (**56b**)	i-Bu	i-Pr	i-Pr	i-Pr	*s*-Bu	i-Pr
enniatin O_3 (**56c**)	i-Bu	i-Pr	i-Pr	i-Pr	i-Pr	*s*-Bu



An unidentified fungus, BCC 2629, isolated from a spore attached to the synnema of *Hirsutella formicarum* (the asexual state of *Cordyceps unilateralis*) on an ant

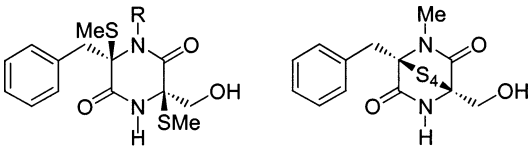
FIGURE 10. *Verticillium hemipterigenum.*

(from Khao Sok National Park, Surat Thani, southern Thailand) produced many enniatins, including four novel hydroxy analogues, enniatins L (**57**), M_1 (**58a**), M_2 (**58b**), and N (**59**).[55]



57 : $R^1 = R^2 = H$
58a : $R^1 = Me$, $R^2 = H$
58b : $R^1 = H$, $R^2 = Me$
59 : $R^1 = R^2 = Me$

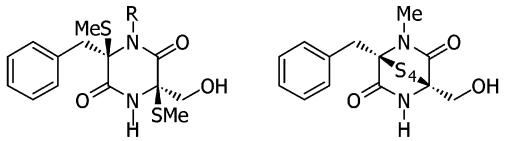
During the early investigation of *V. hemipterigenum* BCC 1449, two new diketopiperazines, **60** and **61**, were isolated together with enniatins from ethyl acetate extracts of the culture filtrate grown in PDB.[56] Upon changing the liquid medium to YES, there was a significant enhancement of diketopiperazine production, which led to the isolation of two additional new compounds, vertihemiptellide A (**63**) and B (**64**), and two known compounds, **62** and **65**.[57] The symmetrical dimeric compound **63** and its *N*-demethyl analogue, **64**, possess a hitherto unknown skeleton where two diketopiperazines are linked via two dithio bridges. The unusual structure of **63** was confirmed by X-ray crystallographic analysis, which also established the absolute configuration of this compound. The dimers **63** and **64** exhibited moderate antituberculous activity (MIC 12.5 μg/mL) and cytotoxicity.



60 : R = Me
62 : R = H

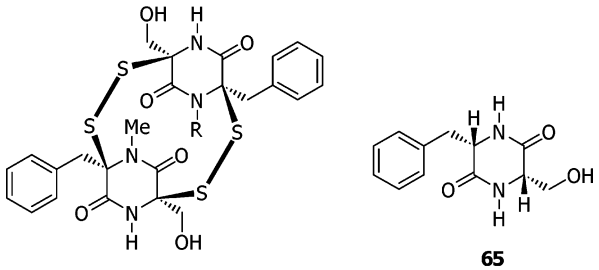
61

Chemical investigation of a different strain of *Verticillium hemipterigenum*, BCC 2370 (collected from Heo Narok waterfall, Khao Yai National Park, central Thailand),



60 : R = Me
62 : R = H

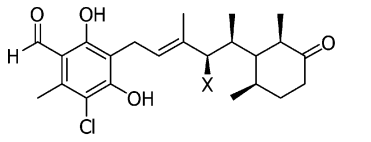
61



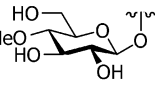
65

63 : R = Me
64 : R = H

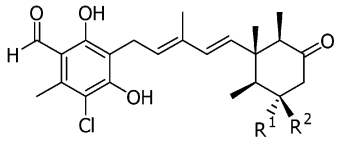
however, led to the isolation of a novel ascochlorin glycoside, vertihemipterin A (**66**), its aglycone, **67**, and a new analogue, 8′-hydroxyascochlorin (**68**), together with five known compounds in this class.[58]



66 : X =



67 : X = OH



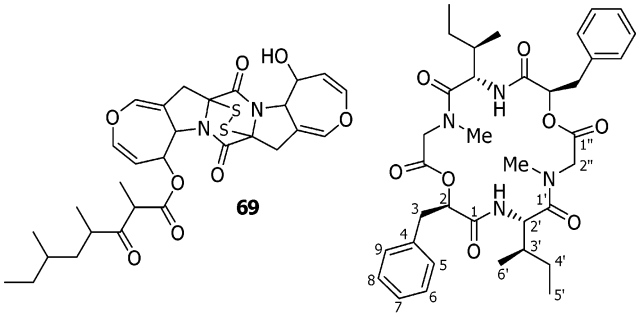
68 : $R^1 = OH$, $R^2 = H$

The entomopathogenic fungus *Hirsutella kobayasii* (a new species known only from Thailand) BCC 1660 (Figure 11) was found to produce a small amount of epidithiodike-

FIGURE 11. *Hirsutella kobayasii* on a cricket.

FIGURE 12. *Aschersonia tubulata* on scale insects.

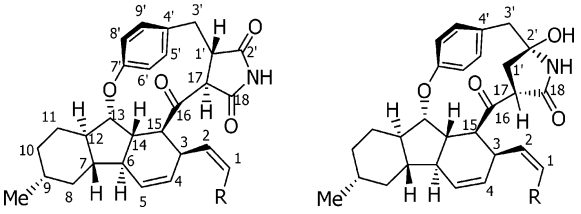
topiperazine **69**. The piperazine **69** exhibited potent antimycobacterial activity (MIC value of 0.15 ng/mL). Unfortunately, further biological testing of this highly promising anti-TB agent plus the chemical work to establish the complete stereostructure was not possible because of the lack of material. For unknown reasons, in the later fermentation batches, strain BCC 1660 ceased production of the piperazine **69**, instead, it provides a less active cyclohexadepsipeptide, hirsutellide A (**70**), which exhibited antimycobacterial activity with an MIC of 600−1200 ng/mL and weak antimalarial activity.[59]



69 **70**

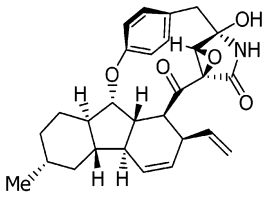
Hirsutella nivea (known only from Thailand on leafhoppers) BCC 2594 (collected from Khao Yai National Park), provided five new alkaloids, hirsutellones A−E (**71**−**75**).[60] Structures of the two major constituents, **71** and **72**, are related to antifungal substances GKK1032B and GKK1032A_2, respectively, which bear four additional methyl groups attached to C-3, -5, -7, and -11.[61] The difference in relative stereochemistry of compounds **71**−**75** to that of GKK1032s is on the tricyclic ring, where the opposite configuration at the C-13 position was observed. Pyrrocidines A and B, isolated from an unidentified filamentous fungus, also possess a similar molecular framework.[62]

Hirsutellones exhibited significant antituberculous activity (MIC of 0.78 μg/mL for **71**, **72**, and **73**) while showing little or no cytotoxicity to Vero cells (IC_{50} > 50 μg/mL for **71** and **72** and 12 μg/mL for **73**) and cancer cell lines. Especially, hirsutellone A (**71**) exhibited a high selectivity index (anti-TB/cytotoxicity) and deserves further investigation as an antituberculous lead.



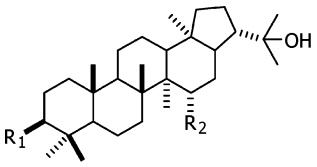
71: R = H
74 : R = Me

72 : R = H
75 : R = Me

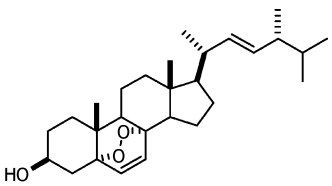


73

Aschersonia species are pantropical asexual states of *Hypocrella* infecting only scale insect nymphs. *Aschersonia tubulata* is known from Australia, Sri Lanka, and Thailand. Known (**76** and **77**) and new (**78**) hopane triterpenes together with an ergosterol endoperoxide, **79**, were found to be metabolites of *Aschersonia tubulata* BCC 1785 (Figure 12). Compounds **76** and **78** exhibited antimycobacterial activity (both with MICs of 12.5 μg/mL), while they were nontoxic to Vero cells (at 50 μg/mL).[63] Interestingly, results from screenings of other *Aschersonia* species shows that dustanin (**76**) and trihydroxyhopane (**77**) are consistently found in this genus; hence, it might be possible to use these hopanoids as chemotaxonomic markers for *Aschersonia*. In addition, the endoperoxide **79** was isolated from *Aschersonia* sp. BCC 1785; its corresponding glycoside was already reported to be found in *Cordyceps sinensis*.[23]



76, R_1 = H, R_2 = OH
77, R_1 = OH, R_2 = OH
78, R_1 = OAc, R_2 = OH



79

The long known anthraquinone dimers, (+)rugulosin and skyrin, were found in mycelia of *Aschersonia samoensis* strains BCC 1616, BCC 2015, and BCC 2061 (Figure 13). These two dimers showed selective cytotoxicity toward insect cells, suggesting that the fungus *A. samoensis* might be useful for future utilization as a pest control agent.[64] Another known dimer, the xanthone TMC-315A2, previously isolated from the fungus *Ceuthospora* sp. and patented for its use of prevention and control of osteoporosis,[65] was also isolated from *Aschersonia tamurai* BCC 1726.

FIGURE 13. *Aschersonia samoensis* on scale insects.

Conclusion

The work described in this Account demonstrates that insect fungi not only act as controllers of insect populations, but they are also sources of novel and sometimes structurally unusual chemical compounds. Moreover, types or amounts of chemical constituents produced by a specific fungus strain can be entirely different depending upon the nutrients used or technique employed during fermentation. Because the number of known species is only a small fraction of the estimated total number of the world's fungi, it is not unreasonable to assume that research on insect fungi has a long way to go before reaching maturity.

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