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A 3-Vinyl Cephem Derivative, a Useful Intermediate in the Synthesis of Cepham Antibiotics, from *Aspergillus awamori* Associated with Banana Fruit

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Aspergillus awamori was isolated from a diseased banana fruit, *Musa acuminata* cv. Ambul. The fungus was fermented in potato dextrose broth and on potato dextrose agar media and the fungal media were extracted with EtOAc. Chromatographic separation of the EtOAc extracts furnished 4-methoxybenzyl 7-phenylacetamido-3-vinyl-3-cephem-4-carboxylate (**1**), along with three naphtho-γ-pyrones, flavasperone (**2**), foncesinone A (**3**) and aurasperone A (**4**), and three alkaloids, aspernigrin A (**5**), pestalamide C (**6**) and nigragillin (**7**). Compound **1**, a known key intermediate in the chemical synthesis of cepham antibiotics, was isolated from a natural source for the first time. Compound **1** is the first 3-vinyl cephem derivative of microbial origin.

Keywords: 3-Vinyl cephem, *Aspergillus awamori*, *Musa acuminata*, Fungal metabolites, Cepham antibiotics.

New and more powerful drugs are necessary to combat various infectious diseases. Bioactive compounds originating as natural products can play a prominent role in this regard. Fungi have been historically found to be a promising source for the isolation of bioactive compounds [1]. The accidental discovery of penicillin, a broad-spectrum antibiotic during World War II paved the way for scientists worldwide to discover hidden bioactive compounds in the fungal world. The fungal kingdom includes many species with unique and unusual biochemical pathways. These pathways have produced a variety of secondary metabolites including important pharmaceuticals such as penicillin, cyclosporine, statins, and potent poisons such as aflatoxins and trichothecenes [2]. In a continuation of our studies directed towards the search for bioactive compounds from Sri Lankan flora, we studied the secondary metabolites produced by a fungus associated with fruits of the banana *Musa acuminata* cv. Ambul (local name: Ambul banana), which is the most widely cultivated banana variety in Sri Lanka [3]. Fruits of *Musa* sp. are very popular fruits that are consumed globally and are possibly the most consumed fruits in the world. When the fruit is ripe, it is however, more susceptible to fungal attacks [4].

Polyketides are the most common fungal secondary metabolites [2]. Among the polyketides, naphtho-γ-pyrones are the most common secondary metabolites produced by *Aspergillus* spp. [5]. It has been reported that some of these compounds possess antibacterial, antifungal [6], antitumor, cytotoxic [7] and acute toxic [8] activities.

In this article we describe the isolation and identification of an *Aspergillus* species associated with a diseased fruit of *M. accuminata* cv. and the isolation and structure elucidation of a unique 3-vinyl cephem derivative (**1**), along with six known secondary metabolites (**2**–**7**).

A pure culture of the *Aspergillus* strain was isolated from the inner side of a sterilized diseased banana fruit peel on potato dextrose agar medium (PDA) with the fungus being identified as *A. awamori* based on the sequencing of the internal transcribed spacer (ITS) region of rDNA. A number of endophytic fungi and bacteria, including *Aspergillus* spp., have been isolated from various parts of bananas [9].

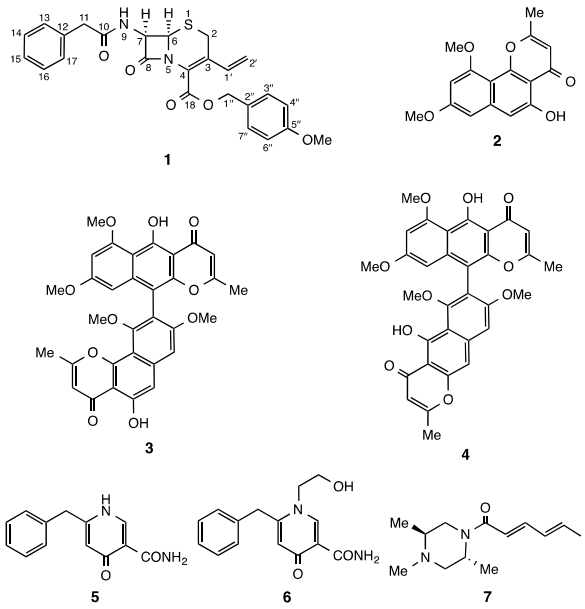


Figure 1: Structures of compounds **1**–**7**.

Fermentation of *A. awamori* was carried out on PDA and in potato dextrose broth (PDB) media for 4 weeks. The incubation media, as well as the mycelium of PDB, were individually extracted with EtOAc. These extracts were combined after TLC analysis and chromatographed over normal silica gel, reversed phase (C_{18}) silica gel, Sephadex LH-20 and reversed phase HPLC to give compounds **1**–**7** (Figure 1). Compounds **2**–**4** were identified as naphtho-γ-pyrones, flavasperone (**2**) [10], fonsecinone A (**3**) [11] and aurasperone A (**4**) [11,12], while compounds **5**–**7** were

characterized as the alkaloids, aspernigrin A (**5**) [13], pestalamide C (**6**) [14], and nigragillin (**7**) [15] by comparison of the spectral data with those reported. Compound **1** was determined to be a 3-vinyl cephem derivative.

Compound **1** was obtained as an amorphous powder. The molecular formula, $C_{25}H_{24}N_2O_5S$, was determined from the high resolution FABMS, which showed a pseudo-molecular ion at *m*/*z* 465.1493 (Calcd 465.1484 for $C_{25}H_{25}N_2O_5S$). As a result, fifteen degrees of unsaturation were expected from the molecular formula. The ^{1}H NMR spectrum of **1** indicated the presence of 24 protons and the characteristic signals of a methoxy group (δ 3.80, 3H, s), a mono-substituted phenyl ring (5H), a *para*-substituted benzene ring {δ 6.88 (2H, d, *J* = 7.0 Hz) and δ 7.27 (2H, d, *J* = 7.0 Hz)} and a vinyl group {δ 7.06 (1H, d, *J* = 17.5, 11.0 Hz), 5.43 (1H, d, *J* = 17.5 Hz) and 5.31 (1H, d, *J* = 11.0 Hz)}. In addition, the H-H COSY spectrum revealed the presence of a three-proton network for –HX–HC–HC– {δ 6.18 (d, *J* = 9.0 Hz), 5.80 (dd, *J* = 9.0, 5.0 Hz), 4.94 (d, *J* = 5.0 Hz, respectively}, which was eventually assigned to HN(9)–HC(7)–HC(6) (*vide infra*). The remaining 6 protons were assigned to three sets of methylene protons, which were all observed as AB doublets at δ 3.45/3.61 (*J* = 18.0 Hz), 3.61/3.67 (*J* = 18.0 Hz) and 5.17/5.22 (*J* = 18.0 Hz). The ^{13}CNMR spectrum of **1** displayed 25 carbon signals (the peaks for the *ortho* and *meta* carbons of the benzene rings were counted as two signals each). These carbons were classified into 8 quaternary, 12 methine, 4 methylene and one methoxy with the aid of the DEPT spectrum. The NMR data, in conjunction with the presence of two nitrogen atoms and one sulfur atom, were reminiscent of a β-lactam ring containing structure.

Table 1: NMR spectral data ($CDCl_3$) for compound **1** (mult. *J* in Hz).

No	^{13}C	^{1}H
2	24.0	3.45 (d, 18.0), 3.61 (d, 18.0)
3	124.2	–
4	126.3	–
6	57.5	4.94 (d, 5.0)
7	59.3	5.80 (dd, 9.0, 5.0)
8	164.6	–
9	–	6.18 (d, 9.0)
10	171.1	–
11	43.4	3.61 (d, 18.0), 3.67 (d, 18.0)
12	133.7	–
13/17	129.5	7.27 (brd, 7.0)
14/16	129.2	7.36 (brd, 7.0)
15	127.7	7.32 (brt, 7.0)
18	161.7	–
1′	131.9	7.06 (dd, 17.5, 11.0)
2′	117.7	5.43 (d, 17.5, 11.0), 5.31 (d, 11.0)
1″	67.9	5.17 (d, 12.0), 5.22 (d, 12.0)
2″	126.9	–
3″/7″	130.7	7.27 (δ, 7.0)
4″/6″	114.0	6.88 (δ, 7.0)
5″	159.9	–
6″-OCH_3	55.3	3.80 (s)

Figure 2 shows HMBC correlations observed for compound **1**, which helped to link the fragment structures described above. The mono-substituted phenyl group was extended to the phenylacetyl group (δ_C 43.3 for C-11, 171.1 for C-10), which was connected with the amino group at C-7 to form an amide linkage. The *para*-substituted benzene group was connected to the methoxy group (δ_C 55.3 for MeO) and one of the methylene carbons (δ_C 67.9) to form a *para*-methoxybenzyl alcohol moiety, which was used to make an ester linkage (δ_C 67.9 for C-1″; δ_C 161.7 for C-18) with the carboxyl group at C-4. The unique vinyl group was unambiguously located at the C-3 position based on the HMBC correlations shown

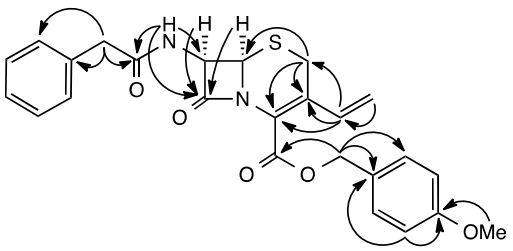
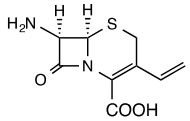


Figure 2: Key HMBC correlations of compound **1** (H→C).

in Figure 2. Location of the sulfur atom at the 1-position was supported by a rather unique upfield chemical shift of C-2 (δ_C 23.4), which ruled out the placement of other hetero atoms such as nitrogen and oxygen at the 1-position and was close to those of 3-cephem compounds [16]. The other nitrogen atom was required to make bonds with C-4, C-6 and C-8 to build up a β-lactam ring fused to a dihydrothiazine ring (3-cephem ring system) so that the structure satisfied the introduction of another two degrees of unsaturation. Thus, the structure of **1** was determined to be 4-methoxybenzyl 7-phenylacetamido-3-vinyl-3-cephem-4-carboxylate (4-methoxy benzyl 7-(2-phenylacetamido)-8-oxo-3-vinyl-5-thia-1-aza-bicyclo[4.2.0]oct-2-ene-2-carboxylate), as shown in Figure 1. This compound has been synthesized, but has not been isolated from a natural source. The complete assignment of NMR data for **1** is listed in Table 1, since neither ^{1}H nor ^{13}C NMR data of **1** have been reported in the literature.

7-Amino-3-vinyl-3-cephem-4-carboxylic acid (7-AVCA) (**8**) (Figure 3) is a key intermediate in the synthesis of orally active third-generation cephalosporin antibiotics such as cefixime and cefdinir [17]. 7-AVCA can be synthesized starting from deacetyl cephalosporin C, 7-aminocephalosporanic acid, or 7-phenylacetamido-3-chloromethyl-3-cephem-4-carboxylate [17]. However, the conversion of these starting materials to 7-AVCA requires several steps, including the manipulation of the vinyl group at the C-3 position. Compound **1** has the vinyl substituent and does not require any step for the introduction of the vinyl group. Thus, the isolation of **1** as a microbial product is of great significance, since it is anticipated that the synthesis of 7-AVCA can be achieved only in two steps (e.g., treatment with trifluoroacetic acid for the removal of the *p*-methoxybenzyl group and enzymatic hydrolysis with penicillin G amidase) [17]. It is worthy of identifying the genes that are associated with the introduction of the vinyl group and the biosynthetic mechanism for the introduction of the vinyl group. Once the genes for the biosynthesis of **1** are identified, it will open the way to produce the 3-vinyl cephem compound in quantity by fermentation through a genetic engineering study. To our knowledge this is the first paper on the production of cephEm type compounds by *Aspergillus* sp., although some *Aspergillus* sp. are known to produce penicillins [18].



8

Figure 3: Structure of 7-AVCA **8**.

Compounds **2–6** were tested for brine shrimp [19], DPPH radical scavenging [20] and antifungal activity against *Cladosporium cladosporioides* [21], phytotoxicity against *Lactuca sativa* [22], and α-amylase inhibitory activity [23]. Brine shrimp toxicity of

compounds **2** and **3** (LD_{50} value (50% mortality of brine shrimps): 50 and 9 ppm, respectively) is reported in our previous paper [24]. Compounds **4–6** displayed negligible toxicity (LD_{50} value, >100 ppm) in the assay. Compounds **2–6** did not show any significant activity in the DPPH radical scavenging, antifungal activity against *C. cladosporioides*, phytotoxicity against *Lactuca sativa* and α-amylase inhibitory activity assays. Compound **7** is reported to show considerable DPPH radical scavenging activity [25].

Experimental

General: ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX-500 (500 MHz for ^{1}H and 125 MHz for ^{13}C) spectrometer. ^{1}H chemical shifts are reported with reference to the internal standard TMS (0.000 ppm), while ^{13}C-chemical shifts are referenced to $CDCl_3$ (77.00 ppm). HRMS-FAB spectra were obtained on a JEOL JMS-700 spectrometer with 3-nitrobenzylalcohol as the matrix. Silica gel (Art No. 7734 and 9385, Merck), RP-silica gel (Art No. 1.13900.250, 40-63 μm, Merck) and Sephadex LH-20 (Art No. 20100, Fluka) were used for chromatography. RP-HPLC was carried out with a Shimdzu LC-6A apparatus attached with an ODS column (30 cm x 1 cm i.d.) monitored with a UV detector (254 nm).

Isolation and identification of A. awamori from Musa acuminata cv. Ambul: Ripe fruit of the banana were collected from the Central Province of Sri Lanka in September 2011. A diseased banana fruit was washed with running water, followed by ethanol, sterilized with 2.5% NaOCl and washed with distilled water, respectively, 3 times. A segment from the inner part of the peel of the sterilized fruit was placed on a Petri dish containing PDA medium and incubated in the dark at 25°C for one week. Emerging fungi were isolated after 5 days and sub-cultured to obtain a black colored pure culture, which was identified as *Aspergillus awamori* through molecular means using the internal transcribed (ITS) region of the DNA gene (PCR and DNA sequencing was carried out by the GeneTech Institute, Sri Lanka). The sequence matched 100% with that of *A. awamori* SRRC 332 (GenBank Accession No. AY373840.1). Photographic evidence of the banana fruits and *Aspergillus awamori* strain (IFS/SB/1/2014) were deposited at the Institute of Fundamental Studies.

Fermentation, extraction and isolation of compound 1: A pure culture of *Aspergillus awamori* was inoculated on PDA medium in 40 Petri dishes (10 cm) and 20 1L Erlenmeyer flasks each containing 400 mL PDB medium. Inoculated Petri dishes and flasks were allowed to stand incubating at room temperature. After 4 weeks, the PDA media was extracted with EtOAc using a sonicator to give the EtOAc extract (1.07 g). The PDB medium was filtered and the filtrate was extracted with *n*-hexane and EtOAc to give the *n*-hexane extract (1.00 g) and the EtOAc extract (1.15 g). The residual mycelium was extracted with EtOAc using a sonicator to give the EtOAc extract (0.88 g). TLC analysis indicated that the major constituents in the three extracts were identical. Hence, the EtOAc extracts were combined and subjected to chromatographic separation over silica gel (*n*-hexane-CH_2Cl_2-MeOH and *n*-hexane-EtOAc-MeOH – in increasing order of polarity), reverse phase C_{18} silica gel (50-5% of H_2O-MeOH), Sephadex LH-20 (MeOH) and reverse phase HPLC (15% H_2O–MeOH, flow rate 1.0 mL/min, ODS) to give the cephem derivative (**1**, 10.6 mg). Similar fractionation afforded flavasperone (**2**, 7.7 mg), foncesinone A (**3**, 13.6 mg), aurasperone A (**4**, 17.0 mg), aspernigrin A (**5**, 7.6 mg), pestalamide C (**6**, 8.6 mg) and nigragillin (**7**, 6.0 mg).

4-Methoxybenzyl 7-phenylacetamido-3-vinyl-3-cephem-4-carboxylate (1)

White amorphous solid.

^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz): Table 1.

HRFABMS *m/z* 465.1493 $[M+H]^+$ (465.1484 Calcd for $C_{25}H_{25}N_2O_5S$).

Biological assays: Brine shrimp toxicity [19], DPPH radical scavenging activity [20], antifungal activity against *Cladosporium cladosporioides* [21], phytotoxicity against *Lactuca sativa* [22], and α-amylase inhibitory activity [23] were examined as reported previously.

Supplementary data: ^{1}H, ^{13}C NMR, H-H COSY and HMBC spectra of **1** are available.

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