3-Oxo-γ-costic acid fungal-transformation generates eudesmane sesquiterpenes with in vitro tumor-inhibitory activity


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Chiliadenus montanus
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Abstract

While select eudesmane sesquiterpenes exhibit anti-neoplastic activity, tumor-inhibition for costic-acids has not been established. Here biological activity of 3-oxo-γ-costic acid (1), previously isolated from Chiliadenus montanus, as well as new sesquiterpenes (2–5) and the known derivative, 3-oxo-eudesma-1,4,11(13)-trien-7-1061-H-l2-oic acid (6), all produced from 1 by the fungus Athelia rolfsii, are reported. Structures were elucidated using MS and NMR spectroscopy with activity-screening utilizing human colon- and lung-tumor lines, Caco-2 and A549 respectively. Compound 1 exhibited anti-proliferative activity against Caco-2 (IC50 39 M) and 2 was active against A549 (IC50 74 M) suggesting therapeutic potential for the original substrate and a bio-transformed product.

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Nature produces a diverse array of metabolites employed in chemical signaling and defense. Such plant, marine and microbial chemicals are the basis for many of the anti-neoplastic agents currently available on the market. For example, the plant-derived, anti-cancer vinca alkaloids, vincristine and vinblastine interfere with proper microtubule function as does the terpene derivative paclitaxel. While many studies have demonstrated anti-tumor activity for sesquiterpenes, only a small fraction of eudesmane-type sesquiterpenes have been investigated for anti-tumor activity. To broaden functional-group diversity for a given set of natural products, targeted substrates can be subjected to enzymatic biotransformations. Such microbe-mediated chemical conversions employ biological catalysts to transform non-host metabolites into an array of novel metabolites with alternative and/or additional functional-group features. For the anti-malaria drug artesiminis, fungal-mediated chemical modifications have resulted in the generation of potent new antimalarial agents with greater in vivo stability. For the triterpene saponin ginsenoside, fungal transformations have yielded new anti-tumor metabolites with higher efficacy than original parent compounds.

While the genus Chiliadenus is a member of the biologically active plant family Asteraceae, anti-tumor activity for eudesmane-type sesquiterpenes isolated from Chiliadenus montanus, has not been reported. To probe structure-biological activity relationships for related chemical derivatives with subtle functional group modifications, a microbial biotransformation approach was employed with 3-oxo-γ-costic acid as the exogenous substrate. The fungal strain, isolated from an Egyptian soil source, was identified via a DNA sequence tag. To provide structure-biological activity data, the anti-proliferative activity of bio-transformed metabolites were assayed against human colon (Caco-2) and lung (A549) tumor cell lines.

Out of six terrestrial fungus strains assayed, Athelia rolfsii was the most enzymatically active for metabolite transformations of 3-oxo-γ-costic acid, based on tlc analysis of fungal extracts (data not shown). The A. rolfsii nucleotide sequence was deposited in GenBank; a phylogenetic relationship was conducted via a rooted tree using a neighbor-joining method.

Chromatographic fractionation and purification was performed for the organic layer of a liquid-liquid extraction of medium in which A. rolfsii was present; 3-oxo-γ-costic acid (1) exogenously introduced into the medium was previously isolated from C. montanus. The substrate was bio-transformed into new sesquiterpene...
acid products (2-5) and one known compound, 3-oxoederma-
1.4,11(13)-trien-7βH-12-oic acid (6) \(^ {17} \) (Fig. 2).

Compound 2 was isolated as a colorless oil, and the IR spectrum showed absorption bands at 3500 (broad), 1719 and 1650 cm\(^{-1}\) (Fig. 3). In the NOESY spectrum, the correlations of H-9 (deduced from chemical shifts as well as coupling constants and 2H) and C-12 (42.0); H-13 a,b (159.7); H-6 (124.6) and C-5 (159.7), C-7 (42.0); H-13 a b (δH 5.78, 6.40) and C-7 (42.0), C-11 (142.9), C-12 (δC 170.1), H-14 (δH 1.37) and C-1 (δC 76.3), C-5 (δC 159.7), C-9 (δC 36.2), C-10 (δC 41.6); and H-15 (δC 198.7) and C-3 (124.6) with H-6 (δH 2.53) indicated that the carbohydrate type sesquiterpene except for the presence of two additional hydroxy-
tons. Moreover, the correlation of H-14 (δH 1.37) with carbon signals in the HMBC spectrum at δC 76.3 (C-1) and 72.8 (C-2), respectively (Table 1).

The sequence and connectivities from C-1 to C-10 were estab-
lshed by HMBC analysis (Fig. 3). Signature correlations were observed between H-1 (δH 3.82) and C-3 (δC 198.7), C-5 (δC 159.7); H-6 (δH 2.32) and C-4 (δC 124.6), C-5 (δC 159.7), C-7 (δC 42.0); H-13 a,b (δH 5.78, 6.40) and C-7 (δC 42.0), C-11 (δC 142.9), C-12 (δC 170.1), H-14 (δH 1.37) and C-1 (δC 76.3), C-5 (δC 159.7), C-9 (δC 36.2), C-10 (δC 41.6); and H-15 (δC 198.7) and C-3 (δC 124.6) and C-5 (δC 159.7). The stereochemistry of 2 was deduced from chemical shifts as well as coupling constants and confirmed by NOESY data in consultation with a Dreiding model (Fig. 3). In the NOESY spectrum, the correlations of H-9 (δH 1.83) with H-2 (δH 4.40), H-6 (δH 2.76), and H-7 (δH 2.53) indicated that these were situated on the same face and were assigned as α-protons. Moreover, the correlation of H-14 (δH 1.37) with H-6 (δH 2.32) and H-8 (δH 1.92) suggested a β-orientation for these protons. The small coupling constant between H-1 (δH 3.8) and H-2 (δH 4.40) indicated protons with α-orientations. Based on these data 2 was identified as 1,2-dihydroxy-3-oxo-γ-costaic acid, a new natural compound.\(^ {18} \)

Compound 3 possessed the same skeleton and functionalities as 2, except for the absence of the hydroxyl group at C-2 that was supported by the \(^ {1} \)H NMR signals at δH 2.65 (δd J = 16.5, 5.5) and 2.58 m for H-2x and H-2β, respectively; the H-2 protons also correlated with the methylene carbon at δC 42.5 (C-2) in the HMBC spectrum. All protonated and quaternary carbons were assigned with the aid of HMQC and HMBC experiments (Fig. 3).

The HMBC was used to place the hydroxyl group at C-1 based on a correlation of its proton at δH 3.84 (dd, J = 13.0, 4.8) with C-9 (δC 37.7) and C-14 (δC 163.3). Other correlations were observed, including H-15 at δH 1.77 with C-3 (δC 197.2), C-4 (δC 130.1) and C-5 (δC 160.4), locating the carbonyl group at C-3. Additionally, correlations were observed between H-13a, b (δH 5.76, 6.41) with C-7 (δC 39.0) and C-12 (δC 170.2). The NOESY spectrum indicated interactions between H-14β and H-6 (δH 2.09), H-8 (δH 1.68) and H-9 (δH 2.20) establishing a β-orientation of these protons (Fig. 3). Moreover, correlations between H-7α, H-8 (δH 1.83) and H-9 (δH 1.42), indicating an α-orientation of these protons. The correlation between H-9α and the hydroxylated proton at C-1 confirmed the α-orientation of this proton. From the above results, compound 3 was identified as 1-hydroxy-3-oxo-γ-costaic acid, a new natural compound.\(^ {19} \)

Compound 4 possesses the same skeleton and functionalities as 2 except for the absence of the hydroxyl group at C-1; this proposed structure was consistent with \(^ {1} \)H NMR signals at δH 2.16 (dd) and 1.70 (m) for H-1α and H-1β respectively HMQC correlated with a methylene carbon signal at δC 43.3 (C-1). The location of groups and connectivities from C-1 to C-10 were established by HMBC experiments (Fig. 3). Correlations were observed between H-1 (δH 2.16, 1.70) and C-2 (δC 68.6), C-5 (δC 162.6), C-9 (δC 37.3), C-14 (δC 27.7); H-13 (δH 5.76, 6.39) and C-7 (δC 41.5), C-12 (δC 170.1); H-15 (δH 1.84) and C-3 (δC 200.7) and C-5 (δC 162.6). NOESY correlations between H-14 (δH 1.23) and H-6 (δH 2.31) and H-9 (δH 1.71) was consistent with a β-orientation of these protons. The α-configuration of the hydroxylated proton (H-2) was deduced from a correlation with H-9α (δH 2.00). Thus 4 was identified as 2-hydroxy-3-oxo-γ-costaic acid, a new natural compound.\(^ {20} \)

Compound 5 was isolated as a colorless oil. The EIMS showed a molecular ion peak [M]+ at m/z 262, in agreement with a molecular formula C_{12}H_{18}O_{4}. HREIMS determination of the 262 ion peak established a molecular formula of C_{12}H_{18}O_{4} (calcd. 262.1933; found 262.1926). The \(^ {1} \)H NMR spectrum revealed the presence of
five singlet signals at $\delta_H$ 6.06 (H-1), 5.79, 6.42 (H-13a,b), 1.28 (H-14) and 1.98 (H-15); aliphatic methylene protons were identified at $\delta_H$ 2.97 (d, $J = 13.1$, H-6a), $\delta_H$ 2.23 (t, $J = 13$, H-6b), $\delta_H$ 1.77 (m, H-8x,y) and $\delta_H$ 1.41 (m, H-9x,y) (H-9a,b). DEPT multiplicities confirmed the molecular formula. Carbon signals were assigned as two methyls at $\delta_C$ 24.7, 10.8; four methylenes at $\delta_C$ 33.4, 26.7, 39.2, 126.1; two methines at $\delta_C$ 41.0, 125.6 and seven quaternary signals at $\delta_C$ 170.0 and 183.0 (both carbonyls). Correlations were observed between $\delta_H$ 6.06 (H-1) and $\delta_C$ 145.0 (C-2), $\delta_C$ 183.0 (C-3), $\delta_C$ 162.0 (C-5), $\delta_C$ 39.2 (C-9) and $\delta_C$ 24.7 (C-14) (Fig. 4). Additionally, correlations were observed between $\delta_C$ 162.0 (C-5) and $\delta_H$ 2.97, 2.23 (H-6), $\delta_H$ 1.28 (H-14), $\delta_H$ 1.98 (H-15). Correlations were also observed between $\delta_H$ 5.79, 6.42 methylene protons (H-13a,b) with $\delta_C$ 41.0 (C-7) and $\delta_C$ 170.0 (C-12). The stereochemistry was deduced from the NOESY spectrum showing correlations between $\delta_H$ 1.28 (H-14b) with $\delta_H$ 2.23 (H-6) and $\delta_H$ 1.94 (H-9), establishing a $\beta$-orientation for these protons (Fig. 4). Thus, 5 was identified as 3-oxo-$\gamma$-costic acid, 1,2-ene-2-ol, a new natural compound.  

### Table 1

$^1$H NMR and $^{13}$C NMR spectral data for 2-5 (600 MHz, δ-ppm).

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<th>No</th>
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<th>$C$ ($\delta$ in Hz)</th>
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<td>1</td>
<td>3.82 d (2.7)</td>
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<td>2</td>
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</tr>
<tr>
<td>4</td>
<td>–</td>
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</tr>
<tr>
<td>5</td>
<td>–</td>
<td>159.7 s</td>
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<tr>
<td>6</td>
<td>2.32 t (12.4)</td>
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<td>8</td>
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<td>27.0 t</td>
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<td>15</td>
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Fig. 3. Selected HMBC (→) and NOESY (↔) correlations for 2 (R1-2 = OH), 3 (R1 = H & R2 = OH) and 4 (R1 = OH & R2 = H).

Fig. 4. Selected HMBC (→) and NOESY (↔) correlations of 5 (R = OH) and 6 (R = H).

### Table 2

IC$_{50}$ values for Caco-2 and A549 cell lines.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Caco-2 ($\mu$M)</th>
<th>A-549 ($\mu$M)</th>
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<tr>
<td>6</td>
<td>66</td>
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</tr>
</tbody>
</table>

Fig. 5. Morphological cytotoxicity includes cell rounding, shrinking and monolayer integrity loss compared to solvent controls (150× magnification).
To evaluate the anti-proliferative activity of the isolated eudesmane-type sesquiterpenes, human colon (Caco-2), and lung (A549) tumor cell lines were assessed using a cell viability assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Anti-proliferative activity was highest with 1 in the Caco-2 line and activity was not observed against A549 cells except for 2 (Table 2). Bright field phase contrast morphological assessment confirmed selective cytotoxicity with cell shrinkage and/or monolayer disruption compared to vehicle controls (Fig. 5). All assayed metabolites exhibited a concentration-dependent anti-proliferative effect against caco-2 and A549 cells (Suppl. Fig. 1A, B), however no discernable structure biological activity pattern is observed. Moreover, biological activity appears to be tumor-type dependent. The activity of 1 against Caco-2 compared with 2 suggests that the hydroxyl group on C-1 and/or C-2 impacts cytotoxic activity.

Acknowledgments

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2017.06.057.

References

7. Lee IS, Elsohly HN, Croom EM, Hufford CD. J Nat Prod. 1983, 55]. Cell monolayers were treated in quadrate with trypsin-EDTA solution. All cell incubations were maintained in a humidified incubator with 5% CO2 at 37°C.
12. The fungus Athelia rolfsii was identified morphologically and by molecular markers using internal transcribed spacer regions 1 and 4 with primers, 5'-TCCGTAGGTGCACTGCGG-3' and 5'-TCCTCCGGTATTGATATGC-3', respectively; for protocol see Henry et al. 2000 [Henry T, Peter CI, Steven HH. J. Clin. Microbiol. 2000, 38, 1510].
13. Fungal cultures were pre-incubated in PDA medium for 1 week at 28°C with a rotatory shaker at 120 rpm and then a spor suspension was inoculated into Czapek dox medium (composed of (g/L): NaNO3, 2.0; K2HPO4, 1.0; KCl, 0.5; MgSO4, TH2O, 0.5; FeSO47H2O, 0.01 and sucrose 20.0). After 3 days 3-oxo-γ-cyfolic acid (1) (20 mg dissolved on 200 μL methanol) was added to the suspension culture (100 mL × 2). Cultures were filtered after 14 days substrate incubation and filtered medium was CH2Cl2 extracted and concentrated in vacuo.
14. The fungal nucleotide sequence was deposited in GenBank under accession number KX685358.
15. The phylogenetic relationship was conducted by constructing a rooted tree using neighbor– joining method in MEGA6.
16. The crude extract was subject to reversed phase semi-preparative HPLC with an isocratic MeOH/CH3OH solvent system (3 mL/min) to give products 2–6 in a pure form.
18. 2-Dihydroxy-3-oxo-γ-cyfolic acid (2, 2.2 mg), a colorless oil; m/z 252.1984 (rel. int.) = 262.1926 [M], calcd. 262.1933 for C15H18O5; 13C NMR (150 MHz, CDCl3), see Table 1.
19. Hydroxy-3-oxo-γ-cyfolic acid (3, 3 mg), a colorless oil; m/z 250.1994 (rel. int.) = 260.0060 [M], calcd. 260.0066 for C15H18O5; 13C NMR (150 MHz, CDCl3), see Table 1.
20. Hydroxy-3-oxo-γ-cyfolic acid (4, 3 mg), a colorless oil; m/z 250.1994 (rel. int.) = 260.0060 [M], calcd. 260.0066 for C15H18O5; 13C NMR (150 MHz, CDCl3), see Table 1.
21. Hydroxy-3-oxo-γ-cyfolic acid (5, 5 mg), a colorless oil; m/z 250.1994 (rel. int.) = 260.0060 [M], calcd. 260.0066 for C15H18O5; 13C NMR (150 MHz, CDCl3), see Table 1.
22. Human tumor cell lines Caco-2 and A549 were obtained from the American Type Culture Collection (ATCC) and maintained as a monolayer culture in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, 4 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate. Monolayers were passaged at 70–90% confluence using a trypsin-EDTA solution. All cell incubations were maintained in a humified incubator with 5% CO2 at 37°C.
23. Caco-2 or A549 cells (5000 cells/well) were seeded onto 96-well plates and left overnight to form a semi-confluent monolayer. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Carbosynth, UK) was employed as a dye to monitor cell viability based on mitochondrial dehydrogenase reduction by metabolically active cells to insoluble formazan crystals [Mosmann, T. J. Immunol. Methods. 1983, 65, 55]. Cell monolayers were treated in quadruplicate with vehicle (DMSO, 0.1% v/v), increasing concentrations of test samples (6.25–100 μm) or doxorubicin hydrochloride as positive control anticancer drug for an exposure time of 48 hrs. At the end of exposure, MTT solution in PBS (5 mg/ml) was added to all wells and left to incubate for 90 min. The formation of formazan crystals was visually confirmed using phase contrast microscopy. DMSO (100 μL/well) was added to dissolve the formazan crystals with shaking for 10 min after which the absorbance was read at 492 nm against a no-cell blank on a Sunstar™ microplate reader (Tecan Austria Gmbh, Grödig, Austria). Cell proliferation was calculated comparing the averages OD values of the response curve fit to non-linear regression model using GraphPad Prism V 6.