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Sesterterpene sulfates as isocitrate lyase inhibitors from tropical sponge *Hippospongia* sp.

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Abstract—Two sesterterpene sulfates (**1**–**2**) were isolated from tropical sponge *Hippospongia* sp. and their inhibitory activities against isocitrate lyase (ICL) from the rice blast fungus *Mgnaporthe grisea* were evaluated. Compound **3** was obtained by hydrolysis of compound **1**. Compounds **1** and **3** were found to be potent ICL inhibitors, which inhibited appressorium formation and C_2 carbon utilization in *M. grisea*. Our results suggest that ICL plays crucial role in appressorium formation of *M. grisea* and is a new target for the protection of rice blast disease.

In microorganisms, the glyoxylate bypass of the tricarboxylic acid (TCA) cycle provides the means to grow on C_2 compounds by converting them into C_4 dicarboxylic acids.[1] This is achieved through the activity of two unique enzymes, isocitrate lyase (ICL) and malate synthase (MLS). The oxaloacetate supplied by the bypass maintains the TCA cycle by replacing intermediates that are removed for biosynthesis. This is an archetypal anaplerotic reaction.[2,3] The carbon conserving glyoxylate pathway is present in most prokaryotes, lower eukaryotes, and plants, but has not been observed in vertebrates.[4] In microorganisms, it provides a means to survive on fatty acids as the sole carbon source and in plants it serves to utilize seed lipids for growth. Bacterial and fungal mutants lacking ICL are unable to grow on acetate as the sole carbon source.[2,3]

Mgnaporthe grisea (Hebert) Barr (anamorph: *Pyricularia grisea*) is a typical heterothallic ascomycete and the causal agent of rice blast, one of the most destructive diseases of cultivated rice worldwide.[5,6] *M. grisea* causes plant infection by means of a specialized infection structure called an appressorium. Recently, it has been reported that the genes of the glyoxylate cycle are highly induced, when *M. grisea* infects rice.[7,8] Δ*icl* mutants are less virulent than an isogenic wild-type strain of *M. grisea* and impaired in virulence-associated functions such as germ tube emergence, appressorium development, and cuticle penetration.[9] Therefore, ICL could be promising target for the control of rice pathogenic fungal infection and development of antifungal agents.

Our group has been interested in the search for biologically active secondary metabolites from marine sponges. During our continuing program with this aim, we encountered the marine sponge *Hippospongia* sp. from Federated States of Micronesia whose crude extract exhibited significant inhibitory activity toward *M. grisea* ICL. Bioassay-guided separation of the crude extract using various chromatographic techniques yielded two sesterterpene sulfates as potent ICL inhibitors. Herein we report the isolation and biological activities of these compounds.

The specimens of *Hippospongia* sp. (family Spongidae) were collected by hand using SCUBA at 10–20 m depth from Chuuk Atoll, Federated States of Micronesia, in June 2003 and July 2005. Frozen sponge (760 g) was

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repeatedly extracted with MeOH (1.5 L × 2) and CH_2Cl_2 (1.5 L). The extract was filtered and concentrated under reduced pressure to afford 87.7 g of crude extract. The residue was partitioned between H_2O and *n*-BuOH to yield 21.4 g of organic-soluble material. The *n*-BuOH layer was re-partitioned between 15% aqueous MeOH and *n*-hexane. The residue of aqueous MeOH layer (18.2 g) was subjected to C_{18} reversed phase flash chromatography using gradient mixture of MeOH and H_2O. The fraction eluted with 30% aqueous MeOH was dried (3.36 g) and separated by reversed phase HPLC to give 2.28 g of compound **1** as major products. A portion (470 mg) of the fraction eluted with 20% aqueous MeOH (4.75 g) in flash chromatography was separated by reversed phase HPLC to yield 105 and 7.2 mg of compounds **1** and **2**, respectively.

Based on the results of combined spectroscopic analyses, the structures of these compounds were defined as halisulfate 1 (**1**) and halisulfate 5 (**2**). The spectral data of these compounds were in good agreement with those reported previously.[10] These two metabolites were previously reported as a phospholipase A_2 inhibitor and antimicrobial constituent.[10,11] Compound **1** (57 mg) was treated as described by Kernan and Faulkner.[10] After workup, the residue was subjected to chromatography over silica gel (EtOAc/Hexane = 15:85) to yield hydrohalisulfate 1 (**3**) (41 mg) (Fig. 1).

The cloning and purification of ICL and MLS from the genomic DNA of *M. grisea* Guy 11 were carried out as described previously.[12–14] The compounds **1**–**3** were evaluated for their inhibitory activities toward *M. grisea* ICL and MLS according to a previously documented procedure.[13,15] The inhibitory potencies (IC_{50} values) of the tested compounds were compared with that of a known ICL inhibitor, 3-nitropropionic acid (Table 1).[16] The IC_{50} value of ICL and MLS from Guy 11 by 3-nitropropionic acid was 92.4 and 1570.8 μM, respectively, which was similar to the value reported for ICL and MLS from *Aspergillus fumigatus*.[15] As shown in Table 1, compounds **1**–**3** also had weak inhibitor activity against MLS, but were potent inhibitory to ICL. These results suggest that halisulfates are relatively specific inhibitors against ICL. In addition, compounds **1** and **3** exhibited 7- (IC_{50} = 12.6 μM) and 6-fold (IC_{50} = 15.0 μM) stronger ICL inhibitory activities than that of 3-nitropropionic acid (IC_{50} = 92.4 μM), respectively.

Table 1. Inhibitory effect of sesterterpene sulfates on the activity of isocitrate lyase (ICL) and malate synthase (MLS) from *M. grisea* Guy 11

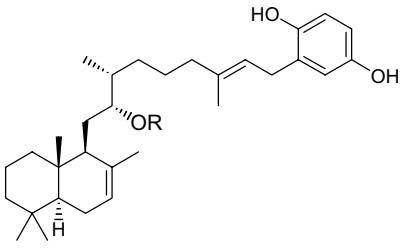
Compound	IC_{50}[a] (μM)	
	MLS	ICL
1	226.8	12.6
2	1145.3	67.4
3	273.6	15.0
3-Nitropropionate	1570.2	92.4

[a] IC_{50} values were calculated from the concentration at which 50% enzyme activity was inhibited by compounds. 3-Nitropropionate was used as a reference inhibitor of ICL and MLS.

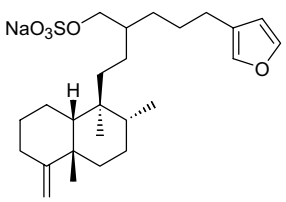
Interestingly, compound **3**, which was prepared by hydrolysis of the sulfate ester at the C-12 position of compound **1**, was found to have similar inhibitory activity compared with **1**. In contrast, substitution of a hydroquinone moiety for furan moiety (compound **2**) resulted in a decrease in the ICL inhibitory activity (IC_{50} = 67.4 μM). These results suggest that the hydroquinone moiety is important for the ICL inhibitory activity of halisulfate compounds.

To investigate the influence of ICL inhibitors on the appressorium formation in *M. grisea* Guy 11, the development of appressoria in germinating conidia was monitored on the hydrophobic surface of GelBond film according to a previously documented procedure.[13,17,18] In the appressorium formation assay, the conidia of isogenic knockout mutant I-10 (Δ*icl*) were germinated and mycelial growth continued without the formation of infectious structure (appressorium), whereas 98% of the conidia formed appressoria in wild-type rice pathogenic fungus Guy 11 (data not shown). Treatment of strain Guy 11 with compounds **1**–**3** reduced the appressorium formation ability of the fungus in a dose-dependent manner (Fig. 2). It is important to note that the inhibition of appressorium formation in *M. grisea* Guy 11 treated with compounds **1** and **3** (at 50 μM) is comparable to the behavior of I-10 (Δ*icl*).

The strategy for survival during appressorium-mediated infection in a nutrient-free environment entails a metabolic shift in the fungi's carbon source to C_2 substrates generated by β-oxidation of fatty acids.[7,8] Under these conditions, glycolysis is decreased and the glyoxylate shunt is significantly upregulated to allow anaplerotic



1 R = SO_3Na, Halisulfate 1
3 R = H, Hydrohalisulfate 1



2 Halisulfate 5

Figure 1. Structures of sesterterpene sulfates.

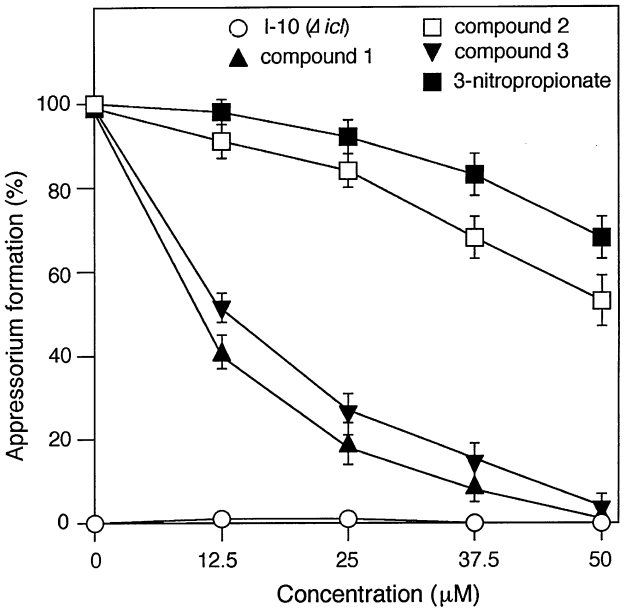


Figure 2. Effect of sesterterpene sulfates on the appressorium formation of wild-type rice pathogenic fungus *M. grisea* Guy 11 and its isogenic knockout mutant I-10 (Δ*icl*). Conidial suspension of Guy 11 (1×10^5 conidia/mL) with different concentrations of sesterterpene sulfates was placed on the hydrophobic side of GelBond and incubated in a moistened box at 24 °C for 14 h. 3-Nitropropionate was used as a reference inhibitor of ICL.

maintenance of the TCA cycle and assimilation of carbon via gluconeogenesis.[9,16,17] Therefore, we investigated the effect of compounds **1**–**3** on the fungal growth and survival, when *M. grisea* in media containing either glucose or sodium acetate as sole carbon source. A minimal growth medium containing variable concentrations of halisulfates was inoculated with *M. grisea* and inhibition was evaluated based on the minimum inhibitory concentration (MIC).[13,19] In this study, both wild-type Guy 11 and Δ*icl* mutant I-10 grew normally on glucose. However, I-10 failed to grow in acetate, whereas the Guy 11 grew normally (data not shown). As shown in Table 2, compounds **1**–**3** had weak inhibitory effect on the Guy 11 grown in glucose, but were inhibitory to Guy 11 grown in acetate, albeit at high concentration. These results indicate that halisulfates are good starting candidates for structure based ICL inhibitor design.

Table 2. Inhibitory effect of sesterterpene sulfates on *M. grisea* Guy 11 grown in glucose or acetate as sole carbon source

Compounds	MIC (μM)[a]	
	Glucose	Acetate
1	701.4	87.7
2	853.4	106.7
3	842.8	105.4
3-Nitropropionate	>3361.3	2941.2

[a] Conidia (2.5×10^5 conidia/ml) were incubated for 6 days at 28 °C in a minimal growth medium containing variable concentrations of test compound and 1% glucose or 1% sodium acetate as sole carbon source. The MIC was defined as the lowest concentration of compound at which no growth was observable. 3-NP, 3-nitropropionate (positive control).

In conclusion, sesterterpene sulfates were isolated and evaluated for their activities against ICL from the rice blast fungus *M. grisea*. These compounds were found to be strong ICL inhibitors, which inhibited appressorium formation and C_2 carbon utilization. This is the first report of ICL inhibitors from marine natural products. Since the enzymes of the glyoxylate cycle are not found in mammals, sesterterpene sulfates are good starting candidates for antifungal agent design.

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12. The wild type rice pathogenic fungus *Mgnaporthe grisea* Guy 11 and its isogenic knockout mutant I-10 (Δ*icl*) were provided by Dr. N.J. Talbot of the University of Exeter, UK.
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14. In the similar manner described in Ref. 13, cDNA of *M. grisea* Guy 11 was used as a template for the amplification of the *MLS* gene by PCR. Two synthetic primers (5′-ATGGCTTCCACAGAGTCCAT-3′, forward; 5′-GGCCCGCTTCGAAATTGTAA-3′, reverse) were synthesized based on the nucleotide sequence of *M. grisea* 70-15 (GenBank Accession No. XM366737). The N-terminal His-tagged MLS protein was purified using Ni–NTA column chromatography (Qiagen) according to the

manufacturer's instructions and the N-terminal His-tag was cleaved with EnterokinaseMax (Invitrogen).
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18. Conidial suspension of *M. grisea* Guy 11 (1×10^5 conidia/mL) with different concentrations of sesterterpene sulfates was placed on the hydrophobic side of GelBond and incubated in a moistened box at 24 °C for 14 h. For ICL inhibitor addition, we prepared concentrated stock solutions (10 mg/mL) in dimethylsulfoxide and added them to conidial suspension at appropriate dilutions (final solvent concentration <1%).
19. *Mgnaporthe grisea* Guy 11 (2.5×10^5 spores/ml) was inoculated in a minimal growth medium (carbon source 10 g/L, $NaNO_3$ 6 g/L, KH_2PO_4 1.5 g/L, $MgSO_4 \cdot 7\,H_2O$ 0.5 g/L, KCl 0.5 g/L, trace element 0.1%, and vitamin solution 0.1%) containing either sodium acetate or glucose as sole carbon source. Stock solutions of test compounds were prepared in dimethylsulfoxide and stored at −20 °C. Each stock solution was diluted with minimal growth medium to prepare serial dilutions in the range of 400–0.195 μg/ml prior to use. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of compound at which no growth was observable after being incubated for 6 days at 28 °C.