New pimarane diterpenes and other antimycobacterial metabolites from *Anisochilus verticillatus*

Roshan R. Kulkarni, Ketaki Shurpali, Vijay M. Khedkar, Vedavati G. Puranik, Dhiman Sarkar and Swati P. Joshi*

*Division of Organic Chemistry, CSIR-National Chemical Laboratory, Pune 411008, India; Combi-Chem Bio-Resource Centre, CSIR-National Chemical Laboratory, Pune 411008, India; Centre for Materials Characterization, CSIR-National Chemical Laboratory, Pune 411008, India*

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Phytochemical investigation of the acetone extract of the aerial parts of *Anisochilus verticillatus* afforded a new 8,9-secopimarane diterpene (1), two new isopimarane diterpenes (2, 3) and the known ursolic acid (4), α-amyrin (5), β-amyrin (6), stigmast-5-en-3-one (7) and hydroxychavicol (8). Structures of the new compounds were elucidated with the help of 1D and 2D nuclear magnetic resonance spectroscopic data, and single crystal X-ray crystallography of compound 3. Compounds 2 and 8 inhibited *Mycobacterium tuberculosis* H37Rv with an IC$_{50}$ of 11.3 (IC$_{90}$ of 20.0 µg/mL) and 12.5 µg/mL, respectively. Correspondingly, molecular docking studies with Extra Precision Glide revealed a correlation between score and biological activity for these compounds to describe the molecular basis for the most significant SAR results.

**Keywords:** *Anisochilus verticillatus*; diterpenes; antimycobacterial activity; alanine racemase; Lamiaceae

1. Introduction

*Anisochilus* Wall. Ex Benth. is a genus comprising 16 species of herbs and shrubs, 14 of which are found in India, with three being present in the state of Maharashtra – *Anisochilus carnosus*, *Anisochilus verticillatus* and *Anisochilus eriocephalus* (Suddee & Paton 2009). *A. verticillatus* Hook. F. (= *Anisochilus adenanthus* Dalzell & Gibson) is a herb endemic to Maharashtra (Singh et al. 2001).

Phytochemical investigation was carried out on the acetone extract of the aerial parts of *A. verticillatus*. This afforded a new 8,9-secopimarane diterpene (1), two new isopimarane diterpenes (2, 3) (**Figure 1**) and the known ursolic acid (4), α-amyrin (5), β-amyrin (6), stigmast-5-en-3-one (7) and hydroxychavicol (8). Structures of the new compounds were elucidated with the help of 1D and 2D NMR spectroscopy and single crystal X-ray crystallography of 2.
Compounds 1–8 were evaluated for antimycobacterium activity and the results motivated us to evaluate their binding mode to the target proteins related to tuberculosis. In order to understand mechanistically the observed inhibition pattern, we performed the computational molecular docking studies on the active compounds 1 and 8, with alanine racemase.

2. Results and discussion

Compound 1, molecular formula C_{20}H_{32}O_{3} assigned based on a pseudomolecular peak at m/z 319.2279 [M − H]−, in HR-ESI-MS operating in negative mode corresponding to five indices of hydrogen deficiency. The \(^{1}\)H NMR spectrum (Table S1) showed the presence of four methyl singlets at \(\delta_{\text{H}}\) 1.00, 1.05, 1.06 and 1.11 while the \(^{13}\)C NMR spectrum (Table S1) showed a monosubstituted double bond (CH\(_{2}\) \(\delta_{\text{C}}\) 110.4, CH \(\delta_{\text{C}}\) 145.3). This indicated towards a pimarane, isopimarane or a rosane-type of diterpene. Heteronuclear multiple bond correlation (HMBC) of the methyl groups at \(\delta_{\text{H}}\) 1.00, 1.06 and 1.11 with the methine carbon at \(\delta_{\text{C}}\) 73.8 indicated that compound 1 was similarly substituted as compound 3. This methine was assigned to be C-6 based on COSY correlation between \(\delta_{\text{H}}\) 4.35 (H-6) and 1.88 (H-5) (Figure S5), the latter confirmed by the HMBCs of C-5 (\(\delta_{\text{C}}\) 51.9) with H\(_{3}\)-18, H\(_{3}\)-19 and H\(_{3}\)-20 (Figure S5). The quaternary carbon at \(\delta_{\text{C}}\) 107.7 was assigned to position 9 based on HMBC with H\(_{3}\)-20 (\(\delta_{\text{H}}\) 1.11). A downfield shift of C-9 indicated attachment of two oxygen functions with one as a hydroxyl group and another forming an 1, 4-epoxy bridge with C-6. However, this necessitated cleavage of the C8–C9 bond. The structure was deduced as follows; analysis of the COSY spectrum revealed an isolated 5-6-7 linkage (Figure S5). The H-5 showed a COSY correlation with the methine at \(\delta_{\text{H}}\) 4.35 (H-6). The latter showed a COSY correlation with the
methylen protons at δ_H 3.30 and 2.33 (δ_C 49.3). Hence, the methylene at δ_C 49.3 was assigned to be C-7. Three-bond HMBC of H_2-14 (δ_H 3.05) with this methylene confirmed the assignment. This confirmed the C5-C6-C7 linkage and excluded the possibility of δ_C 73.8 being at C-7, which would place the epoxy bridge between C-9 and C-7. The carbonyl carbon at δ_C 213.1 was assigned as C-8 based on the HMBC with H-6. This confirmed the 8,9 bond cleavage and the location of the 1,4-epoxy bridge to be between C-6 and C-9 (Figure S5). The NOESY correlation of H-6 with H_2-19 and H_2-20 confirmed β-orientation of the epoxy bridge (Figure S5). A possible α-orientation was ruled out based on a comparison between MM2 stabilised models for the two possibilities (Figure S6). The observed NOESY data were consistent with the β-orientation of the epoxy bridge. Observation of a NOESY correlation between H_2-11 and H-16 confirmed isopimarane-type of skeleton for 1 (Figure S6), as a pimarane skeleton would lead to the separation of more than 5 Å between them which would not yield a NOESY peak. From these spectral analyses, compound 1 was identified to be a new diterpene, 8,9-secoisopimara-6,9-epoxy-9-hydroxy-8-one, with a novel 8,9-secopimarane skeleton. To the best of our knowledge, this is the first report on the 8,9-secoisopimarane skeleton. Literature survey revealed that with one exception (Kubo et al. 2003), the isolated isopimaranes belong to the normal series (Hussein et al. 1999; Shibuya et al. 1999; Stampoulis et al. 1999; Politi et al. 2002; Topçu et al. 2002; Lekphrom et al. 2010; Liang et al. 2013). Thus 1 most likely belonged to the normal series and not ent series.

Compound 2 showed a pseudomolecular peak at m/z 345.2397 [M + Na]^+ in HR-ESI-MS, indicating molecular formula C_{20}H_{34}O_{3}, corresponding to four indices of hydrogen deficiency. The IR spectrum showed absorptions at 3418, 1634 and 1464 cm\(^{-1}\), indicating presence of hydroxyl and olefinic groups. The \(^1\)H NMR spectrum (Table S1) showed four tertiary methyl singlets at δ_H 1.00, 1.22, 1.42 and 1.44. The \(^{13}\)C NMR (Table S1) showed a monosubstituted double bond (CH\(_2\) δ_C 108.9, CH δ_C 151.1). This indicated towards a pimarane, isopimarane or a rosane-type of diterpene. HMBC of the methyl groups at δ_H 1.00, 1.42 and 1.44 with the quaternary carbon at δ_C 77.2 (C-5) was consistent with pimarane or isopimarane-type of skeletons. The \(^{13}\)C NMR data of compound 1 were similar to those of the previously isolated pimarlan-8β-ol (Carvalho et al. 2006) and isopimarane-8β-ol (Lorimer & Weavers 1987). Analysis for \(^{13}\)C NMR data of the two known compounds revealed \(^{13}\)C NMR shifts of carbons in the vicinity of C-13 to be diagnostic in distinguishing between the pimarane and isopimarane types with trans-fused B and C rings. In the isopimaranes, the methyl group at C-17 resonates at ~δ_C 25.0 while in pimaranes it is downfield shifted to ~δ_C 32.0. Similarly, the monosubstituted double bond in isopimaranes resonates at ~δ_C 151.0 (C-15) and ~δ_C 108.0 (C-16), while in pimaranes they resonate at ~δ_C 147.0 and 112, respectively. Other significant differences observed between isopimarane and pimarane diterpenes were among C-11 (δ_p-δ_p = 2), C-12 (δ_p-δ_p = +2) and C-14 (δ_p-δ_p = 2). This analysis revealed 2 to be an isopimarane diterpene with the methyl being axial. Quaternary carbons at δ_C 77.2 and 74.7 and methine carbons at δ_C 72.7 and 49.0 indicated that two of the three ring junction carbons were substituted by hydroxyl groups (Table S1). The methine at δ_C 72.7 (δ_H 4.11) was assigned as C-6 based on HMBCs of δ_H 4.11 with the methylene at δ_C 43.6 (C-7) (Figure S11) and with the quaternary carbons at C-5 (δ_C 77.2), C-8 (δ_C 74.7) and C-10 (δ_C 40.1). An HMBC of H_3-20 (δ_H 1.42) with the methine at δ_C 49.0 placed it at position 9 (Figure S11). The observation of a Nuclear Overhauser Effect SpectroscopY (NOESY) correlation between H-6 (δ_H 4.11) and H_3-18 (δ_H 1.00) confirmed β-orientation of the hydroxyl group at C-6. Similarly, a NOESY correlation between δ_H 1.73 (either H-9 or H-7 or both) and H-14 (δ_H 1.43) confirmed a β-orientation for the hydroxyl group at C-8 (Figure S11) as well as a trans-fusion at C-8 and C-9. A cis-fusion at C-8 and C-9 would be contradictory with the \(^{13}\)C NMR shifts at positions 12, 14, 15, 16 and 17 (Barrero et al. 2003). Based on the observation noted for compound 1, compound 2 was identified to be a new natural product, isopimaran-5,6,8-triol.
Compound 3 showed a molecular ion peak at \( m/z \) 338.2456 [M]\(^+\) in HR-EI-MS which led to the molecular formula \( C_{20}H_{34}O_4 \), corresponding to four indices of hydrogen deficiency. The IR spectrum showed the presence of hydroxyl (3419 cm\(^{-1}\)) and olefinic (1634, 1453 cm\(^{-1}\)) groups. The \(^1\)H NMR spectrum (Table S1) showed four methyl singlets at \( \delta_H 0.99, 1.28, 1.47 \) and 1.54. The \(^{13}\)C NMR spectrum (Table S1) showed a monosubstituted double bond (\( CH_2 \delta_C 108.9, CH \delta_C 151.0 \)). The methyl group at \( \delta_C 25.0 \) and the chemical shifts of the monosubstituted double bond indicated compound 3 to be an isopimarane skeleton. The presence of three quaternary carbons at \( \delta_C 77.2, 78.7 \) and 79.8 indicated 3 to be similar to 2 with all the ring junction carbons at positions 5, 8 and 9 substituted by hydroxyl groups. The methine at \( \delta_C 73.7 \) indicated a hydroxyl substitution at one of the methylene groups. This methine was assigned C-6 based on a COSY correlation of \( \delta_H 4.31 \) (\( \delta_C 73.7 \)) with \( H_2-7 \) at \( \delta_H 1.60 \) and 2.37 (\( \delta_C 38.5 \)), the latter being assigned position 7 based on HMBC of methylene at \( \delta_C 38.5 \) with one of the H-14 at \( \delta_H 1.95 \) (Figure S17). The NOESY correlation between \( H_3-19 \) (\( \delta_H 1.47 \)) and \( H_3-20 \) (\( \delta_H 1.54 \)) confirmed their \( \beta \)-orientation (Figure S17), while a NOESY correlation between \( H-6 \) at \( \delta_H 4.31 \) and \( H_3-18 \) (\( \delta_H 0.99 \)) confirmed a \( \beta \)-orientation of the hydroxyl group. Anti-configuration of the hydroxyl groups at C-8 and C-9 with a \( \beta \)-orientation of C-8 hydroxyl group was revealed by single crystal (grown in 10% acetone in hexane) X-ray crystallographic studies (Table S2). X-ray crystallography also revealed an anti-orientation of the C-5 hydroxyl group and \( H_2-20 \) (CCDC no. 932686) (Figure S16). Based on the same argument as discussed for 1 and 2, compound 3 also most likely belonged to the normal series. Thus 3 was identified as a new natural product, isopimarane-5,6,8,9-tetraol.

Compound 4 was identified as ursolic acid (Seebacher et al. 2003), 5 and 6 as a non-separable mixture of \( \alpha \) and \( \beta \) amyrin (Migas et al. 2005), 7 as stigmast-4-en-3-one (Barla et al. 2006) and 8 as hydroxychavicol (Shimoni et al. 2003) by comparison of the literature NMR data and mass spectra.

Compounds 1–8 were evaluated for their inhibition of *Mycobacterium tuberculosis* H37Ra (Singh et al. 2011). Compound 8 inhibited *M. tuberculosis* H37Ra with an IC\(_{50}\) of 12.5 \( \mu \)g/mL while 2 exhibited an IC\(_{50}\) of 11.3 \( \mu \)g/mL (IC\(_{90}\) of 20.0 \( \mu \)g/mL). Under the same conditions, isoniazid which was used as a positive control showed an IC\(_{90}\) of 0.05 \( \mu \)g/mL.

The molecular docking approach was used to evaluate whether 2 and 8 were good ligands to the target proteins related to tuberculosis. The study revealed that experimental results are in agreement with the predicted binding affinities for alanine racemase (Lee et al. 2013). The antibiotic \( D \)-cycloserine was used to compare the binding modes of 2 and 8. It was observed that both 2 and 8 exhibited binding modes similar to those observed for \( D \)-cycloserine. Compound 2 occupied a large portion of the binding site compared to 8, resulting in additional thermodynamic interactions and higher proximity to the active site residues consequently showing slightly better docking score (−8.50) than compound 8 (−8.23). The best binding modes of \( D \)-cycloserine, 2 and 8 at alanine racemase’s active sites are displayed in Figures S18, S19 and S20, respectively, and their corresponding docking scores are listed in Table S3.

The per-residue ligand–receptor interaction analysis revealed favourable electrostatic and strong van der Waal’s interactions which were responsible in major parts for the binding of these ligands with alanine racemase (Table S4). Noticeably favourable hydrogen bonding interactions also contributed to the stability of the ligand–receptor complex of compounds 2 and 8 (Table S4). The per-residue interaction energy break up observed for 2 and 8 portrayed a very similar energetic distribution with the active site residues as seen for \( D \)-cycloserine, suggesting that they could follow the same binding mechanism as observed for \( D \)-cycloserine to exert the antimycobacterial activity. The stabilities of conformations with the highest scores for 2 and 8 in complex with alanine racemase were confirmed in the Molecular Dynamic Simulation using the Desmond program (DE Shaw, NY, USA) for a period of 1 ns. Post-facto analysis of the MD trajectory complexes did not show any alterations in the orientation of ligand into the active site.
Compounds 2 and 8 could form a potential starting point for development of novel drugs targeting alanine racemase.

3. Experimental

3.1. General experimental procedure

Optical rotations were measured with a JASCO P-1020 polarimeter (Easton, MD, USA). IR spectra were measured in chloroform using a PerkinElmer FT-IR spectrometer (Waltham, MA, USA). The $^1$H and $^{13}$C NMR spectra were measured using a BrukerAvance III Ultra Shield NMR instrument ($^1$H: 500 MHz, $^{13}$C: 125 MHz, Billerica, MA, USA). HR-ESI-MS were recorded with a Thermo-Scientific Q-Exactive spectrometer (Waltham, MA, USA) and HR-EI-MS were recorded with an MSI-Autoconcept mass spectrometer (Mass Spectrometry Instruments Ltd., West Yorkshire, UK). Single crystal X-ray diffraction was carried out using a Bruker SMART APEX CCD diffractometer with Mo $K_{\alpha}$ radiation. Column chromatography (CC) was performed using silica gel mesh 230–400 (Thomas Baker, Ltd., Mumbai, India), TLC plates procured from Merck Ltd. (Whitehouse Station, NJ, USA). Isoniazid and 2, 3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) were purchased from Sigma Aldrich (St. Louis, MO, USA). M. tuberculosis H37Ra (ATCC 25177) was obtained from MTCC (Chandigarh, India).

3.2. Plant material

Whole, healthy and flowering A. verticillatus plants were collected from Purandar Fort area, District Pune, on 21 October 2008. A herbarium was deposited in Botanical Survey of India, Western Circle, Pune (No. SPJ-1). Cleaned aerial parts were shade dried and powdered.

3.3. Extraction and isolation

The powdered plant (1.13 kg) was extracted using acetone ($3L \times 3 \times 14$ h) at room temperature. The acetone-soluble parts were filtered and concentrated under reduced pressure to yield a greenish acetone extract (39.7 g), 38.0 g of which was separated by CC with elution gradient acetone in petroleum ether to collect 18 fractions (AV1–AV18). The fraction AV2 (11.8 g) was further separated by CC using an acetonitrile gradient of 0.5% to 3% in chloroform to collect four fractions (AV2a–d). The fraction AV2c (287.2 mg) was separated by CC using a gradient of acetonitrile from 0.5% to 1% in chloroform to isolate the mixture of compounds 5–7. They were further separated by preparative TLC using benzene as a developing system in one direction and 1% ethyl acetate–benzene in the reverse direction to isolate compound 7 (12 mg) and compounds 5 and 6 (15 mg) as a non-separable mixture. The fraction AV3 (800 mg) was purified by CC using acetonitrile gradient (0.5% to 3%) in chloroform to collect eight fractions (AV3a–h). Compound 4 was isolated from the fraction AV3c by preparative TLC using 2% acetonitrile in chloroform and 10% ethyl acetate in cyclohexane as developing systems. The fraction AV3g contained compound 1 (6 mg) and was purified by repeated preparative TLC using 2% acetonitrile in chloroform and 4% ethyl acetate in benzene as developing systems. The fraction AV4 (1.8 g) was separated by CC eluting with an acetonitrile gradient (1% to 4%) in chloroform to collect 13 fractions (AV4a–m). From the combined fractions AV4i, AV4j and AV4h, 2 (12 mg) was purified by CC in 3% acetonitrile in chloroform followed by preparative TLC using 20% acetone in cyclohexane as a developing system. From the fractions AV4k, AV4l and AV4m, compound 3 (120 mg) was separated by crystallisation. The fraction AV5 (4.0 g) was separated by CC eluting with an acetonitrile gradient (1% to 15%) in chloroform to collect 15 fractions (AV5a–o). From fractions AV5n
and AV5o, compound 8 (10 mg) was isolated by preparative TLC using 10% ethyl acetate in benzene as developing system.

3.3.1. 8, 9-Secopimara-6, 9-epoxy-9-hydroxy-8-one (1)
Yellow amorphous; $^1$H (CDCl$_3$, 400 MHz): see Table S1; $^{13}$C NMR CDCl$_3$, 100 MHz): see Table S1; HR-ESI-MS [M – H]$^-$ m/z 319.2279 (calculated for C$_{20}$H$_{31}$O$_3$, 320.2273).

3.3.2. Isopimaran-5, 6, 8-triol (2)
White amorphous; $[\alpha]^D_{25}$ + 6.86 (c = 0.81, acetone); IR (CHCl$_3$) $\nu_{\text{max}}$ 3419, 1634 and 1453 cm$^{-1}$; $^1$H NMR (CDCl$_3$, 400 MHz): see Table S1; $^{13}$C NMR (CDCl$_3$, 100 MHz): see Table S1; HR-ESI-MS [M + Na]$^+$ m/z 345.2397 (calculated for C$_{20}$H$_{34}$O$_3$Na, 345.2406).

3.3.3. Isopimaran-5, 6, 8, 9-tetraol (3)
White crystals; mp 200.7\(^\circ\)C; $[\alpha]^D_{25}$ − 27.93 (c = 1.00, acetone); IR (CHCl$_3$) $\nu_{\text{max}}$ 3419, 1634 and 1453 cm$^{-1}$; $^1$H NMR (CDCl$_3$, 400 MHz): see Table S1; $^{13}$C NMR (CDCl$_3$, 100 MHz): see Table S1; HR-EI-MS [M]$^+$ m/z 338.2456 (calculated for C$_{20}$H$_{34}$O$_4$, 338.2457).

3.4. Antimycobacterial activity and molecular docking study
Compounds 1–8 were evaluated in vitro for their inhibitory effect against M. tuberculosis H37Ra. Compound 8 inhibited M. tuberculosis H37Ra with an IC$_{50}$ of 12.5 $\mu$g/mL, while 2 exhibited an IC$_{50}$ of 11.3 $\mu$g/mL (IC$_{90}$ of 20.0 $\mu$g/mL).

Molecular docking was performed using Glide (Grid-Based Ligand Docking With Energetics) (Friesner et al. 2004; Halgren et al. 2004) module available in the SchrÖdinger molecular modelling package (SchrÖdinger, Inc., USA) installed on a Linux workstation, having an AMD 3600 + processor, 3 GB physical memory and with a CentOS platform.

The crystal structure of alanine racemase from M. tuberculosis (PDB 1XFC) was obtained from Protein Data Bank (www.rcsb.org). The 3D structures of compounds 1 and 8 were built using builder panel in Maestro. Both the compounds (2 and 8) were docked into the active site defined using the Grid generation protocol utilising the extra precision (XP) Glide scoring function to estimate protein–ligand binding affinities. The output file is analysed using Maestro’s Pose Viewer utility.

4. Conclusion
This work led to the isolation of isopimarane-types of diterpenes as well as a new skeleton, 8,9-secoisopaimare, for the natural products. Compounds 2 and 8 being active against tuberculosis in whole cell assays also gave a good binding to the target alanine racemase. Further investigations on the hydroxychavicol and the isopimarane series of natural products should lead to more potent antimycobacterial compounds.

Supplementary material
Supplementary material relating to this paper is available online, alongside Tables S1–S4 and Figures S1–S20.
Disclosure statement
No potential conflict of interest was reported by the authors.

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