Acremochlorin O and Other Prenylated Chlorophenol Antimicrobial Metabolites from the Fungus Acremonium sp. Strain MNA-F-1

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Abstract

Chemical investigation of the ethyl acetate extract of the fungus Acremonium sp., derived from the inner tissue of anise roots, led to the isolation and characterization of one new chlorinated compound, acremochlorin O (1), together with five related derivatives (2–6) and an alkaloidal metabolite, ilicicolin H (7). Structure elucidation of the isolated compounds was determined based on extensive 1D/2D NMR spectroscopic analyses and HR-ESI-MS measurements. The absolute configuration of acremochlorin O (1) was established using an electronic circular dichroism (ECD) experiment. All isolated compounds were evaluated for their antibacterial activity against Staphylococcus aureus, Escherichia coli and Mycobacterium tuberculosis, where several compounds revealed potent activities against tested Gram-positive strains.

Keywords
Fungal endophyte; Acremonium sp.; prenylated chlorophenols; antitubercular.

Introduction

The genus Acremonium has previously been reported from plant, marine or soil sources and its chemical exploration afforded the identification of more than 350 secondary metabolites [1]. These compounds belong mainly to polyketides, terpenoids, peptides, meroterpenoids, alkaloids and steroids [1]. The biological activities reported for Acremonium fungal extracts and fractions include cytotoxic, anti-inflammatory, antimicrobial, antiviral, enzyme-inhibitory and insecticidal activities [1].
Despite the presence of numerous effective antimicrobial drugs, the increasing emergence of antimicrobial resistance has dramatically decreased the choices among the available medications or their combinations [2]. Thus, an elevated risk of resistant microbial infections is anticipated and occurring including those building biofilms that increases the health burden, hospitalization periods and mortality rates [3]. Therefore, an increasing research endeavor is directed toward exploring natural products for new antimicrobials or potential candidates that can be used as an adjuvant therapy with conventional antibiotics to regain their efficacy and combating the resistant pathogens. Motivated by many previous studies describing fungal-derived antimicrobial metabolites [4], we herein report the isolation and structure elucidation of prenylated chlorophenol secondary metabolites from *Acremonium sp.*, derived from the inner tissues of anise roots. In addition, the isolated compounds were assessed for their antimicrobial activities. Herein, we describe the results of chemical and biological prospection.

![Chemical structures of 1–7.](image)

**Figure 1.** Chemical structures of 1–7.
Results and discussion

Structure Elucidation

In the course of our ongoing research targeting potent antimicrobial and cytotoxic metabolites, this study investigated an extract obtained from cultivation of *Acremonium* sp. on solid-state rice medium. Chemical investigation unveiled a new prenylated chlorophenol compound (1) along with six related derivatives (Figure 1) namely; ascofuranone (2) [5], chlorocylindrocarpol (3) [6], 4',5'-dihydro-4'-hydroxyascochlorin (4) [5,7], ilicicolins C (5) [7], D (6) [7] and H (7) [8]. Herein, we report the isolation and structure characterization of 1 along with the results of assessing all isolated compounds for their antimicrobial activity.

Compound 1 was isolated as a pale yellow amorphous solid. The molecular formula of 1 was established to be C_{25}H_{33}ClO_{6} based on its HR-ESI-MS that revealed a protonated molecular ion at \textit{m/z} 465.2045 [M+H]^+ (calculated 465.2038) indicating nine degrees of unsaturation. In addition, its HR-ESI-MS exhibited a protonated molecular ion peak cluster at \textit{m/z} 465.2045 and \textit{m/z} 467.2023 in a ratio of 3:1 supporting the presence of a single chlorine atom in its structure [9]. The $^{13}$C NMR, HMBC and HSQC spectra of 1 (Table 1, Figure 2) disclosed the presence of twenty-five carbon resonances that can be differentiated into two carbonyl carbon atoms at $\delta_C$ 193.4, designated as an aldehyde group based on its direct correlation to a deshielded singlet proton signal at $\delta_H$ 10.15, and at $\delta_C$ 170.8 that was designated as an acetyl group based on its HMBC correlation to a singlet methyl group ($\delta_H$ 2.08). In addition, the $^{13}$C NMR spectral data of 1 (Table 1) showed the presence of nine other unprotonated $sp^2$ carbons ($\delta_C$ 162.3, 156.6, 137.8, 136.5, 134.3, 114.5, 113.7, 113.4, 81.9), four methine carbon atoms ($\delta_C$ 127.5, 121.2, 80.9, 79.6), four secondary ($\delta_C$ 39.2, 37.1, 26.2, 22.1) and six primary $sp^3$ carbon atoms ($\delta_C$ 25.5, 22.7, 21.2, 16.3, 14.6, 11.1). The $^1$H NMR
and \(^1\)H-\(^1\)H COSY data of 1 (Table 1, Figure 2) suggested the presence of one geranyl moiety supported by the presence of two olefinic protons at \(\delta_H\) 5.42 (tdt, \(J = 7.1, 2.4, 1.2\) Hz, H-14) and \(\delta_H\) 5.21 (ddt, \(J = 8.5, 7.2, 1.3\) Hz, H-10), each was correlated to a methylene and an olefinic methyl group at \(\delta_H\) 2.12 (m, H-13)/\(\delta_H\) 1.60 (d, \(J = 1.2\) Hz, H-22) and at \(\delta_H\) 3.39 (d, \(J = 7.1\) Hz, H-9)/\(\delta_H\) 1.78 (d, \(J = 1.3\) Hz, H-23), respectively.

A literature search of 1 based on the interpreted structural features proposed it to be a prenylated chlorophenol merosesquiterpenoid derivative and its spectral data revealed a close resemblance to those reported for ascofuranol [5]. The main difference in spectral data between 1 and ascofuranol was the presence of an additional acetyl group in 1 as indicated by \(^1\)H and \(^{13}\)C NMR data (Table 1) explaining its higher molecular weight by 42 atomic units compared to ascofuranol.

Further confirmation to the depicted structure of 1 (Figure 1) was provided by the HMBC spectrum (Figure 2) which revealed key correlations from an aromatic methyl group at \(\delta_H\) 2.61 (s, H-7) to the aldehyde carbon at \(\delta_C\) 193.4 (C-8) and three unprotonated \(sp^2\) carbons at \(\delta_C\) 113.7 (C-1), 113.4 (C-5) and 137.8 (C-6) indicating its existence at C-6. In addition, the HMBC spectrum of 1 confirmed the position of the acetyl group at C-18 by the revealed key correlations from H-18 at \(\delta_H\) 5.00 (dd, \(J = 6.9, 4.2\) Hz) and a singlet methyl group at \(\delta_H\) 2.08 (s, H-25) to an acetocarbonyl carbon at \(\delta_C\) 170.8 (C-24). The relative configuration of 1 was determined by ROESY (Figure 2) which revealed key ROE correlations between H-16, Ha-17 and H-18 indicating that they are cofacial while the acetyl group is located on the opposite face of the molecule.

Compound 1 revealed an optical rotation value equal but with an opposite sign to that reported for its deacetylated congener, \((-\)uesto)-ascofuranol, to which the \((16S,18S)\) configuration had been assigned [7,10]. To determine the absolute configuration of 1, its ECD spectrum was acquired and compared to the calculated spectra for both \((16S,18S)\) and \((16R,18R)\) isomers (Figure 3). The comparison of experimental and
simulated Boltzmann-averaged ECD spectra revealed the absolute configuration of 1 as (16R,18R). According to the aforementioned results and by comparison with the reported literature, compound 1 was identified as a previously undescribed natural compound, chemically determined to be 18-O-acetylascofurnaol and was trivially named acremochlorin O.

Figure 2. Key $^1$H-$^1$H COSY, HMBC and ROESY correlations of 1.

Figure 3. Comparison of experimental (black) and simulated Boltzmann-averaged (red: (16R,18R), similarity factor: 0.90; green: (16S, 18S), similarity factor: 0.02) ECD spectra of compound 1 ($\Delta$-value: 0.88).

It is noteworthy to point out that acremochlorin O (1) could be traced back in the total fungal extract and its subsequent fractions, supporting its identity as an authentic fungal metabolite and not an artefact produced during chromatographic separations.
The purification scheme avoided any exposure to acetic acid which adds to the authenticity of 1. However, no ascofuranol could be detected in the total fungal extract, which indicates the acetylated derivative to be the produced exclusively instead of its non-acetylated congener ascofuranol. This undoubtedly defined acremochlorin O (3-O-acetylascofuranol, 1) as an authentic fungal product.

**Table 1.** $^1$H and $^{13}$C NMR data of 1.

<table>
<thead>
<tr>
<th>pos.</th>
<th>$\delta_C$, type</th>
<th>$\delta_H$ (multi, $\nu$ [Hz])</th>
<th>pos.</th>
<th>$\delta_C$, type</th>
<th>$\delta_H$ (multi, $\nu$ [Hz])</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>113.7, C</td>
<td></td>
<td>15</td>
<td>134.3, C</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>162.3, C</td>
<td></td>
<td>16</td>
<td>80.9, CH</td>
<td>4.32 t (7.7)</td>
</tr>
</tbody>
</table>
| 3    | 114.5, C       |                               | 17   | 37.1, CH$_2$   | $\alpha$ 1.73 ddd (14.0, 7.7, 4.2)  
|      |                |                               |      |                | $\beta$ 2.45 ddd (14.0, 7.7, 6.9)         |
| 4    | 156.6, C       |                               | 18   | 79.6, CH       | 5.00 dd (6.9, 4.2)           |
| 5    | 113.4, C       |                               | 19   | 81.9, C       |                               |
| 6    | 137.8, C       |                               | 20   | 22.7, CH$_3$   | 1.23 s                       |
| 7    | 14.6, CH$_3$   | 2.61 s                        | 21   | 25.5, CH$_3$   | 1.24 s                       |
| 8    | 193.4, CO      | 10.15 s                       | 22   | 11.1, CH$_3$   | 1.60 d (1.2)                 |
| 9    | 22.1, CH$_2$   | 3.39 d (7.1)                  | 23   | 16.3, CH$_3$   | 1.78 d (1.3)                 |
| 10   | 121.2, CH      | 5.21 ddt (8.5, 7.2, 1.3)      | 24   | 170.8, CO      |                               |
| 11   | 136.5, C       |                               | 25   | 21.2, CH$_3$   | 2.08 s                       |
| 12   | 39.2, CH$_2$   | 2.02 m                        | 2-OH | 12.70 s        |                               |
| 13   | 26.2, CH$_2$   | 2.12 m                        | 4-OH | 6.50 br s      |                               |
| 14   | 127.5, CH      | 5.42 tdt (7.1, 2.4, 1.2)      |      |                |                               |

*a Measured in chloroform-$d$ at 125 MHz for $^{13}$C and 500 MHz for $^1$H.

**Biological evaluation**

In our ongoing search for antibacterial and particularly antitubercular natural products, compounds 1–7 were assessed in a minimal inhibitory concentration assay against *S. aureus* ATCC 29213, *E. coli* ATCC 25922 and *M. tuberculosis* H37Rv. None of the tested compounds revealed inhibitory effect against *E. coli*. However, ascofuranone (2) revealed moderate antibacterial activity against *S. aureus* with MIC$_{90}$ of 25 µM,
whereas a cremochlorin O (1), ilicilons D (6) and H (7) exhibited weak inhibitory effects with MIC\textsubscript{90} values of 62.5, 50 and 100 µM, respectively. All tested compounds had no growth inhibitory effects against \textit{M. tuberculosis} except for 7 which displayed a weak activity with an MIC\textsubscript{90} of 50 µM.

**Conclusion**

The fungal genus \textit{Acremonium} has been previously derived from different plant organs [11-12]. In the present study, we isolated and identified several prenylated chlorophenol meroterpenoidal metabolites, with some revealing moderate to weak activities against \textit{S. aureus} and/or \textit{M. tuberculosis}. Halogenated natural compounds have been widely recognized for different bioactivities such as anti-inflammatory [13] and antimicrobial against different microbial pathogens including \textit{M. tuberculosis} such as spiromastixone R [14] and chlorflavonin [15], respectively. In conclusion, this study valorizes the repertoire of natural products produced by the genus \textit{Acremonium} in particular those belonging to the chlorophenol meroterpenoids with an overview about their bioactivity against various human microbial pathogens [14,15]. One of these compounds spiromastixone R was trichlorinated and revealed potent activity against \textit{S. aureus}, \textit{Bacillus subtilis} and \textit{B. thuringiensis} with MIC values ranging from 0.5 to 1 µg/mL [14]. In addition, some chlorinated natural products have shown antimicrobial effects against \textit{M. tuberculosis} as exemplified by the dihydroxyflavone chlorflavonin that was isolated from the endophytic fungus \textit{Mucor irregularis} [15] and chlorinated coumarins, isolated from the polypore mushroom \textit{Fomitopsis officinalis} [16].

**Experimental**

3.1 General experimental methods
Optical rotation measurement was performed in acetonitrile (Uvasol, Merck, Darmstadt, Germany) at 20 °C using Anton Paar MCP-150 polarimeter (Seelze, Germany). ECD spectra were recorded on a J-815 spectropolarimeter (JASCO, Pfungstadt, Germany). The nuclear magnetic resonance (NMR) spectra were recorded on Avance III 500 spectrometer (1H NMR, 500 MHz; and 13C NMR, 125 MHz, Bruker). UltiMate 3000 Series UHPLC (Thermo Fischer Scientific, Waltman, MA) equipped with an amaZon speed ESI-ion trap-MS (Bruker, Billerica, MA) was employed to record the ESI-MS spectra using a C18 Acquity UPLC BEH column (2.1 × 50 mm, 1.7 μm) (Waters, Milford, MA). The HPLC conditions used were: gradient elution using solvent A (0.1% aqueous formic acid) and solvent B (0.1% formic acid in acetonitrile), gradient was set at 5% B for 0.5 min mounting to 100% B in 19.5 min then isocratic at 100% B for 5 min, the flow rate was 0.6 mL/min, and the diode-array detector (DAD) was set at 190-600 nm. Agilent 1200 Infinity Series HPLC instrument (Agilent Technologies, Santa Clara, CA) was used for high-resolution electrospray ionization mass spectrometry (HRESIMS) analyses using a C18 Acquity UPLC BEH column (2.1 × 50 mm, 1.7 μm) (Waters) and applying the same solvent composition and elution system as above. UV-Vis detection (200–640 nm) was connected to a maXis ESI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) using the following parameters: scan range 100–2500 m/z, capillary voltage 4500 V, dry temperature 200 °C. The final purification steps of metabolites were performed on Agilent 1100 preparative HPLC (Agilent, Waldbronn, Germany). All chemicals were procured by Carl Roth GmbH (Karlsruhe, Germany) and AppliChem GmbH (Darmstadt, Germany) whereas solvents for HPLC runs were purchased from Merck (Darmstadt, Germany).

3.2 Isolation and Identification of Fungal Material
Anise roots were collected from El-Orman botanical gardens (Giza, Egypt). According to the standard protocols [17,18], the fungal strain was isolated from the inner tissues of the sliced root parts, in which the roots were rinsed thoroughly with distilled water then subjected to surface sterilization by dipping in 70% ethanol for 2 min and small pieces from the inner tissues were cut using a sterilized blade and placed onto a malt agar plate (15 g/L malt extract, 15 g/L agar, 0.2 g/L chloramphenicol to suppress bacterial growth, pH adjusted to 7.4-7.8 using 10% NaOH). By regular inspection of the incubated plate at 25 °C, the investigated fungal strain was observed to grow out of the fruit tissues. Then, pure fungal strains were grown by repeated re-inoculation on fresh culture media. The fungal strain was identified as Acremonium sp. MNA-F-1 using a molecular biological protocol by DNA amplification and sequencing of the ITS region as previously described [19]. The sequencing data was submitted to the GenBank with an accession code: OQ930617.

3.3 Cultivation, Extraction and Purification

Ten Erlenmeyer flasks (1 L) each containing 100 g rice and 110 mL water were set aside overnight, then autoclaved for 20 min at 121 °C and used for the fungal fermentation at 25 °C for six weeks under static conditions. For harvesting, each flask was soaked in EtOAc (3 × 500 mL), and the culture mass was mechanically cut into small pieces by a spatula. The flasks were set aside overnight then shaken for 8 h at 150 rpm. The combined EtOAc extracts were then evaporated in vacuum at 40 °C to yield brown oily residue. The obtained residue (4.5 g) was suspended in 90% aqueous methanol and partitioned with n-hexane. The two phases were separated and dried under vacuum yielding 2.5 and 1.7 g of the n-hexane and 90% aqueous methanol phase, respectively. The defatted 90% MeOH extract (1.7 g) was subjected to vacuum liquid chromatography (VLC) using normal phase silica gel as a stationary phase and
applying a gradient elution using \( n \)-hexane:EtOAc (100:0 to 30:70) followed by dichloromethane:methanol (100:0-0:100) yielding 9 major fractions (F1-F9) that were analyzed using LC-MS. Selected fractions were then purified accordingly, where fraction F3 (270 mg; \( n \)-hexane:EtOAc (1:1)) was re-fractionated on Sephadex LH20 column chromatography eluted with dichloromethane:methanol (1:1) yielding three subfractions (SF3-1 to SF3-3). Subfraction (SF3-2) was purified using preparative reversed phase HPLC applying isocratic elution using 80% acetonitrile in water as a mobile phase to yield 7 (21 mg, \( t_R = 8.5 \) min). On the other hand, fraction F4 (120 mg; \( n \)-hexane:EtOAc (4:6)) was similarly purified on preparative HPLC using 70% acetonitrile in water yielding an inseparable mixture of 5 and 6 (16.2 mg, \( t_R = 9 \) min.). Fraction F5 (83 mg; \( n \)-hexane:EtOAc (3:7)) was purified using 65% acetonitrile in water to obtain 1 (11 mg; \( t_R = 10 \) min) and 2 (9 mg; \( t_R = 11.5 \) min). Fraction 6 (220 mg; DCM:MeOH (9:1)) was fractionated using 55% acetonitrile in water to afford 3 (28 mg; \( t_R = 8 \) min) and 4 (13 mg; \( t_R = 11 \) min).

3.3.1 Spectral data

Acremochlorin O (1): light yellow amorphous solid; 8.7 mg; \([\alpha]_{D}^{20} +7.0 \) (c 0.1, methanol); UV-Vis (MeOH): \( \lambda_{\text{max}} \) (log \( \varepsilon \)) = 203.0 (1.1), 229 (0.8), 294 (0.6), 345 (0.4) nm; ECD (c = 3.16\times10^{-4} \) M; MeOH) \( \lambda \) [nm], \((\Delta\varepsilon)\) 373 (1.4), 310 (1.0), 268 (−3.2), 243 (1.1), 218 (−4.6), 196 (4.2); NMR data (\(^1\)H NMR: 500 MHz, \(^{13}\)C NMR: 125 MHz in chloroform-\( d \)) see Table 1; HR-(+)ESI-MS: \( m/z \) 465.2045 [M+H]\(^+\) (calcd. 465.2038 for C\(_{25}\)H\(_{34}\)ClO\(_6\)\(^+\)).

3.4 Determination of the Minimal Inhibitory Concentration against Different Pathogenic Bacteria

For the testing against \( M. \) tuberculosis H37Rv, the Minimal Inhibitory Concentration (MIC) was determined in 96-well microtiter plates containing a total volume of 100 \( \mu \)L employing a resazurin reduction assay. Briefly, a 96-well plate was prepared
containing 7H9 medium supplemented with 10% ADS (0.81% NaCl, 5% BSA, 2% dextrose), 0.5% glycerol, and 0.05% tyloxapol. Compounds were two-fold serially diluted with the highest tested concentration of 100 µM. A M. tuberculosis culture was pre-grown to an OD_{600 nm} of approx. 0.4–0.6 by shaking at 37 °C in PETG square bottles (ThermoFisher Scientific, Braunschweig, Germany) containing 10 mL supplemented 7H9 medium. The cell density was adjusted to an OD_{600 nm} of 0.08 (10^6 CFU/mL and 5 × 10^4 CFU were added to each well). Rifampicin and DMSO were used as a positive and solvent control, respectively. The 96-well plates were incubated for 5 days at 37 °C and 5% CO_2 in humidified atmosphere. Afterwards, 10 µL of a 100 mg/mL resazurin solution was added to each well and carefully re-suspended. After another 24 h at room temperature, the cells were fixed by adding 100 µL of a 10% formalin solution to each well. The readout was performed using a TECAN plate reader at 535 nm excitation and 590 nm emission. The growth was calculated in relation to the solvent control being 100% growth. For testing the antibacterial activity against S. aureus (ATCC 29213) and E. coli (ATCC 25922), single colonies obtained from agar plates were grown in Mueller-Hinton broth (MHB) at 37 °C shaking at 120 rpm to reach an optical density of approx. 0.4. The cell suspension was adjusted to 10^6 CFU/mL, of which 50 µL was seeded into a prepared 96-well polystyrene round-bottom plate containing test compounds diluted in MHB in a 1:1 serial dilution ranging from 100 µM to 0.78 µM. The plates were incubated at 37 °C statically for 24 h, and readout was performed using the BacTiter Glo assay (Promega) following the manufacturer’s instructions. Briefly, BacTiter Glo reagent was added to a white flat-bottom 96-well plate, and an equal volume of bacteria suspension was added to each well and mixed carefully. After 5 min, the luminescence was measured using a TECAN plate reader. The growth was calculated concerning the vehicle (DMSO) and sterile control.
Moxifloxacin and cefuroxime were used as a positive and negative control, respectively.

3.8 Computational details

Conformer distribution was calculated using Spartan’10 V1.1.0 [20] on the PM6 level of theory [21]. Density-functional theory (DFT) computations were carried out utilizing the Gaussian program package, Revision C.01 [22]. Geometry optimizations were performed on the B3LYP/6-31G(d,p) level of theory with tight cutoffs on forces and step size [23-26]. To simulate the effects of Methanol, the IEFPCM solvent model was used [27]. Conformers were determined as minima by frequency calculations. Double conformers and conformers 4.5 kcal/mol above the lowest energy conformer were discarded. Time-dependent DFT calculations were performed on the same level of theory. SpecDis 1.71 was used to create a Boltzmann-averaged spectrum and to compare the simulated and experimental spectra [28].

Supporting Information

Supporting Information File 1:

HRESIMS profiles and NMR spectroscopic data of 1.

File Format: .pdf

Title: Supplementary Information.

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