Cyclopentenones, Scaffolds for Organic Syntheses Produced by the Endophytic Fungus Mitosporic Dothideomycete sp. LRUB20

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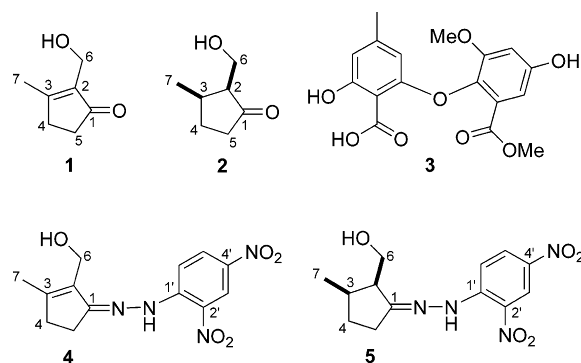
Received April 3, 2006

Two new natural products, 2-hydroxymethyl-3-methylcyclopent-2-enone (**1**) (synthetically known) and *cis*-2-hydroxymethyl-3-methylcyclopentanone (**2**), and a known compound, asterric acid (**3**), were isolated from the endophytic fungus mitosporic Dothideomycete sp. LRUB20, which was isolated from the stem of the Thai medicinal plant *Leea rubra*. Compound **2** was separated and identified in the form of its 2,4-dinitrophenylhydrazone derivative (**5**). Compounds **1**, **3**, and hydrazone **5** exhibited mild antimycobacterial activity, both with MIC values of 200 μg/mL. Compounds **1**, **3**, and **4** were inactive (at 50 μg/mL) toward Vero, KB, NCI-H187, and BC cell lines. Hydrazone **5** showed only mild cytotoxicity against the Vero cell line with an IC_{50} value of 21.7 μg/mL; however, it was inactive toward KB, NCI-H187, and BC cell lines. Endophytic fungi may be a source for the production of building blocks for organic syntheses.

Thailand is located in a tropical area, furnishing great biodiversity ranging from higher organisms (plants and animals) to microorganisms. Thai microorganisms isolated from unique habitats, e.g., actinomycetes from acidic peat swamps,[1] fungal endophytes from Thai medicinal plants,[2,3] entomopathogenic fungi from insects,[4] and bacteria from marine origins,[5] have been shown to be sources of bioactive compounds. Endophytic fungi are a potential source for the production of a diverse array of bioactive metabolites, some of which are promising candidates for drug development.[6] Interestingly, particular fungal endophytes are able to produce bioactive molecules previously isolated from higher plants, including useful drugs, e.g., Taxol and camptothecin.[7] Furthermore, plant metabolites may actually originate from associated endophytes.[2,8] The present work demonstrates that, in addition to being a source of bioactive substances, endophytic fungi also produce useful chemical scaffolds for organic syntheses. Two new natural products, 2-hydroxymethyl-3-methylcyclopent-2-enone (**1**) (synthetically known) and *cis*-2-hydroxymethyl-3-methylcyclopentanone (**2**), and a known compound, asterric acid (**3**), were isolated from the fungal endophyte mitosporic Dothideomycete sp. strain LRUB20, which was isolated from the stem of the Thai medicinal plant *Leea rubra* Blume ex Spreng. (Leeaceae).

Purification of the culture broth extract of the endophytic fungus strain LRUB20 by repeated Sephadex LH-20 column chromatography furnished 2-hydroxymethyl-3-methylcyclopent-2-enone (**1**) and asterric acid (**3**). Spectroscopic data of asterric acid (**3**) were in good agreement with reported data.[9] Since compound **2** could not be separated from **1** by column chromatography (Sephadex LH-20 and Si gel) and HPLC (C_{18} reversed phase), it was separated and identified in the form of its 2,4-dinitrophenylhydrazone derivative (**5**).

2-Hydroxymethyl-3-methylcyclopent-2-enone (**1**) was obtained as a colorless oil. Comparison of the ^{1}H and ^{13}C spectra of **1** with



those of 2-hydroxymethyl-3-methylcyclopent-2-enone, a common chemical scaffold in organic synthesis,[10] suggested that **1** was 2-hydroxymethyl-3-methylcyclopent-2-enone. It should be noted that δ_C (173.6) of the C-3 double bond in **1** was significantly shifted downfield, similar to the carbonyl resonances for carboxylic acids or esters. Compound **4**, i.e., a 2,4-dinitrophenylhydrazone derivative of **1**, was prepared, and the δ_C (158.2) of C-3 was 15.4 ppm shifted upfield. ^{1}H−^{1}H COSY and HMBC spectra allowed assignment of protons and carbons in **1** and **4**. Furthermore, X-ray single-crystal analysis of 2,4-dinitrophenylhydrazone **4** confirmed the structure of **1** (see Supporting Information).

As mentioned earlier, compound **2** was obtained as an inseparable mixture with compound **1**. Subsequently, the cyclopentenone mixture of **1** and **2** was transformed to the corresponding 2,4-dinitrophenylhydrazones **4** and **5**, which were separated on a Sephadex LH-20 column. The ESITOFMS spectrum indicated the molecular formula $C_{13}H_{16}O_5N_4$ for hydrazone **5**. The ^{1}H−^{1}H COSY spectrum of **5** revealed the connectivity from H-2 through H-7, while the HMBC spectrum showed correlations from H-2, H-4, H-5, and H-6 to C-1 and from the NH proton to C-1, C-1′, C-2′, and C-6′, establishing the gross structure of **5**. The NOESY spectrum of **5** showed intense cross-peaks between the H-6 methylene and H-7 methyl and between the H-7 methyl and H-4β, suggesting a *cis* orientation of the methyl and methylene groups. On the basis of these data, compound **5** was a hydrazone derivative of *cis*-2-hydroxymethyl-3-methylcyclopentanone (**2**). While the *cis* isomer of 2-hydroxymethyl-3-methylcyclopentanone (**2**) is a natural

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10.1021/np060148h CCC: $33.50 © 2006 American Chemical Society and American Society of Pharmacognosy
Published on Web 08/04/2006

product, its *trans* isomer is widely used, together with cyclopentenone **1**, as an intermediate in the chemical synthesis of natural products.[11]

Among the compounds isolated, 2-hydroxymethyl-3-methylcyclopent-2-enone (**1**), asterric acid (**3**), and hydrazone **5** exhibited mild antimycobacterial activity, with a minimum inhibitory concentration (MIC) value of 200 μg/mL. Compounds **1**, **3**, and **4** were inactive (at 50 μg/mL) toward Vero, KB, NCI-H187, and BC cell lines. Hydrazone **5** showed only mild cytotoxicity against the Vero cell line, with an IC_{50} value of 21.7 μg/mL; however, it was inactive toward KB, NCI-H187, and BC cell lines. Cyclopentenones in nature are rare and occur as 2-cyclopentenones with an odd-number carbon alkyl chain attached at C-5 or as 2,3-dimethyl-2-cyclopentenone commonly found in volatile components in food.[12] Due to interesting antibacterial and antifungal activities reported,[12] synthetic chemists have shown interest in the syntheses of cyclopentenones.[13] In the present work, despite the fact that fungal metabolites **1** and **2** are not attractive in bioassays (anticancer and antimycobacteria), they are useful scaffolds for organic synthesis.[10,11] Green chemistry may play a crucial role in future chemical syntheses, and the use of microbial building blocks for subsequent use in organic syntheses provides fundamental advantages in comparison to chemical methods.[14] The term "white biotechnology" has been recently introduced for microbial production of building blocks, and various 2-oxocarboxylic acids are examples of chemical scaffolds from microbial fermentation.[14] In the present study, the fungal metabolite 2-hydroxymethyl-3-methylcyclopent-2-enone (**1**) could be obtained with high yield up to 0.4 g from 5 L of culture broth when culturing the fungus LRUB20 in a particular culture medium (data not shown). Therefore, the production of **1** in multigram scale is practically possible. Unfortunately, the important chiral derivative **2** is generally found as a minor component in all culture media investigated. On the basis of this investigation, endophytic fungi may be useful for the production of building blocks for organic syntheses.

Experimental Section

General Experimental Procedures. Optical rotation was measured on a JASCO DIP370 polarimeter, and UV spectra were recorded on a Cary 1E UV−vis spectrophotometer. IR spectra were measured on a Perkin-Elmer 2000 spectrometer. NMR experiments were carried out on a Bruker DRX 400 NMR spectrometer, operating at 400 MHz for proton and 100 MHz for carbon spectra. The ESITOFMS spectra were obtained using a Micromass LCT mass spectrometer.

Isolation of Endophytic Fungus. An apparently healthy twig was collected from a Thai medicinal plant, *Leea rubra* Blume ex Spreng. (family Leeaceae), in the forest areas of Pitsanulok Province, Thailand. The sample was cleaned under running tap water and then air-dried. The cleaned twig was cut into a 5 cm piece, and the fragment was surface-sterilized as described by Schulz and co-workers[15] with some modifications. The fragment was sequentially immersed in 70% EtOH for 1 min, NaOCl solution (6% available chlorine) for 5 min, and sterile distilled H_2O for 1 min (two times). Using a sterile blade, the twig fragment was then cut into small pieces and placed on sterile water agar plates for incubation at 30 °C. The hyphal tip of the endophytic fungus growing out from the plant tissue was removed using 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 Fungus. Endophytic fungus isolate LRUB20 was identified on the basis of both morphology on PDA and banana leaf agar and analysis of the DNA sequences of the 18S ribosomal RNA gene and ITS region of the ribosomal RNA gene. Total DNA was extracted from fungal mycelia grown in potato dextrose broth using DNeasy plant mini kit (Qiagen). Primers NS1 (GTAGTCATATGCTTGTCTC) and NS8 (TCCGCAGGTTCACCTACGGA)[16] were used to amplify the 18S rRNA gene from total DNA extracts. The thermal cycle program was as follows: 3 min at 95 °C followed by 30 cycles of 50 s at 95 °C, 40 s at 42 °C, and 2 min 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 NS1, NS2, NS3, NS4, NS5, NS6, NS7, and NS8.[16] Primers ITS5 (GGAAGTAAAAGTCGTAACAAGG) and ITS4 (TCCTCCGCTTATTGATATGC)[16] were used to amplify the ITS1−5.8S−ITS2 region from total DNA extracted. The thermal cycle program was as follows: 3 min at 95 °C followed by 30 cycles of 50 s at 95 °C, 40 s at 45 °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[17] was used to search for similar sequences in the GenBank. DNA sequence similarity was determined by the ClustalW (1.82) multiple sequence alignment program.[18] Phylogenetic relationship was estimated using PAUP* (v 4.0 b10).[19]

Endophytic fungus isolate LRUB20 grown on PDA had a gray velvety colony and produced characteristic brown spores. On banana leaf agar it did not develop any fruiting body after cultivation at 25 °C for 2 months. A GenBank search for similar sequences showed that the 18S rRNA gene sequence of LRUB20 had 96% sequence identities to those of *Acrospermum compressum* and *A. gramineum* in the family Acrospermaceae, subphylum Pezizomycotina, phylum Ascomycota. Phylogenetic analysis of LRUB20 with 33 known species hit using maximum parsimony placed it in the same clade with *A. compressum* and *A. gramineum* with 100% bootstrap support. Microscopic morphology of LRUB20 differs apparently from that of *Dactylaria* (the anamorph of *A. compressum*) and *Virgariella* (the anamorph of *A. graminum*).[20,21] On the basis of the current Ascomycota systematics,[22] LRUB20 is tentatively classified as a mitosporic species member of Dothideomycetes et Chaetothyriomycetes incertae sedis, class Dothideomycetes, subphylum Pezizomycotina. In an attempt to classify LRUB20 to lower taxonomic level, the ITS1−5.8S−ITS2 sequence was used as query sequence. The highest ITS sequence similarity to known species hit was only 78% to *Mycoleptodiscus terrestris*, a mitosporic Magnaporthaceae, Sordariomycetes incertae sedis, subphylum Pezizomycotina, suggesting that LRUB20 is potentially a new species. The 18S rRNA gene sequence and ITS sequence of LRUB20 have been submitted to GenBank with accession number DQ381536 and DQ384608, respectively. The culture of isolate LRUB20 has been deposited at the MIM Laboratory, Department of Microbiology, Mahidol University, Thailand.

Bioassays. Cytotoxicity was determined by employing the colorimetric method described by Skehan and co-workers.[23] The reference compound, ellipticine, exhibited activity toward the Vero, KB, NCI-H187, and BC cell lines with IC_{50} ranges of 0.2−0.3 μg/mL. Antimycobacterial activity was assessed against *Mycobacterium tuberculosis* H37Ra using the microplate Alamar Blue assay (MABA).[24] The mycobacterium *M. tuberculosis* H37Ra was cultured in Middlebrook 7H9 broth. The standard drugs isoniazid and kanamycin sulfate showed MIC values of 0.040−0.090 and 2.0−5.0 μg/mL, respectively.

Extraction and Isolation. The endophytic fungus mitosporic Dothideomycete sp. LRUB20 was cultured in a malt Czapek culture medium (5 L) for 21 days at 25 °C. Fungal cells were separated from the culture broth by filtration, and the broth was subsequently extracted with EtOAc (equal volume, × 3), yielding 1.5 g of a crude extract. The extract was subjected to a Sephadex LH-20 column (3 × 60 cm), eluted with MeOH, to yield 10 fractions (A1−A10). Fraction A7 was crystallized (MeOH), yielding 198 mg of asterric acid (**3**). Fraction A5 was further purified by a Sephadex LH-20 column (2.5 × 52 cm), and nine fractions (B1−B9) were obtained. Fractions B3 and B4 were combined and subjected to Sephadex LH-20 column chromatography (1.5 × 43 cm) to afford seven fractions (C1−C7). Fraction C5 contained 83 mg of 2-hydroxymethyl-3-methylcyclopent-2-enone (**1**), while fraction C6 was a mixture of compound **1** and compound **2**. Further separation by Sephadex LH-20, Si gel, and HPLC (C_{18} reversed phase) failed to separate **2** from **1**. This inseparable mixture of cyclopentenones was then derivatized to their corresponding 2,4-dinitrophenylhydrazone. The inseparable mixture (30 mg) was dissolved in MeOH (with catalytic amount of formic acid), and 2,4-dinitrophenylhydrazine (50 mg) was added. The reaction mixture was kept stirring at room temperature for 24 h, then separated using a Sephadex LH-20 column, to yield hydrazones **4** (16.2 mg) and **5** (12.8 mg). Hydrazone **4** was crystallized from MeOH for X-ray crystal analysis; however, hydrazone **5** failed to crystallize from any solvent system.

2-Hydroxymethyl-3-methylcyclopent-2-enone (1): colorless oil; UV (MeOH) λ_{max} (log ϵ) 207 (3.69), 304 (2.99) nm; IR (KBr) ν_{max} 3423 (br), 2925, 1689, 1644, 1437, 1388, 1341, 1307, 1066, 1006 cm^{-1};

^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.30 (2H, s, H-6), 2.54−2.56 (2H, m, H-4), 2.37−2.40 (2H, m, H-5), 2.11 (3H, s, H-7); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 210.6 (C, C-1), 173.6 (C, C-3), 138.6 (C, C-2), 54.9 (CH_2, C-6), 34.4 (CH_2, C-5), 32.0 (CH_2, C-4), 17.2 (CH_3, C-7); ESITOFMS *m/z* 149.0586 (calcd for $[C_7H_{10}O_2 + Na]^+$, 149.0578).

Compound 4: red solid; mp 183−186 °C; UV (MeOH) λ_{max} (log ϵ) 214 (4.45), 255 (4.45), 285 (4.21), 385 (4.65) nm; IR (KBr) ν_{max} 3503, 3423, 3299, 3108, 2914, 2340, 2108, 1615, 1583, 1512, 1497, 1415, 1329, 1252, 1134, 1060 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 10.91 (1H, s, N*H*), 9.15 (1H, d, J = 2.6 Hz, H-3′), 8.31 (1H, dd, J = 9.6, 2.6 Hz, H-5′), 7.84 (1H, d, J = 9.6 Hz, H-6′), 4.53 (2H, s, H-6), 2.74 (4H, m, overlapping signals of H-4 and H-5), 2.07 (3H, s, H-7); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 168.1 (C, C-1), 158.2, (C, C-3), 144.7 (C, C-1′), 137.6 (C, C-4′), 135.0 (C, C-2), 130.1 (CH, C-5′), 129.0 (C, C-2′), 123.7 (CH, C-3′), 115.9 (CH, C-6′), 56.1 (CH_2, C-6), 34.8 (CH_2, C-4), 25.5 (CH_2, C-5), 15.9 (CH_3, C-7); ESITOFMS *m/z* 307.1050 (calcd for $[C_{13}H_{14}O_5N_4 + H]^+$, 307.1042).

Compound 5: red solid; mp 169−171 °C; $[\alpha]^{28}_{D}$ −78.9 (*c* 0.60, MeOH); UV (MeOH) λ_{max} (log ϵ) 227 (4.70), 251 (4.59), 366 (4.86) nm; IR (KBr) ν_{max} 3443 (br), 3312, 3107, 2958, 2931, 2873, 1619, 1589, 1518, 1504, 1423, 1335, 1310, 1269, 1135, 1066, 919, 832 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 10.86 (1H, s, N*H*), 9.15 (1H, d, J = 2.6 Hz, H-3′), 8.32 (1H, dd, J = 9.5, 2.6 Hz, H-5′), 7.80 (1H, d, J = 9.5 Hz, H-6′), 4.01 (1H, dd, J = 11.5, 4.0 Hz, H-6b), 3.83 (1H, dd, J = 11.5, 7.2 Hz, H-6a), 2.70 (1H, ddd, J = 18.0, 9.0, 1.5 Hz, H-5β), 2.43−2.50 (1H, m, H-5α), 2.41−2.48 (1H, m, H-2), 2.19−2.26 (1H, m, H-4α), 1.91−2.01 (1H, m, H-3), 1.49−1.59 (1H, m, H-4β), 1.20 (3H, d, J = 6.5 Hz, H-7); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 169.8 (C, C-1), 144.8 (C, C-1′), 138.0 (C, C-4′), 130.2 (CH, C-5′), 130.0 (C, C-2′), 123.6 (CH, C-3′), 115.9 (CH, C-6′), 62.2 (CH_2, C-6), 54.2 (CH, C-2), 35.7 (CH, C-3), 31.6 (CH_2, C-4), 28.1 (CH_2, C-5), 18.5 (CH_3, C-7); ESITOFMS *m/z* 309.1190 (calcd for $[C_{13}H_{16}O_5N_4 + H]^+$, 309.1199).

Acknowledgment. Financial support from The Thailand Research Fund (TRF) to N.S. (grant no. DBG4780003) is gratefully acknowledged. N.C. also thanks the TRF for the award of Senior Research Scholar (grant no. RTA4880008). A student grant from the Higher Education Commission Grants for Graduate Dissertation (Chulalongkorn University) to P.C. is acknowledged. We thank Prof. O. E. Eriksson, Prof. T. W. Flegel, and Dr. J. Worapong for suggestions on fungal identification.

Supporting Information Available: X-ray data for compound **4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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NP060148H