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Biotransformation of Cycloastragenol by *Cunninghamella blakesleeana* NRRL 1369 Resulting in a Novel Framework

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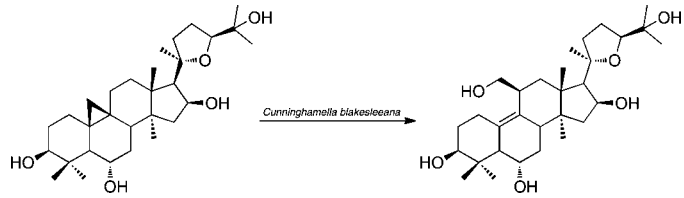
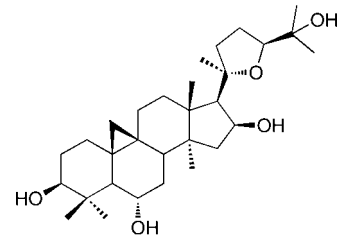
ABSTRACT



The microbial transformation of cycloastragenol by the fungus *Cunninghamella blakesleeana* NRRL 1369 was investigated. Unlike the original compound, the metabolite was found to possess an interesting triterpenic skeleton derived via an exceptional transformation involving ring cleavage and methyl group migration. The structure of the new metabolite was elucidated by 1-D (^{1}H, ^{13}C) and 2-D NMR (COSY, HMBC, HMQC, NOESY) techniques and MS analyses.

The formulations prepared from the roots of *Astragalus* species are widely used in Chinese traditional medicine, especially in the treatment of cancer (leukemia and uterine cancer) and immune system disorders.[1] *Astragalus* species have exhibited cardiotonic, analgesic, sedative, hepatoprotective, antiviral, and immunostimulant activities.[2−6] During studies to determine the molecules responsible for these therapeutic effects, many cycloartane-type molecules have been isolated from a wide range of *Astragalus* species. Cycloastragenol (**1**, CA) is the main aglycon of many cycloartane-type glycosides found in the *Astragalus* genus. Although CA is a minor metabolite mostly found in its glycosidic form, it has been shown to possess a remarkable pharmacological activity. It extends T cell proliferation by increasing telomarase activity showing that it may also help delay the onset of cellular aging.[7] Additionally, it was shown that the exposure of CD8 T lymphocytes from HIV-infected human donors to CA, reasonably retarded telomere shortening, increased proliferative potential, and importantly, enhanced cytokine/chemokine production and antiviral activity.[8] By this study, Fauce and his co-workers proved a new approach, for the first time, to use a pharmacological telomerase-based therapy to enhance immune function, thus directly addressing the telomere loss immunopathologic facet of chronic viral infection. Indeed, recently, CA has been introduced to the market as a new generation antiaging molecule.



Cycloastragenol (**1**)

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The natural product drug discovery process involves the isolation of new molecules from natural products, the investigation of their biological activities, and producing more active molecules from them. The last process includes chemical modifications, and microbial transformation is a very useful approach in this area. The use of whole microorganisms that mimic the mamalian metabolism to carry out selective transformation reactions make the economically and ecologically friendly microbial transformations advantageous.[9]

With the aim of generating new and more potent saponin derivatives, CA was subjected to microbial transformation. On the basis of the literature survey, the most commonly used microorganisms for the biotransformation of saponins, *Nocardia* sp. NRRL 5646, *Mycobacterium* sp. NRRL 3683, *Mycobacterium* sp. NRRL 3805, and *Cunninghamella blakesleeana* ATCC 8688a (NRRL 1369) were chosen.[10−12] All three microorganisms were screened for their biotransformation effectiveness on the basis of both the chemical diversity and the yield of the products in the extracts using the analytical scale method described below. The best preliminary results were observed with *C. blakesleeana*, and further studies were carried out by using this fungus for the microbial transformation.

The biotransformation process was conducted at two scales; analytical and preparative. Stock cultures of *C. blakesleeana* stored at +4 °C were revived on PDA (potato-dextrose-agar) slants at 30 °C. 2% inoculum derived from the suspension of 4-day-old cultures with Tween 80 (0.1%) was used in the biotransformation process. A one-stage fermentation protocol was followed where CA was fed to the biotransformation media 72 h after the inoculation. The biotransformation media contained 2% glucose, 0.5% yeast extract, 0.5% NaCl, 0.5% K_2HPO_4, and 0.5% (w/v) peptone (pH 6.0). Biotransformation processes were conducted using 250 mL flasks containing 50 mL of media and 10 mg of CA. Both submerged (30 °C, 200 rpm) and surface (30 °C) culture conditions were tested in the analytical scale, taking 2 mL samples on days 0, 2, 4, 7, 10, 14, and 21. Samples were centrifuged at 9000 rpm for 15 min. The supernatants were then extracted with ethyl acetate. The TLC profiles of the samples showed more consumption of CA in the submerged culture transformation processes. As a result, the preparative-scale studies continued with 10 flasks at 30 °C and 200 rpm for 7 days. In the preparative scale, the centrifuged fermentation broths were extracted with both ethyl acetate and *n*-butanol (each three times). The organic layers were then combined and evaporated under vacuum to yield 335.9 mg.

The EtOAc extract was then applied to a reversed-phase column (C18, 75 μm, 50 g). The extract had a major metabolite together with a couple of minor metabolites. The fraction containing the major metabolite, compound **2**, was further purified with silica gel column chromatography (40 g, 5:5; EtOAc/hexane). Compound **2** was then lyophilized to afford 8.9 mg of pure metabolite, making the biotransformation yield approximately 9%.

The structures of the metabolite (**2**) were characterized by MS and NMR analyses (Agilent Jet Stream ESI 6460 LC-MS; Bruker Autoflex III (smart beam) MALDI TOF/TOF; VARIAN AS400 NMR). The LC−ESI−MS spectrum showed a major ion peak at *m*/*z* 529.1, and the MALDI TOF/TOF spectrum showed a major ion peak at *m*/*z* 529.432 ($C_{30}H_{50}O_6Na$) that were assigned to $[M + Na]^+$. Together with the ^{13}C NMR data of the molecule, the MS data supported a molecular formula of $C_{30}H_{50}O_6$. ^{13}C and ^1H data of compound **2** are given in Table 1.

Table 1. ^{13}C and ^1H NMR Data of Compound **2** Recorded in CD_3OD (100 and 400 MHz, Respectively)

C\H	δ_C (**2**) (ppm)	δ_H (2) (ppm), *J* (Hz)
1	29.6	1.74 m, 2.71 m
2	32.8	1.86 m, 1.46 m
3	78.4	3.35 dd (4.8, 11.2)
4	42.5	
5	57.8	1.77 d (6.8)
6	68.8	3.74 m
7	37.2	1.30 m, 1.62 m
8	41.8	2.34 m
9	133.1	
10	135.8	
11	39.4	2.94 m
12	33.6	1.62 m, 2.11 d (14.4)
13	45.7	
14	46.2	
15	45.1	1.95 dd (4.0, 8.0), 1.48 m
16	74.4	4.65 ddd (6.4, 8.0, 8.0)
17	58.9	2.33 d (8.0)
18	20.3	1.16 s
19	69.1	3.47 dd (11.2, 5.2), 3.40 t (11.2)
20	88.4	
21	28.6	1.28 s
22	35.4	2.62 ddd (10.8, 10.8, 10.8), 1.45 m
23	26.9	2.04 m, 1.67 m
24	82.6	3.76 dd (6.0, 8.4)
25	72.5	
26	26.6	1.13 s
27	27.6	1.25 s
28	26.5	1.17 s
29	14.8	0.67 s
30	19.6	0.79 s

The absence of AX system signals in the upfield region deriving from the characteristic 9,19-cyclopropane ring was directly noticed from the ^1H NMR spectrum implying a ring cleavage. Seven tertiary methyl groups in the upfield and

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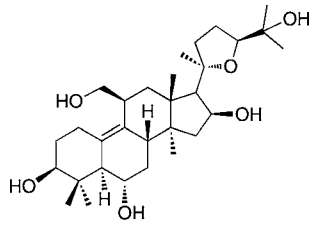
characteristic signals belonging to the H-3, H-6, H-16, and H-24 oxymethyne protons in the lowfield showed no significant alteration compared to the starting compound, CA.



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In the ^{13}C NMR spectrum, in addition to the oxymethyne signals and the quaternary carbon resonances of C-20 and C-25, an oxymethylene group at δ 69.1 and two olefinic carbons at δ 133.1 and 135.8 were observed. Full assignments of the proton and carbon signals of **2** were secured by $^1H-^1H$ DQF-COSY and HMQC spectra. In the HMQC spectrum, no correlation of the double bond carbons with any proton indicated a tetrasubstituted olefinic system.

The isolated lowfield proton signals resonating at an uncrowded region of the spectrum were used as the starting point for the COSY experiments. Detailed inspection of these signals revealed the spin systems shown in Figure 1. The

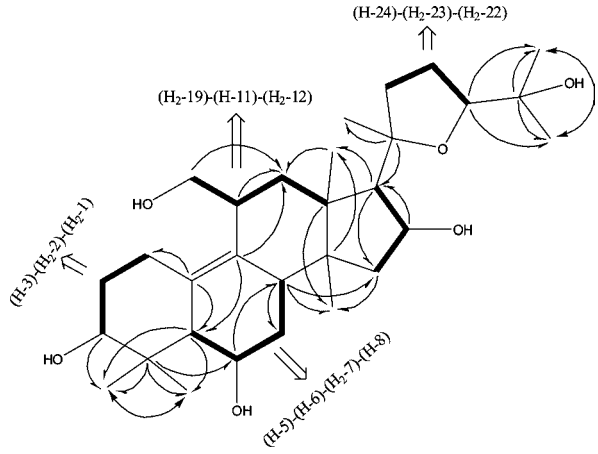

Figure 1. Spin systems deduced from the COSY spectrum and key long distance correlations from the HMBC spectrum.

significant difference in **2** was a new spin system including the aforementioned oxymethylene group (H_2-19 → H_2-12).

In order to establish the interfragment relationship, a heteronuclear multiple-bond correlation experiment (HMBC) was performed, which not only connected the fragments but also helped in locating the double bond and the new spin system resulting from the cleavage of the cyclopropane ring (Figure 1).

Thus, the long-distance correlations from C-9, C-11, C-18, and C-19 to H-12, C-9 to H-5, and C-10 to H_2-1 and H-5 indicated that the double bond was located between C-9 and C-10 and C-19 migrated over to C-11.

In order to prove the extraordinary rearrangement, compound **2** was acetylated in pyridine−acetic anhydride yielding two products. The major one (**2a**) showed three acetyl group signals, whereas the other exhibited four acetyl groups (**2b**). The ^1H NMR and COSY spectrum of **2b** indicated downfield shifts for H-3 (δ 4.67), H-6 (δ 4.95), H-16 (δ 5.39), and H_2-19 (δ 3.92 and 4.00), while **2a** showed exact chemical shifts for all the protons except H-16 signal difficult to acetylate due to the steric hindrance of the tetrahydrofuran ring. On the basis of these results, the presence of the C-19 oxymethylene group was evident, verifying the proposed structure.

The relative stereochemistry of the chiral centers was assigned by a combination of 2D-NOESY data, analysis of coupling constants, and molecular modeling studies performed on **2**. From the NOE data, it was obvious that **2** had the same configurations at the chiral centers C-3, C-5, C-6, C-8, C-16, C-17, C-20, and C-24 as CA, and only the stereochemistry at C-11 remained to be determined. In regard to the stereochemistry of C-11, the minimum energy conformations of α and β oriented H-11 were calculated with a 3-D computer-generated model. In the case of α-oriented C-19, H-8, H-11, and H_3-18 are required to show NOE interactions due to their close proximity on the β-plane (H-11/H_3-18: 2.6 Å; H-11/H-8: 3.6 Å), whereas no correlation is predicted in the case of H-11β (H-11/H_3-18: 4.1 Å; H-11/H-8: 4.0 Å). On the basis of the missing correlations between H-11 and H_3-18/H-8, the β-orientation of the methyleneoxy group at C-11 was concluded.

Based on a substructure search, we realized that there was only one secondary metabolite possessing an oxymethylene group at C-11 together with a double bond between C-9 and C-10. This metabolite, named podacarpaside, was reported from a cycloartane rich species, *Actaea podocarpa*.[13] The reported NMR data of the rearranged positions was also substantiated our structural findings. In the podacarpaside study, Ali and his co-workers proposed a biosynthetic rearrangement route that is driven by initial oxygenation of the α-equatorial C-11 hydrogen, followed by acid-catalyzed rearrangement of the strained cyclopropane into cyclobutane analogue and hydrolytic ring cleavage resulting methyl migration and generation of the C9−C10 double bond. In a similar fashion, a biomimetic reaction of humulene being converted to dactylol also relies upon a more oxidized intermediate.[14] As 11-hydroxy-CA was available as a reference compound (the biotransformation product obtained with *Glomerella fusarioides* ATCC 9552; unpublished data), we screened all of the samples, which were harvested in previously mentioned days to follow up the biotransformation products of *C. blakesleena*, on HPTLC to find out whether such an initiation through oxidation at C-11 was the case for CA. In view of the fact that there was no trace of 11-hydroxy-CA in the collected samples, we propose an alternative rearrangement mechanism that is more consistent with a single P450 reaction: (i) Initially, a secondary radical

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formation at C-11 followed by a ring expansion yielding a cyclobutyl radical (C-10 → C-9 → C-11 → C-19). (ii) Cleavage of the cyclobutyl ring resulting C9−C10 double bond and primary radical which is then trapped by OH radical to give the oxymethylene group (C-19) at the C-11 position.

Cunninghamella species are known to show parallelism with the mamalian liver microsomal monooxygenase system. In the previous studies, it was reported that *Cunninghamella* species can carry out the transformation reactions in the mamalian models, organs, and cells.[15−19] It was reported that *C. blakesleeana* has a broad enzyme system including aminopyrine *N*-demethylase, aniline hydroxylase, NADH-DCIP reductase for acetylation, methylation, and glycosidation.[20] Another study with *C. blakesleeana* proves its selective reaction abilities in making a shortcut in the 31-step process of the cortisone production.[21]

In this study, a rearrangement to give a novel triterpene framework via a ring cleavage (9,19-cyclopropane ring) followed by a methyl migration was reported for the first time, demonstrating once more the potential of the microbial systems and *C. blakesleeana* for the transformation of bioactive molecules.

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Supporting Information Available: TLC chromatograms, 1- and 2D- NMR spectra, LC−MS spectrum, MALDI-TOF spectra, and minimum energy conformers of **2** and NMR spectra and data for **2a**. This material is available free of charge via the Internet at http://pubs.acs.org.

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