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RESEARCH COMMUNICATION

Natural Products

Bioactive cyclo-(*S*-Pro-*R*-Leu) from *Aspergillus flavus*, the marine endophytic fungus from brown alga, *Dictyota kunthi*

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Abstract: The marine environment is regarded as a rich source of bioactive secondary metabolites, and some of these compounds have been shown to be useful for novel drug discovery. In recent years, research on the chemistry of endophytic fungi from marine algae has shown them to be an important source of secondary metabolites with a wide range of biological activities. The objective of this study was to describe the isolation and identification of bioactive compounds obtained from *Aspergillus flavus,* hosted in the brown alga, *Dictyota kunthi,* together with *β*-glucuronidase bioassay of them. This fungal strain was cultivated on a large scale and extracted with ethyl acetate (EtOAc). Using various column chromatographic and spectroscopic techniques, we isolated and characterized two metabolites, cyclo-(*S*-Pro-*R*-Leu), 1 and genistein 2. Cyclo-(*S*-Pro-*R*-Leu) inhibited *β*-glucuronidase with an IC_{50} value of 83.9 ± 0.1 µM when compared to glucosaccharo-1,4-lactone (IC_{50} = 48.4 ± 1.3 µM). This appears to be the first report of the isolation of cyclo-(*S*-Pro-*R*-Leu) from this entophyte. To the best of our knowledge, this is the first report of significant *β*-glucuronidase inhibitory activity of this compound. From these findings, we can confirm that the endophyte from the brown alga, *Dictyota kunthi,* produces a *β*-glucuronidase inhibitory metabolite.

Keywords: *β*-glucuronidase, *Aspergillus flavus,* cyclo-(*S*-Pro-*R*-Leu), *Dictyota kunthi,* marine endophytes.

INTRODUCTION

Due to the discovery of unique chemical structures and their bioactivity, the search for novel chemicals from the marine environment has rapidly increased in recent years (Teixeira *et al*., 2019). Endophytic fungi live in the inner tissues or even cells of their hosts (Zhang *et al*., 2009). Secondary metabolites of algal-derived endophytic fungi have proven to be a good source of novel bioactive secondary metabolites for the development of pharmaceuticals and agrochemicals, showing activities such as anticancer, antibiotic, antiviral, antioxidative, and kinase inhibitory or activating activities (Sarasan *et al*., 2017; Handayani *et al*., 2019). In continuation of our studies towards the discovery of biologically active compounds from the Sri Lankan seaweeds and their endophytes (Haroon *et al*., 2013; Haniffa *et al*., 2019) we isolated the fungal strain *A. flavus*, from the brown alga, *D. kunthi,* collected from the south coast of Sri Lanka. This fungal strain was cultivated on a large scale and extracted using EtOAc. We extracted and characterized two metabolites, 1 and 2, using various column chromatographic techniques. Compounds 1 and 2 were evaluated *in vitro* for *β*-glucuronidase inhibitory activity and compound 1 was found to have significant activity. This appears to be the first report of significant *β*-glucuronidase inhibitory activity of this compound. The activity of *β*-glucuronidase rises in various conditions, including cancer, some hepatic disorders, and AIDS (Awolade *et al*., 2020). Therefore, development of specific inhibitors of *β*-glucuronidase could be useful. The present study describes the isolation and identification of cyclo-(*S*-Pro-*R*-Leu), 1 and genistein 2 together with *β*-glucuronidase bioassay for screening of 1. To our knowledge, this is the first report of the isolation of cyclo-(*S*-Pro-*R*-Leu) from this endophytic fungus.

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MATERIALS AND METHODS

General procedure

A Büchi 535 melting point instrument was used to determine melting points. A Jasco DIP-360 digital polarimeter was used to quantify optical rotation in MeOH. Bruker AMX 300 and 500 MHz NMR spectrometers were used to record ^{1}H-NMR spectra, while the same instrument was used to conduct ^{13}C-NMR studies at 75.45 and 100 MHz, respectively. Chemical shifts (**δ**) were measured in ppm relative to TMS, with coupling constants *J* measured in Hz. A Jeol JMS HX 600 mass spectrometer was used to detect EI MS.

Isolation and identification of the fungal strain

D. kunthi, a brown alga, was collected at Kirinda on the southern coast of Sri Lanka. The endophytic fungi were isolated from the blade of the seaweed using the method developed to isolate endophytes from higher plants by Petrini (Fokkema & Heuvel, 1986), with certain modifications (Haroon *et al*., 2013). The endophytic strain (DK-11) was identified as *A. flavus*, by S.R. Premaratne (Institute of Fundamental Studies, Natural Products Programme, Kandy, Sri Lanka). A subculture has been deposited at the Natural Products Laboratory, Institute of Fundamental Studies, Kandy.

Cultivation of Aspergillus flavus

The growth medium (Czapex Dox) for *A. flavus* was prepared by mixing the following chemicals (g/L) in filtered (No. 3 Whatman filter paper) natural seawater (500 mL/L) and distilled water (500 mL/L): K_2HPO_4 (1.0 g), $MgSO_4$ $7H_2O$ (0.5 g), KCl (0.5 g), $FeSO_4$ $7H_2O$ (0.01 g), sugar (30 g), and peptone (30 g). Culture medium (6.0 L) was prepared and distributed (250 mL, each flask) among 24 Erlenmeyer flasks of 1000 mL capacity (each), and autoclaved at 121 °C for 30 min. Then, agar blocks inoculated with mycelia were added into a 1000 mL flask containing 250 mL of culture medium and incubated at 28 °C with a control (without fungal strain) for 14 ds.

Extraction and isolation of pure compounds

After 14 ds, 250 mL of EtOAc was added into each flask containing culture media and mycelium. They were mixed thoroughly using a sonicator and the EtOAc layer was separated from each flask and evaporated using a rotary evaporator at 50 °C. The EtOAc extract was dried in a vacuum oven to afford a white residue (1.5 g). Flash column chromatography (CC) on silica gel (230 - 400 mesh size) produced two pure compounds from the EtOAc extract. This column was initially eluted with gradient polarities of n-hexane and EtOAc, which afforded five fractions (F -1 to F-5) and their weights were given as. F-1 (0.3 g), F-2 (0.1 g), F-3 (0.7 g), F-4 (10 mg), and F-5 (20 mg). Repeated flash silica gel (SiO_2) column chromatography of F-3 (20% EtOAc:hexane) yielded pure compound 1 (40 mg, 2.6%, 15% Acetone:hexane). Fraction F-4, when subjected to column chromatography, yielded compound 2 (2 mg, 0.13%, 20% Acetone : hexane).

β-glucuronidase inhibition assay

The *β*-glucuronidase inhibition assay was performed according to the method developed by Collins *et al.* (Collins *et al*., 1997; Haroon *et al*., 2013), with slight modification.

Characterization of compounds

Cyclo-(S-Pro-R-Leu) (1)

A white amorphous solid (40 mg, 2.6%) mp 170 °C; $[\alpha]^{27}_{D}$ -105.5° (c = 0.5, EtOH) UV (MeOH), λ_{max} at 242 nm; IR (KBr) ν_{max} 1432, 1635, 1670, 2955, 3261 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$); δ_H 0.92 (3H, d, *J* = 6.5 Hz, H_3-12), 0.96 (3H, d, *J* = 6.5 Hz, H_3-13), 1.51(1H, m, Ha-10), 1.74 (1H, m, H-11), 1.85 (2H, m, H_2-4), 2.06 (1H, m, Hb-10), 2.13 (1H, m, Ha-5), 2.33 (1H, m, Hb-5), 3.53-3.6 (2H, m, H_2-3), 3.91 (1H, dd, *J* = 9.5, 3.4 Hz, H-9), 4.15 (1H, t, *J* = 7.7 Hz, H-6), 6.16 (1H, s, NH); ^{13}C NMR (75 MHz, $CDCl_3$); δ_C 21.2 (C-13), 22.7 (C-4), 23.2 (C-12), 24.6 (C-11), 28.0. (C-5), 38.6 (C-10), 45.4 (C-3), 53.4 (C-9), 59.4 (C-6), 166.2 (C-7), 170.3 (C-1); CI MS (positive mode) *m/z* 211 $[M + H]^+$.

Genistein (2)

A pale yellow powder (2 mg, 0.13%) mp 286°C lit (Aida *et al.*, 1995) 285 °C; ^{1}H NMR (500 MHz, CD_3OD); δ_H 6.21 (1H, d, *J* = 2.2 Hz, H-8), 6.31 (1H, d, *J* = 2.2 Hz, H-6), 6.84 (2H, d, *J* = 8.6 Hz, H-3'/5'), 7.35 (2H, d, *J* = 8.5 Hz, H-2'/6'), 8.04 (1H, d, H-2); EI MS 70 eV, *m/z* (rel.int, %): 270 (100) $[M^+]$, 153 (56), 137(10), 118 (21).

RESULTS AND DISCUSSION

Chemistry of isolated compounds

Repeated column chromatography on flash silica gel yielded two metabolites from the EtOAc extract of *A. flavus*. Compound 1 was found to be a white amorphous solid with a melting point of 170 °C. The compound's molecular ion peak was found at m/z 211 $[M + H]^+$with the aid of CI-MS (positive mode). The amide NH group absorbed at 3261 cm^{-1} in the infrared spectrum of 1, while the two amide carbonyl groups absorbed at 1635 and 1670 cm^{-1}. In the ^{1}H-NMR spectrum, recorded in $CDCl_3$, the most downfield singlet, that of the amide NH, appeared at δ_H 6.16. The other two downfield sets of protons, H-6 and H-9 appeared as triplet at δ_H 4.15 (1H, t, $J_{6,5}$ = 7.7 Hz) and as double doublet at δ_H 3.91 (1H, dd, $J_{9,10a/10b}$ = 3.4, 9.4 Hz,) respectively. The ^{13}C-NMR broad-band decoupled, HMQC and DEPT spectra of 1, in $CDCl_3$ indicated 11 carbon signals, including 2 methyls, 4 methylenes, 3 methines, and 2 quarternary carbons. The most downfield signals, those of amide carbons, appeared at δ_C 170.3 and 166.2 while the second most downfield carbon sandwiched between NH and CO appeared at δ_C 58.9. The methine carbon attached to C-6 and the methylene carbon attached to C-3 of 1 appeared at δ_C 53.4 and 45.4 respectively. Compound 1 was identified as cyclo-(*S*-Pro-*R*-Leu) by direct comparison with reported ^{1}H- and ^{13}C-NMR spectral data (Adamczeski *et al.*, 1995; Pedras *et al.*, 2005; Liu *et al.*, 2012) (Figure 1). The absolute configuration was determined by the optical rotation, the $[\alpha]^{27}_D$ (c = 0.5, EtOH) value of 1 (-105.5°), well agreeing with reported data, $[\alpha]_D$ = -108 (Adamczeski *et al*., 1995; Li *et al*., 2008). However, according to Yang *et al*. (2009), it showed the optical rotation of [a]D = -78.3.

Compound 2 was found to be a pale yellow powder with a melting point of 286 °C. The molecular ion and base peak of compound 2 was found at m/z 270, which corresponded to the chemical formula $C_{15}H_{10}O_5$, indicating eleven degrees of unsaturation. The most downfield singlet seen δ_H 8.04 in the ^{1}H-NMR spectra recorded in CD_3OD was ascribed to olefinic H. Moreover, H-2'/6' and H-3'/5' were ascribed to the para-disubstituted aromatic ring protons that resonated at δ_H 7.37 (2H, d, *J* = 8.6 Hz) and δ_H 6.84 (2H, d, *J* = 8.6 Hz), respectively.

The presence of tetra - substituted aromatic ring protons was suggested by meta coupling signals at δ_H 6.32 (1H, *J* = 2.2 Hz, H-8) and 6.21 (1H, *J* = 2.2 Hz, H-6) in the ^{1}H-NMR spectrum of 2. Compound 2 was confirmed as genistein based on a direct comparison of physical and spectral data (Aida *et al*., 1995) (Figure 1). Isoflavones are known to occur commonly in higher plants, but they have also been isolated from bacteria, and from a culture of the fungus *Aspergillus niger*.

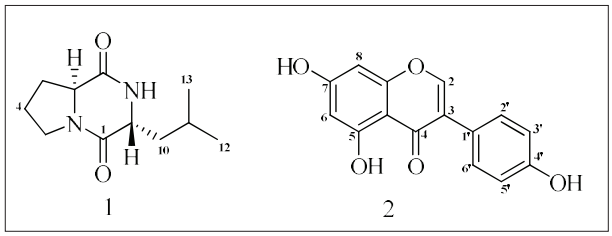

Figure 1: Structures of isolated compounds 1- 2.

Table 1: *β*-glucuronidase inhibition of pure compounds

Compound	IC_{50} (µM) Mean ± S.E.M
1	83.9 ± 0.1
2	-
glucosaccharo-(1,4)-lactone	48.4 ± 1.3

β-glucuronidase inhibitory activity

The compounds 1 and 2 were screened for *β*-glucuronidase inhibitory activity at 200 µM. When compared with the standard glucosaccharo-(1,4)-lactone (IC_{50} = 48.4 ± 1.3 µM), compound 1 showed a significant *β*-glucuronidase inhibitory activity with an IC_{50} value of 83.9 ± 0.05 µM and 2 did not show any activity (Table 1). The substantial inhibitory activity of Compound 1 is most likely due to the enzyme's active site accepting a proton from the CONH. This compound was reported to inhibit the marine pathogenic bacteria, *Vibrio anguillarum* (Fdhila *et al*., 2003) and fungi *Plasmopara viticola* (Musetti *et al*., 2007). Further, it also exhibits antifouling activity against microfoulers (Li *et al*., 2006). To the best of our knowledge, cyclo-(*S*-Pro-*R*-Leu) from algae-associated fungi is uncommon compared to sponge-associated fungi, actinomycetes, and bacteria (Schmidtz *et al*., 1983; Stierle *et al*., 1988; Adamczeski *et al*., 1995; Fdhila *et al*., 2003; Li *et al*., 2006; Li *et*

al., 2008; Yang *et al*., 2009). According to Liu *et al*. (2012), cyclo-(*S*-Pro-*R*-Leu), which has already been shown to be produced by the close species of *Aspergillus fumigates*, showed weak inhibitory activity against the release of *β*-glucuronidase from rat polymorphonuclear leukocytes (PMNLs). However, as per the results of this study, cyclo-(*S*-Pro-*R*-Leu) showed a significant *β*-glucuronidase inhibitory activity with an IC_{50} value of 83.9 ± 0.1 µM. To the best of our knowledge this is the first report of this compound showing significant *β*-glucuronidase inhibitory activity.

CONCLUSION

The present study described the isolation and characterization of cyclo-(*S*-Pro-*R*-Leu) and genistein from EtOAc extract of *A. flavus* hosted in the brown alga*, Dictyota kunthi*. When compared to the standard glucosaccharo-(1,4)-lactone (IC_{50} = 48.4 ± 1.3 µM), the compound cyclo-(*S*-Pro-*R*-Leu) demonstrated significant *β*-glucuronidase inhibitory activity with an IC_{50} value of 83.9 ± 0.1 µM. Based on these findings, we can conclude that the endophyte from *Dictyota kunthi* produces a *β*-glucuronidase inhibitory metabolite.

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