3-Nitropropionic Acid (3-NPA), a Potent Antimycobacterial Agent from Endophytic Fungi: Is 3-NPA in Some Plants Produced by Endophytes?

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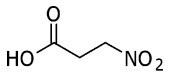
3-Nitropropionic acid (3-NPA, **1**) was found in extracts of several strains of endophytic fungi. 3-NPA (**1**) exhibited potent antimycobacterial activity with the minimum inhibition concentration of 3.3 μM, but was inactive against NCI-H187, BC, KB, and Vero cell lines. Endophytes were found to produce high levels of 3-NPA (**1**), and therefore 3-NPA (**1**) accumulated in certain plants may be produced by the associated endophytes. 3-NPA (**1**) may be used as a chemotaxonomic marker for endophytic fungi. The structure of 3-hydroxypropionic acid, a nematicidal agent, should be revised to 3-NPA (**1**).

Endophytic fungi are rich sources of bioactive compounds,[1−3] and recently several novel bioactive substances have been isolated from these microorganisms.[4−8] We have isolated a number of endophytic fungi from Thai medicinal plants and evaluated biological activities of fungal crude extracts. Consistently, crude extracts of many strains of endophytic fungi exhibited antimycobacterial activity. Chemical exploration of those active crude extracts led to the identification of 3-nitropropionic acid (3-NPA, **1**) as a potent antimycobacterial agent.



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3-NPA (**1**) was obtained from a broth extract of *Phomopsis* sp. strain usia5 after purification with Sephadex LH-20, with a yield of 178 mg/L culture medium. 3-NPA (**1**) was characterized by analyses of its spectroscopic data, as well as by comparison of data with those of an authentic sample (Sigma). ^{1}H NMR analysis of fungal crude extracts revealed that there were seven strains of the isolated endophytes capable of producing 3-NPA (**1**) (Table 1). 3-NPA (**1**) has been widely found in plants[9−14] and some fungi.[15,16] However, in the present work, since endophytic fungi excreted high levels (up to 178 mg/L) of 3-NPA (**1**) to their culture media, the question has been raised on the origin of 3-NPA (**1**) accumulated in plants. Interestingly, a previous report showed that only one from six and eight species of the respective plant genus *Astragalus* (*A. falcatus*) and *Coronilla* (*C. viminalis*) contained 3-NPA (**1**), while three from six species of the genus *Lotus* (*L. angustissimus*, *L. pedunculatus*, and *L. uliginosus*) possessed 3-NPA (**1**), as shown in Table 2.[11] Normally, if a particular secondary metabolite is found in a plant species, it should be found in most or all of those plant genus, e.g., the presence of 3-NPA (**1**) in the genus *Hippocrepis*, *Scorpiurus*, and *Securigera* (Table 2). Further, it is well recognized that certain secondary metabolites may be employed as chemotaxonomic markers in particular plant genera.[17−19] Therefore, there is a question on the origin of 3-NPA (**1**) in *A. falcatus* and *C. viminalis*, the only 3-NPA-producing species in the respective genera *Astragalus* and *Coronilla* (Table 2). It is possible that 3-NPA (**1**) accumulated in *A. falcatus* and *C. viminalis* may not originate from these plants, but may be produced by associated endophytes. In contrast, 3-NPA (**1**) should be a plant metabolite of the genuera *Hippocrepis*, *Scorpiurus*, and *Securigera* (Table 2). Studies on the interaction and correlation between plant hosts and endophytes regarding the production of 3-NPA (**1**) need to be pursued. Furthermore, 3-NPA (**1**) has recently been found to be significantly involved in nitrification processes in leguminous plants.[11] As endophytes are able to produce high levels of 3-NPA (**1**), endophytic fungi may also participate in the nitrification process of the nitrogen cycle.

Recently, Schwarz et al. reported 3-hydroxypropionic acid as a nematicidal principle in endophytic fungi and also reported that several strains of endophytes could produce 3-hydroxypropionic acid.[20] However, comparison of ^{1}H and ^{13}C NMR data[20] with those of 3-NPA (**1**) showed that the structure of the claimed nematicidal agent, 3-hydroxypropionic acid, is more likely to be 3-NPA (**1**). Although 3-NPA (**1**) is found in plants[9−14] and some fungi,[15,16] there have been no reports on the production of **1** by fungi of endophytic origin. During 1999−2004, in addition to our chemical investigation of many types of fungi (insect pathogenic, seed, wood-decaying, and marine fungi),[21] the ^{1}H NMR screening of more than two hundred fungal crude extracts showed that only a few strains of seed fungi (another plant-associated fungi) produced 3-NPA (**1**). The presence of 3-NPA (**1**) in many strains of fungal endophytes reported here (Table 1), together with the previous data on the existence of 3-NPA (mistakenly described as 3-hydroxypropionic acid) in several strains of endophytic fungi, *Phomopsis phaseoli* and *Melanconium betulinum*,[20] suggested

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Table 1. Strains of 3-NPA-Producing Endophytes and Plant Hosts

fungal strains and hosts	location of plant collection
Phomopsis sp. usia5 from leaf of *Urobotrya siamensis* (Opiliaceae)	Nakhonrachasima Province, Thailand
Unidentified fungal strain grsp11 from twig of *Grewia* sp. (Tiliaceae)	Pitsanulok Province, Thailand
Unidentified fungal strain grsp19 from twig of *Grewia* sp. (Tiliaceae)	Pitsanulok Province, Thailand
Phomopsis sp. mfer5 from twig of *Mesua ferrea* (Guttiferae)	Chiangmai Province, Thailand
unidentified fungal strain rlyi1 from twig of *Rhododendron lyi* (Ericaceae)	Pitsanulok Province, Thailand
unidentified fungal strain tasp15 from twig of *Tadehagi* sp. (Leguminosae)	Pitsanulok Province, Thailand
unidentified fungal strain gell14 from twig of *Gmelina elliptica* (Labiatae)	Pitsanulok Province, Thailand

Table 2. Occurrence of 3-NPA in Legumes[11]

species	3-NPA (μmol/g), $n = 3$
Astragalus alpinus	0
A. cicer	0
A. danicus	0
A. falcatus	69.8 (±2.6)
A. lusitanicus	0
A. mexicanus	0
Coronilla coronata	0
C. juncea	0
C. minima	0
C. scorpioides	0
C. vaginalis	0
C. valentina	0
C. valentina glauca	0
C. viminalis	38.5 (±1.8)
Hippocrepis balearica	24.7 (±4.2)
H. comosa	142.0 (±7.5)
H. emerus emerus	60.8 (±3.3)
H. unisiliquosa	61.5 (±5.7)
Lotus alpinus	0
L. angustissimus	6.9 (±0.6)
L. corniculaus	0
L. creticus	0
L. edulis	0
L. pedunculatus	13.8 (±2.2)
L. tenuis	0
L. tetragonolobus	0
L. uliginosus	63.3 (±4.0)
Onobrychis arenaria	0
O. montana	0
Oxytropis campestris	0
O. gaudinii	0
O. jacquinii	0
Scorpiurus muricatus	9.4 (±0.6)
S. vermiculatus	18.0 (±0.9)
Securigera orientalis	22.3 (±0.8)
S. parviflora	47.4 (±12.4)
S. securidaca	41.9 (±1.7)
S. varia	85.9 (±6.3)

that 3-NPA (**1**) may be used as a chemotaxonomic marker for endophytic fungi. In the present work, two *Phomopsis* strains, usia5 and mfer5, were 3-NPA-producing endophytes (Table 1); however other fungal strains could not be taxonomically identified.

3-NPA (**1**) exhibited potent antimycobacterial activity against *Mycobacterium tuberculosis* H37Ra with the minimum inhibition concentration (MIC) value of 3.3 μM. 3-NPA (**1**) at 167.9 μM was inactive against NCI-H187 (small-cell lung cancer), BC (breast cancer), and KB (oral cavity cancer) cell lines. 3-NPA (**1**) was also inactive (at 419.8 μM) toward the Vero cell line. 3-NPA (**1**) is a known inhibitor of the mitochondrial enzyme succinate dehydrogenase (a part of complex II), which links the tricarboxylic acid cycle to the respiratory electron transport chain, and its toxicity has gained acceptance as an animal model of Huntington's disease.[22−25] The mode of action of 3-NPA (**1**) for antimycobacterial activity may be related to the inhibition of mycobacterial succinate dehydrogenase. Recent proteomic studies of *M. tuberculosis* revealed the presence of a succinate-semialdehyde dehydrogenase gene in the bacterium,[26] and therefore 3-NPA (**1**) could be a potential lead chemotype for this enzyme target. It should be noted that 3-NPA (**1**) has been known as a potent neurotoxic agent.[27]

Experimental Section

General Experimental Procedures. NMR experiments were carried out on a Bruker DRX 400 NMR spectrometer, operating at 400 MHz for proton and 100 MHz for carbon. The ESITOFMS were obtained using a Micromass LCT mass spectrometer, and the lock mass calibration was applied for determination of the accurate masses.[28]

Isolation of Endophytic Fungi. Healthy leaves and twigs were collected from six species of Thai medicinal plants (*Urobotrya siamensis*, *Grewia* sp., *Mesua ferrea*, *Rhododendron lyi*, *Tadehagi* sp., and *Gmelina elliptica*) in the forest areas of Chiangmai, Nakhonrachasima, and Pitsanulok Provinces of Thailand. Plant samples were cleaned under running tap water and then air-dried. Before surface-sterilization, the cleaned twigs were cut into pieces 5 cm long. Leaf and twig fragments were surface-sterilized as described by Schulz and co-workers[29] with some modifications. Plant fragments were sequentially immersed in 70% EtOH for 1 min, 5% NaOCl solution for 5 min, and sterile distilled H_2O for 1 min (two times). The surface-sterilized leaf and twig fragments were cut into small pieces using a sterile blade and placed on sterile water agar plates for further incubation at 30 °C. The hyphal tip of the endophytic fungus growing out from the plant tissue was cut by a sterile pasture pipet and transferred onto a sterile potato dextrose agar (PDA) plate. After incubation at 30 °C for 7−14 days, culture purity was determined from colony morphology.

Identification of Endophytic Fungi. Endophytic fungus isolates mfer5 and usia5 were identified on the basis of both morphology of fungi grown on banana leaf agar at 25 °C for 1−2 months and analysis of the DNA sequences of the ITS region of ribosomal RNA gene. Total DNA was extracted from fungal mycelia grown in potato dextrose broth. Primers ITS5 (GGAAGTAAAAGTCGTAACAAGG) and ITS4 (TCCTCCGCTTATTGATATGC) were used to amplify the ITS1-5.8S-ITS2 region from total DNA extracts.[30,31] The thermal cycle program was as follows: 3 min at 95 °C followed by 30 cycles of 50 s at 94 °C, 40 s at 48 °C, and 40 s at 72 °C, with a final extension period of 10 min at 72 °C. The amplified DNA was purified and directly subjected to sequencing reactions using primers ITS5 and ITS4. BLASTN 2.2.10 was used to search for similar sequences in the GenBank.[32] DNA sequence similarity was determined by the ClustalW (1.82) multiple sequence alignment program.[33] Phylogenetic relationship was estimated using PAUP* (v 4.0 b10).[34]

Endophytic fungi isolates mfer5 and usia5 grew on PDA as a white filamentous fungus colony. On banana leaf agar they developed black stromata bearing pycnidia. Only α-conidia were produced by the isolate usia5, while both α-conidia and β-conidia could be observed in the isolate mfer5. These characteristics suggest that they are members of the genus *Phomopsis*, but should be different species. A GenBank search for similar sequences to their ITS regions revealed several species of *Phomopsis* and their teleomorphs, *Diaporthe*, as the closest matches for similar sequences, with sequence identities of 90%−98% for the 18 known species closest hit. This confirmed that mfer5 and usia5 are *Phomopsis*. The mfer5 sequence showed the highest identity (98%) to those of *P. eucommii* and *P. lagerstroemiae*, while the usia5 sequence

exhibited the highest identity (97%) to those of *P. quercina*, *P. vaccinii*, *P. amygdali*, and *P. juniperivora*. The sequence similarity between strains mfer5 and usia5 was 93%. Phylogenetic analysis using maximum parsimony and a neighbor-joining algorithm placed them in two different clades, suggesting that they are different species in the genus *Phomopsis*. The DNA sequences ITS1-5.8S-ITS2 of mfer5 and usia5 have been submitted to GenBank with accession numbers AY907347 and AY907348, respectively. Cultures of *Phomopsis* sp. mfer5 and usia5 have been deposited at the BIOTEC Culture Collection, Thailand, under the registration codes BBC 4196 and BCC 9036, respectively.

Extraction and Isolation of 3-NPA (1). *Phomopsis* sp. usia5 was cultured in a MID[35] culture medium (4 L) for 21 days at 25 °C. Fungal cells were separated from a broth by filtration, and a culture broth was subsequently extracted with EtOAc (equal volume, ×3), yielding 1.2 g of a crude extract. The extract was subjected to a Sephadex LH-20 column (3 × 60 cm), which was eluted with MeOH. Nine fractions (each 90 mL) were obtained, and fraction 5 contained 3-NPA (**1**). A yield of 178 mg/L (culture broth) was obtained for 3-NPA (**1**).

3-Nitropropionic Acid (3-NPA) (1): white solid; ^{1}H and ^{13}C NMR data are identical to those of an authentic sample (Sigma); ESITOFMS (positive ion mode) *m/z* 142.0108 $[M + Na]^+$, calcd for $[C_3H_5O_4N + Na]^+$, 142.0116; ESITOFMS (negative ion mode) *m/z* 118.0143 $[M - H]^-$, calcd for $[C_3H_5O_4N-H]^-$, 118.0140.

Bioassays. Antimycobacterial activity was assessed against *Mycobacterium tuberculosis* H37Ra using the Microplate Alamar Blue Assay (MABA).[36] The mycobacterium *M. tuberculosis* H37Ra was cultured in Middle-brook 7H9 broth. The standard drugs isoniazid and kanamycin sulfate, used as reference compounds for the antimycobacterial assay, showed MIC values of 0.29−0.66 and 3.4−8.5 μM, respectively. The MIC values of the reference compounds were determined in the same experiment as experimental samples. Cytotoxicity was determined by employing the colorimetric method described by Skehan and co-workers.[37] The reference compound, ellipticine, exhibited activity toward the Vero, KB, NCI-H187, and BC cell lines with an IC_{50} range of 0.8−1.2 μM.

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