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Tetrahdroxysqualene from *Rhus taitensis* Shows Antimycobacterial Activity Against *Mycobacterium tuberculosis*

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Abstract

Tuberculosis has become a major health problem, in particular with the emergence of extremely drug resistant tuberculosis (XDRTB). In our search for new therapeutic leads against TB, we isolated a new triterpene (**1**) from the plant *Rhus taitensis* collected in Papua New Guinea. Tetrahydroxysqualene (**1**) was isolated using bioassay-guided fractionation of the methanolic extract of *R. taitensis* leaves and twigs. The structure of tetrahydroxysqualene (**1**) was elucidated on the basis of HRESIMS and 1D and 2D NMR spectra. Tetrahydroxysqualene (**1**) exhibited anti–tuberculosis activity with an MIC of 10.0 μg/mL while showing only modest cytotoxicity.

Tuberculosis, caused by the bacterial pathogen *Mycobacterium tuberculosis*, has become a major global health threat, especially in developing nations.1 TB therapy requires long treatment regimens and patient compliance is difficult,[2] and failure of patients to comply with therapy has led to the emergence of multi drug resistant TB (MDRTB) and extremely drug resistant TB (XDRTB).[2,3] There is an ever-growing need for new therapeutics for treatment of TB, especially for HIV/TB patients.4

Over the past decades, natural products have played an important role as sources of secondary metabolites with potential as lead compounds for drug discovery.[5] In our effort to discover new therapeutic leads against TB, we screened a natural products library constructed of plant extract fractions from Papua New Guinea plants. Plant extracts were fractionated on HP20SS, which is a reversed-phase polystyrene-based adsorbent (See Experimental). The subsequent fractions were formatted into 96-well plates for screening. After screening the HP20SS library, we identified a TB inhibitory HP20SS fraction from *Rhus taitensis* Guill. (Anacardiaceae). A large scale extraction and bioassay-guided fractionation yielded one new triterpene, tetrahydroxysqualene **(1)** as the active component and the known 3*β*,22,25-trihydroxylupane.[6] The latter was not active against TB.

The genus *Rhus*, also known by the common name sumac, contains over 250 described species of flowering plants, and the biological activities of *Rhus* spp. extracts have recently been reviewed.[7,8] More recent reports include detailed studies on the anti-HIV activity of

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compounds from *Rhus chinensis* that inhibit HIV-1 at various points in the HIV-1 lifecycle.[9] Additionally, A new benzofuranic acid was recently described from the leaves of *Rhus alata*.[10] Aqueous stem-bark extracts were found to exhibit anti-inflammatory, analgesic, and hypoglycaemic effects in mice and rats.[11] To the best of our knowledge, there has only been one report on the chemical investigation of *R. taitensis* that yielded triterpenes including 3*β*, 20,25-trihydroxylupane.[6]

Tetrahydroxysqualene (**1**) was isolated as a pale off-white solid and accurate mass measurements showed an $[M + Na]^+$ at *m/z* 497.3610 and calculated for $C_{30}H_{50}O_4Na$, 497.3607. The IR spectrum indicated the presence of an OH (3291 cm^{-1}), and the structure was elucidated using 1D ^1H and ^{13}C NMR, and 2D HSQC, CIGAR, and COSY experiments. The ^{13}C spectrum showed 15 carbon resonances and in conjunction with the MS data indicated that the compound was symmetrical. Analysis of the ^{13}C NMR spectrum provided evidence that **1** possessed two overlapped methyl resonances at δ_C 16.01 and 16.02, six olefinic carbons at δ_C 137.2, δ_C 135.0, δ_C 130.9, δ_C 125.2, δ_C 124.5, and δ_C 134.1, and two oxygenated methylenes at δ_C 60.1 and δ_C 67.7. The other carbon signals were identified as aliphatic methylenes (see Experimental). The ^1H NMR spectrum indicated an isoprenoid backbone with prominent oxygenated methylene signals at δ_H 4.18 and δ_H 4.28, consistent with the carbon chemical shifts, δ_C 67.7 (C-1) and 60.1 (C-13), respectively. Assignment of the hydroxylated carbons at δ_C 67.7 (C-1) and 60.1 (C-13) was based on the steric interactions between the methylene at position 5 which would cause the *cis* methylene to resonate upfield.[12] These two oxygenated methylenes showed correlations in the CIGAR spectrum to the olefinic carbons at δ_C 137.1 (C-2) and at δ_C 130.9 (C-3). COSY correlations from H-3 to the methylene at δ_H 2.16 (H-4) allowed the assignment of the adjacent methylene. Correlations in the CIGAR spectrum were observed from H-4 to C-5 (δ_C, 39.3) and C-6 (δ_C, 134.1) providing a linkage to the next isoprene unit. Since both methyl groups were degenerate, the corresponding long-range correlations did not provide any support for the proposed assignments. However, the H-7, H-8, H-9 spin system could be assigned from the COSY spectrum, and correlations in the CIGAR spectrum from H-9 to C-10 supported the linkage to the next isoprene unit. Since the ^{13}C shifts of methylene carbons in linear isoprenoids alternate between ~26 and ~39, the ^{13}C shift of C-12 (δ 28.3) suggested the point of symmetry and supported the proposed structure (**1**). The four olefins were determined to be *Z* based on the ^{13}C shift of the methyl groups.[12] For example, C-14 and C-15 both resonate at ~16 ppm rather than ~25 ppm, which would be expected for *E* geometry.

Tetrahydroxysqualene (**1**) was active against *M. tuberculosis* $H_{37}Ra$ with an MIC of 10 μg/mL while showing only modest cytotoxicity (EC_{50} 27.5 ± 0.8 μg/mL) toward human T-cells. A report in 1956 showed that squalene exhibited antimycobacterial activity against *M. tuberculosis* in vitro and in vivo.[13] Additionally, two independent reports also showed that squalene inhibited the growth of *M. tuberculosis* $H_{37}Rv$, 99% at 100 μg/mL[14,15] and an MIC of 100 μg/mL.[16] Interestingly, squalene did not inhibit the growth of *M. tuberculosis* $H_{37}Ra$ even at 200 μg/mL. In the same assay, the MIC for rifampicin was between 0.0025 and 0.0079 μg/mL, which was essentially identical to previously reported values against $H_{37}Ra$.[17]

In conclusion, tetrahydroxysqualene (**1**) is a new triterpene that shows promising antimycobacterial activity and warrants additional studies. In particular, tetrahydroxysqualene (**1**) is more potent than squalene. However, a potential mechanism of action will require a more detailed investigation.

Experimental Section

General Experimental Procedures

UV spectra were measured on a Hewlett Packard 8452A diode array spectrophotometer. IR spectra were recorded using a JASCO FT/IR–400 spectrometer. NMR spectra were recorded on a Varian INOVA at 500 MHz for ^{1}H and 125 MHz for ^{13}C using vendor supplied pulse sequences. Residual solvent ($CDCl_3$) signals were used as reference (δ_H 7.24 ppm; δ_C 77.0 ppm). Accurate mass measurements were performed on a Micromass Q-tof Micro using positive ion mode. HPLC was performed on a Beckman System Gold equipped with a 168 PDA detector. Supelco HP20SS and squalene were purchased from Sigma.

Biological Material

Leaves and twigs of *Rhus taitensis* were collected in Papua New Guinea as part of an International Cooperative Biodiversity Group (ICBG) agreement, and the plant was identified by Dr. Osia Gideon and Mr. Pius Piskaut from the School of Natural and Physical Sciences at the University of Papua New Guinea. The specimen was collected at 9° 26.3′ S., 147° 20.8′ E. A voucher specimen (U20197-105) has been deposited at the University of Papua New Guinea Herbarium. A replicate voucher was deposited at the PNG Forest Research Institute.

Extraction and Isolation

For large-scale extraction, air-dried leaves and twigs (108.6 g) of *R. taitensis* were ground and extracted with 250 mL MeOH (3 × 24 hours) at room temperature. The crude extract (1.7 g) was filtered and concentrated in vacuo. Five grams of the crude extract were dissolved in MeOH and mixed with five grams of HP20SS and dried. The mixture was then poured into a column and fractionated using 100% water, 75% H_2O/25% 2-propanol, 50% H_2O/50% 2-propanol, 25% H_2O/75% 2-propanol, and 100% MeOH to yield five fractions designated FW, F1, F2, F3, F4, respectively. The fractions were collected and the solvents evaporated using a centrifugal evaporator.

The active HP20SS-F2 (0.2 g) was separated using Sephadex LH20 (1:1 $CHCl_3$/MeOH). The LH20 fractionation yielded five fractions with fractions designated A to E. Fraction A was the most active against TB and was separated using HPLC. Semi-preparative HPLC was carried out using a 250 × 10 mm column (4.5 mL/min, SiO_2) with a gradient from 95% isooctane/5% 2-propanol to 50% 2-propanol over 35 min. Tetrahydroxysqualene (**1**, 1.8 mg) eluted at 25.6 min. Fraction B (40 mg) was separated using same chromatographic conditions to give 3*β*, 22,25–trihydroxylupane (4.7 mg, RT 17.5 min).

Tetrahydroxysqualene (1): pale off-white solid; UV (CH_3CN) λ_{max} (log ε) 210 (3.76); IR (NaCl disc) υ_{max} 3291, 2917, 1666, 1444, 1382, 1222, 1149, 1006, 800 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.51 (2H, t, *J* = 7.2 Hz, H-3, H-3′), 5.12 (2H, m, H-11, H-11′), 5.10 (2H, m, H-7, H-7′), 4.28 (4H, br s, H-13, H13′), 4.18 (4H, br s, H-1, H-1′), 2.16 (4H, q, *J* = 7.2 Hz, H-4, H-4′), 2.06 (4H, m, H-7, H-7′), 2.01 (4H, m, H-5, H-5′), 1.99 (4H, m, H-12, H-12′), 1.97 (4H, m, H-9, H-9′), 1.58 (12H, br s, H-14, H-14′, H-15, H-15′); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 137.1 (C, C-2, C-2′), 135.0 (C, C-10, C-10′), 134.1 (C, C-6, C-6′), 130.9 (CH, C-3, C-3′), 125.1 (CH, C-7, C-7′), 124.4 (CH, C-11, C-11′), 67.6 (CH_2, C-1, C-1′), 60.1 (CH_2, C-13, C-13′), 39.7 (CH_2, C-9, C-9′), 39.3 (CH_2, C-5, C-5′), 28.3 (CH_2, C-12, C-12′), 26.6 (CH_2, C-8, C-8′), 26.0 (CH_2, C-4, C-4′), 16.01 (CH_3, C-14, C14′ or C15, C15′), 16.02 (CH_3, C-14, C14′ or C-15, C15′); HRESIMS *m/z* 497.3610 $[M + Na]^+$, calcd for $C_{30}H_{50}O_4Na$ 497.3607.

3*β*,22,25–Trihydroxylupane: NMR data matched those previously published.[6] HRESIMS *m/z* 483.3801 $[M + Na]^+$, calcd for $C_{30}H_{52}O_3Na$ 483.3814.

Antimycobacterial Assay

The TB assay was performed in a microtiter plate format modified from Franzblau et al.[18] $H_{37}Ra$ (ATCC#25177) was grown in ADC (Remel) supplemented 7H9 (Difco) medium until it reached log growth (OD_{600} 0.6), washed twice in PBS, briefly sonicated, filtered, aliquoted and frozen at −80 °C until use. A 1:200 dilution of this stock (approximately McFarland standard 2) in the above Difco medium was used to test the compounds. DMSO was used to solubilize and dilute the compounds and was kept at a final concentration of 0.5% in all wells. All treatments were performed in quadruplicate. One μL of **1** in DMSO was added to plates with media and TB at final concentrations ranging from 100 to 0.0001 μg/mL in ½ log dilutions. Rifampicin was added in a similar manner from 25 to 2.5×10^{-6} μg/mL in ½ log dilutions. After four days of humidified incubation at 37 °C, 11 μL of MTT (5 mg/mL) were added, and the plates were incubated overnight. The reduced product was solubilized with 50:45:5 water/DMF/SDS, and the absorbance was measured at 570 nm using a plate reader. Background absorbance was measured in wells that contained all media components, but no bacteria. Background was subtracted from all wells. The percent inhibition was calculated by dividing the absorbance of treated wells by the absorbance of the DMSO control; the result was then subtracted from one and multiplied by 100. MIC values were defined as the lowest concentration that resulted in inhibition of ≥90%.[18]

Cytotoxicity

CEM-TART human T-cells were grown and maintained in RPMI (Hyclone) supplemented with 20% FBS (Hyclone) and antibiotics/mycotics (Hyclone HyQ 100 U/mL PenG, 100 U/mL Streptomycin, 0.025 μg/mL Amphotericin B). One μL of each dilution from the same series and at the same final concentration as used in the TB assay, was added to 20,000 cells in 200 μL media per well of a 96-well plate. All samples were tested in quadruplicate. Doxorubicin (EC_{50} 4.4 ± 1.5 ng/mL) was used as a positive control. After 72 hours 11 μL of 5 mg/mL MTT was added and allowed to incubate for 2 hours. The plates were centrifuged at 500 × g for 10 min. The medium was removed, the product was solubilized with 100 μL of DMSO, and the absorbance was measured at 570 nm using a plate reader. Background absorbance was measured in wells that contained but no cells. Background was subtracted from all wells. The percent inhibition was calculated by dividing the absorbance of treated wells by the absorbance of the DMSO control; the result was then subtracted from one and multiplied by 100.

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