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2018-07-03

Langat , M , Helfenstein , A , Horner , C , Tammela , P , Hokkanen , H , Izotov , D &
Mulholland , D 2018 , ' Pumilol, a Diterpenoid with a Rare Strobane Skeleton from *Pinus*
pumila (Pinaceae) ' , Chemistry & Biodiversity , vol. 15 , no. 10 , e1800056 . <https://doi.org/10.1002/cbdv.201800056>

<http://hdl.handle.net/10138/303737>

<https://doi.org/10.1002/cbdv.201800056>

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Received Date:

Revised Date:

Accepted Date:

Article Type: Full Paper

Pumilol, a Diterpenoid with a Rare Strobane Skeleton from *Pinus pumila* (Pinaceae)

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Abstract

Pumilol (**1**), a strobane diterpenoid, reported herein for the first time was isolated from the bark of *Pinus pumila* (Pall.) Regel (Siberian Dwarf Pine or Japanese Stone Pine), along with seventeen known compounds including serratane triterpenoids, not previously reported from this species, and four ferulate derivatives. The stereostructure of pumilol was established using HRESIMS, NMR, the DP4+ probabilities and by comparison of the experimental and calculated electronic circular dichroism (ECD) spectra. Labda-8(17),13-dien-15-oic acid (**4**), bornyl *trans*-4-hydroxycinnamate (**14**) and *E*-bornyl ferulate (**15**) showed activity against *S. aureus* and/or *E. faecalis* with MIC₉₀ values 12.5-50 µM.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/cbdv.201800056

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Keywords

Pinus pumila, pumilol, strobane, serratane triterpenoid, DP4+ probability, electronic circular dichroism

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Introduction

Pinus pumila (Pall.) Regel (Pinaceae) is a shrub growing to 6 m tall with branches that spread to 10 m. It is distributed in Japan, North Korea, North Mongolia, China and from the Yenisey River to East Siberia at 1000 – 2300 m elevation.^[1] Nakonechnaya *et al.* have investigated the genetic variation within *Pinus pumila* and have shown this species to be one of the most polymorphic of the genus *Pinus*.^[2,3] Previously the oleoresin of *P. pumila*, collected on the eastern shore of Lake Baikal and the Poronai region of Sakhalin oblast, were investigated and a range of monoterpenoids were detected using GLC methods, and labdane, abietane, cembrane and isopimarane diterpenoid acids were isolated from complex mixtures, usually after methylation.^[4-6] Material collected in Khabarovsk, Russia was investigated in this study.

Results and discussion

The dried and powdered stem bark of *P. pumila* was extracted with ethanol at room temperature with agitation. The crude extract was separated using column chromatography over silica gel to yield a diterpenoid, pumilol (**1**) possessing the rare strobane diterpenoid skeleton (Fig. 1). In addition to pumilol, seventeen known compounds including four labdanes: agatholic acid (**2**),^[3,4] 18-*O*-succinylagatholic acid (**3**),^[3] labda-8(17),13-dien-15-oic acid (**4**)^[4-6] and agathic acid (**5**)^[3,4]; four abietanes: dehydroabietic acid (**6**),^[4-6] 15-hydroxydehydroabietic acid (**7**),^[4-6] 15-hydroxy-7-oxodehydroabietic acid (**8**)^[6] and 7 α ,15-dihydroxydehydroabietic acid (**9**)^[7]; one abietane: isopimaric acid (**10**)^[4,5]; three serratane triterpenoids: 3 β -methoxyserrat-14-en-21-one (**11**),^[8] 3 β -methoxy-21 β -hydroxyserrat-14-ene (**12**),^[8] 3 β -methoxy-21 β -acetoxyserrat-14-ene (**13**)^[8]; four borneol ester derivatives: bornyl *trans*-4-

hydroxycinnamate (**14**),^[3] *E*-bornyl ferulate (**15**),^[3] bornyl *cis*-4-hydroxycinnamate (**16**),^[3] *Z*-bornyl ferulate (**17**)^[3,9] and catechin (**18**)^[10] were also isolated (Fig. 1).

Pumilol (**1**) (Fig. 1), was isolated as a colourless oil and HR-ESIMS gave a molecular formula of C₂₀H₃₀O₅ for the compound, (*m/z* 373.1980 [M+Na]⁺, calcd. 373.1990), requiring 6 double bond equivalents. The ¹³C NMR spectrum showed 20 carbon signals,

including 4 methyl, 6 methylene, 3 methine (one olefinic) and 7 quaternary carbons (one carboxyl, one olefinic, one dioxygenated and two oxygenated). Rings A and B were very similar to co-isolated and previously reported diterpenoid acids with a carboxylic acid group at C-18 (δ_C 183.8), methyl groups at C-19 (δ_H 1.16, δ_C 16.1) and C-20 (δ_H 0.68, δ_C 15.7), and C-5 and C-9 methine groups.^[11]

Fig. 1. Structures of compounds isolated from *Pinus pumila*

The ¹H-¹H COSY spectrum showed coupling between the H-5 (δ_H 1.86 m)/2H-6 (δ_H 1.44, m/ δ_H 1.44) and 2H-6/2H-7 (δ_H 1.63 m, 1.92 dt, *J* = 12.1, 2.9) resonances. The two H-7 resonances showed no further coupling. A correlation was seen in the HMBC spectrum between an alkene proton resonance (δ_H 5.50 d, *J* = 1.5) ascribed to H-17 and the C-7 (δ_C 35.4) resonance (Fig. 2a). The H-17 resonance showed correlations with the C-15 methyl (δ_C 16.6), the fully substituted C-14 alkene (δ_C 137.0) and the oxygenated C-13 (δ_C 75.7) resonances. The downfield position of the 3H-15 (δ_H 1.78 d, *J* = 1.5) resonance indicated a vinyl methyl group.

The C-14 resonance showed a correlation with the remaining methyl group resonance at δ_{H} 1.25 (3H-16), the downfield position of this resonance indicating oxygenation at C-13.

The HMBC spectrum showed correlations between 3H-16 and the dioxygenated C-12 resonance (δ_{C} 106.7), and between the C-13 (δ_{C} 75.7) and the two H-11 resonances (δ_{H} 1.79 and 1.66). The 2H-11 proton resonances showed ^1H - ^1H COSY coupling with the H-9 resonance (δ_{H} 2.04) and, in the HMBC spectrum, with the C-8 (δ_{C} 81.6) and C-10 (δ_{C} 35.7) resonances. The ^1H NMR spectrum acquired in DMSO showed two hydroxy group proton resonances (δ_{H} 5.56 and 4.03). The molecular formula showed the presence of five oxygens, but six oxygens are required for the carboxylic acid, oxygenated C-8 and C-13 and dioxygenated C-12, indicating the presence of a C-O-C linkage.

Fig. 2. (a) ^1H - ^1H COSY and key HMBC (H→C) correlations of pumilol (**1**); (b) Key NOESY spectrum correlations pumilol (**1**)

Table 1. ^1H and ^{13}C NMR data of **1** (in CDCl_3)^a

No.	δ_{C} , carbon type	δ_{H} mult, (J in Hz)
1 α	38.2 CH_2	1.14 m
β		1.38 br d ($W_{1/2}$ =24.9 Hz)
2 α	17.4 CH_2	1.63 m
β		1.52 m
3 α	37.2 CH_2	1.83 m
β		1.65 m
4	46.8 C	-
5	50.5 CH	1.86 m
6 α	22.0 CH_2	1.44 m
β		1.44 m
7 α	35.4 CH_2	1.63 m
β		1.92 dt (12.1, 2.9)

8	81.6 C	-
9	62.1 CH	2.04 dd (9.6, 11.4)
10	35.7 C	-
11 α	29.4 CH ₂	1.79 m
11 β		1.66 m
12	106.7 C	-
13	75.7 C	-
14	137.0 C	-
15	16.6 CH ₃	1.78 d (1.5)
16	16.7 CH ₃	1.25 s
17	130.2 CH	5.50 d (1.5)
18	183.8 C	-
19	16.1 CH ₃	1.16 s
20	15.7 CH ₃	0.68 s

^aRecorded at 500 MHz (¹H NMR) or 125 MHz (¹³C NMR).

Two structures were possible: either a 12,13-epoxide, or an 8,12-hemiacetal linkage. Due to the chemical shifts of C-12 and 13, the latter was indicated and using the NOESY spectrum the hydroxyl group proton resonances could be assigned (δ_{H} 5.56, 13-OH and δ_{H} 4.03, 12-OH). Correlations seen in the NOESY spectrum between the 3H-19/3H-20, 3H-20/H-17, H-17/3H-15, 3H-15/3H-16, 3H-16/11 β , 12-OH/13-OH, 13-OH/3H-15 resonances were used to determine the relative configuration (See Table 1 and Fig. 2b). Ring C was seven-membered and similar to that of only three compounds, strobic acid, strobol and strobol, reported with the rare strobane diterpenoid skeleton isolated from *Pinus strobus*^[12,13] and strobols A and B reported from *Siegesbeckia pubescens* (Asteraceae).^[14]

The configuration at C-13 for compound **1** was rationalized by calculating the DP4+ probability. The DP4+ determines probabilities for the stereochemical assignment of isomeric compounds using quantum chemical calculations of NMR shifts.¹⁵ Based on the correlations observed in the NOESY spectrum and a model (Fig. 2b), two possible isomers were likely for compound **1** shown as **1r** and **1s** (Fig. 3). Initially, conformational analysis for **1r** and **1s** were conducted using Spartan software at the MMFF basis set. The most significant conformations of **1r** and **1s** that were under 2 kcal/mol were optimized at the B3LYP/6-311+G** level (gas phase). The optimized representative conformations were subjected to NMR GIAO calculations at the PCM/mPW1PW91/6-311+G**) built onto Gaussian09 software and Boltzmann weighted where applicable.^{15,16} The experimental data and the corresponding shielding tensors were introduced to the Excel

file provided by Grimblat *et al.*, to compute the DP4+ probabilities.¹⁵ The results suggested that the 13*R*-isomer (**1r**) of compound **1** was the likely isomer (Table 2).

Fig 3. Possible isomers for **1**

Table 2. DP4+ probability analysis for two possible isomers of **1**

Probability	Isomer 1r	Isomer 1s
sDP4+ (H data)	91.52%	8.48%
sDP4+ (C data)	99.25%	0.75%
sDP4+ (all data)	99.93%	0.07%
uDP4+ (H data)	56.95%	43.05%
uDP4+ (C data)	96.67%	3.33%
uDP4+ (all data)	97.46%	2.54%
DP4+ (H data)	93.46%	6.54%
DP4+ (C data)	99.97%	0.03%
DP4+ (all data)	100.00%	0.00%

In order to determine the absolute configuration of **1**, its theoretically calculated and experimental ECD curves were compared. A theoretical excitation circular dichroism (ECD) spectrum of **1** was compared to the experimental ECD spectrum of **1**. A conformational search on the 4*R**, 5*R**, 8*R**, 9*R**, 10*S**, 12*S** and 13*R** isomer of **1** using Spartan software to evaluate the conformer distribution at ground state with molecular mechanics force fields (MMFF) was undertaken. MMFF analysis of **1** (of the normal diterpenoid series) gave eleven conformers, three of which were under 3 kcal/mol. The three conformers, consistent with NOESY NMR experimental data, M0001 (0.00 kcal/mol), M0002 (0.16 kcal/mol) and M0003 (0.38 kcal/mol) had Boltzmann distributions of 0.435, 0.335 and 0.229 respectively. Each of these three conformers was subjected to TDDFT calculations using a B3LYP method at 6-31G (d, f) level built into Gaussian09 software.^[16] The ECD curves for the three conformers were Boltzmann weighted and compared to the experimental ECD curve for **1** (Fig. 4). From the comparison, the absolute configurations at the chiral centres of **1** were unambiguously assigned as 4*R*, 5*R*, 8*S*, 9*R*, 10*S*, 12*S* and 13*R*. To confirm this assignment a theoretical

ECD spectrum for the enantiomer (**1a** of *ent*-diterpenoid series) of **1**, also consistent with the NOESY NMR experimental data (4*S**, 5*S**, 8*R**, 9*S**, 10*R**, 12*R**, 13*S**) was obtained. Initially a MMFF conformational search using Spartan software of **1a** yielded eleven conformers as for **1**, three of which were under 3 kcal/mol. The three selected conformers were also subjected to TDDFT calculations using a B3LYP method at 6-31G (d, f) level built into Gaussian09 software and Boltzmann weighted (Fig. 4). The theoretical ECD curve of **1a** was opposite and equal in intensity to that of **1**, thus the structure of pumilol, **1** was confirmed to be the previously undescribed [4*R*,5*R*,9*R*,10*S*]-8*S*,12*S*-epoxy-12*S*,13*R*-dihydroxystrob-15(17)-en-18-oic acid.

Fig. 4 Experimental ECD spectrum (black), conformationally averaged calculated ECD spectrum (solid red) of **1** and conformationally averaged calculated ECD spectrum (dashed red) of **1a**. The experimental ECD spectrum has been normalized from 185-175 nm to ease the comparison of experimental and calculated data.

Antimicrobial properties of compounds **2**, **3**, **4**, **5**, **8**, **14**, **15** and the EtOH extract were evaluated in a two-tiered assay: Firstly, the samples were screened for antimicrobial activity against five bacterial strains (*Enterobacter aerogenes*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*) and one fungal strain (*Candida albicans*) at a concentration of 50 μ M (50 μ g/ml for the EtOH extract) (Supporting information). Secondly, compounds that showed antimicrobial activity > 90% in the primary screening (50 μ M) were assessed at several concentrations to determine their MIC₉₀ values. The antimicrobial activity was evaluated using broth microdilution assay according to CLSI guidelines.^[17]

Compounds **4**, **14** and **15** showed activity against *S. aureus* and/or *E. faecalis* in the primary screen (see the Supporting Information). Compound **14** showed potent activity against *S. aureus* (MIC₉₀ 25 µM, Table 3), whereas compound **15** was also active against *E. faecalis* (MIC₉₀ 12.5 µM). The acetates of both bornyl *p*-coumarate and bornyl ferulate (compounds **14** and **15**) were previously isolated from the resin of *P. pumila*.^[3] The (+)-isomer of compound **14** [(-)-bornyl *p*-coumarate] has previously been reported to be active against gram-positive bacteria, with an MIC of 2 µM against *S. aureus*.^[18] Antimicrobial activity of compound **3**, and its derivatives has also been reported.^[19,20]

Table 3. MIC₉₀ values (µM) against *S. aureus* and *E. faecalis* for the most active compounds, **4**, **14**, and **15**

Compound	<i>S. aureus</i>	<i>E. faecalis</i>
4	- ^a	50
14	25	-
15	25	12.5

^anot determined (not active in the primary screen)

Conclusions

In conclusion, a strobane diterpenoid, seventeen known compounds including serratane triterpenoids and four ferulate derivatives are reported from the bark of *Pinus pumila* (Pall.) Regel (Siberian Dwarf Pine or Japanese Stone Pine). The findings support the presence of the rare strobane diterpenoid in a *Pinus* species. Three compounds isolated in substantial quantities, labda-8(17),13-dien-15-oic acid (**4**), bornyl *trans*-4-hydroxycinnamate (**14**) and *E*-bornyl ferulate (**15**) were showed activity against *S. aureus* and/or *E. faecalis* with MIC₉₀ values 12.5-50 µM.

Experimental section

General

Optical rotations were measured on a JASCO P-1020 polarimeter. ECD spectrum for compound **1** was obtained on an Applied Photophysics Chirascan CD spectrometer using a 1 mm cell and acetonitrile as the solvent. FTIR spectra were recorded using a Perkin-

Elmer (2000) spectrometer. 1D and 2D NMR spectra were recorded in CDCl₃ on a 500 MHz Bruker AVANCE NMR instrument at room temperature. Chemical shifts (δ) are expressed in ppm and were referenced against the solvent resonances at 7.26 and 77.23 ppm for ¹H and ¹³C NMR respectively. HR-ESI mass spectra were recorded on a Bruker MicroToF mass spectrometer using an Agilent 1100 HPLC to introduce samples (University of Oxford). Column chromatography was done using SiO₂ packed to 4 or 1 cm diameter gravity columns. TLC was done on aluminium precoated SiO₂ plates visualised using anisaldehyde reagent.

Plant material

Pinus pumila (Pall.) Regel (Pinaceae) was collected in Khabarovsk, Russia 2009. A voucher specimen, PP10102008 has been retained at the Department of Agricultural Sciences, University of Helsinki, Finland.

Extraction and isolation

Air dried, powdered bark of *Pinus pumila* (Pall.) Regel (47 g) was extracted manually with EtOH at room temperature with agitation. The ethanolic extract was concentrated to give 18.3 g of brown gummy crude extract. This extract was subjected to column chromatography over silica gel (Merck 9385) eluting with hexane with increasing amounts of methylene chloride. Once 100% methylene chloride had been used, step gradient elution using methanol was used to yield fractions (75 ml each), which were combined depending on similarity in pattern on analytical TLC. Fractions 1 – 61 that had been eluted with 100% hexane with gradient elution to 1:1 hexane:methylene chloride gave a mixture of glycerols and fatty acids compounds that were not purified. Fractions 62 – 64 eluted using 2:3 hexane:methylene chloride gave compounds **11** (53 mg), 3 β -methoxyserrat-14-en-21-one^[8] and **13** (5 mg), 3 β -methoxy-21 β -acetoxyserrat-14-ene^[8] on further purification using 1:1 hexane:methylene chloride on 1 cm diameter SiO₂ column chromatography, fractions 68 – 69 eluted using 1:4 hexane:methylene chloride gave compounds **12** (5 mg), 3 β -methoxy-21 β -hydroxyserrat-14-ene,^[8] **15** (4 mg), *E*-Bornyl ferulate^[3] and **17** (133 mg), *Z*-Bornyl ferulate^[3,9] on further purification using 100% methylene chloride on 1 cm diameter SiO₂ column chromatography. Fraction 90 -

110 eluted using 100% methylene chloride gave compounds **14**, Bornyl *trans*-4-hydroxycinnamate^[3] and **16** (20 mg), bornyl *cis*-4-hydroxycinnamate^[3] and fraction 115 – 120 eluted using 1% methanol in methylene chloride gave compound **6** (30 mg), dehydroabiatic acid^[4–6] and **8** (13 mg), 15-hydroxy-7-oxodehydroabiatic acid.^[6] Fractions 129 – 139 eluted using 5% methanol in methylene chloride gave compounds **4** (15 mg), labda-8(17),13-dien-15-oic acid^[4,6], **6**, dehydroabiatic acid^[4–6], **7** (10 mg), 15-hydroxydehydroabiatic acid^[4,6] and **10** (13 mg), isopimaric acid.^[4,5] Fractions 139 – 140 eluted using 10% methanol in methylene chloride gave compound **2** (21 mg), agatholic acid^[3,4] whereas fractions 150 – 157 gave compounds **3** (40 mg), 18-0-succinylagatholic acid^[3] and **5** (18 mg), agathic acid^[3,4] on further purification using 5% methanol in methylene chloride on 1 cm diameter SiO₂ column chromatography. Fraction 150 – 158 eluted using 10% methanol in methylene chloride gave compounds **9** (8 mg), 7 α ,15-dihydroxydehydroabiatic acid^[7] and **18** (13 mg), catechin^[10] on further purification using 5% methanol in methylene chloride on 1 cm diameter SiO₂ column chromatography. Fraction 209 – 220 eluted using 15% methanol in methylene chloride followed by preparative TLC gave compound **1** (8 mg).

Pumilol (1): Colourless oil; $[\alpha]_D^{26} +25.4$ (*c* 0.0114, CHCl₃); IR (neat) $\nu_{\max}$ 3419, 2918, 2850, 1643, 1449, 1385, 1261, 1101 cm⁻¹; for ¹H and ¹³C NMR see Table 1; CD (ACN *c* 0.0005, 0.1 cm path length): 190 ($\Delta\epsilon$ – 18.7), 218 ($\Delta\epsilon$ + 4.9) nm; HR-ESIMS *m/z* 373.1980 [M+Na]⁺ (calcd for C₂₀H₃₀O₅Na, 373.1990)

Computational methods

The conformational search was carried out for pumilol (**1**) and *ent*-pumilol (**1a**) at the molecular mechanics level of theory employing force field (MMFF) basis set incorporated in Spartan (Wavefunction, Irvine, CA) software package. Conformers under 2 kcal/mol were selected for TDDFT calculations. The selected conformers were subjected to TDDFT calculations using a B3LYP method at 6-31G (d,f) level built into Gaussian09 software.^[16] The ECD curves were Boltzmann weighted and compared. DP4+ probabilities of the possible isomers were determined using the protocol provided by Grimblat *et al.*¹⁵

Antimicrobial assays

The compounds were dissolved in DMSO to a stock concentration of 10 mM. For the assay, the stock solutions were further diluted with growth medium [Müller-Hinton broth (MHB) for bacteria, RPMI-1640 for fungi, final DMSO concentration 0.5%]. Five bacterial strains (*Enterobacter aerogenes*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*; see Table 1 in Supporting Information for details), selected according to CLSI guidelines^[17], were first cultivated overnight in MHB and then diluted for the assay to yield an inoculum of 5×10^5 colony forming units (CFU)/ml. *Candida albicans* was grown for 2 days on Sabouraud dextrose agar (SDA) plates, and then inoculated into RPMI-1640 to yield an inoculum of 2.5×10^3 CFU/ml for the assay. The antimicrobial activity was evaluated using broth microdilution assay according to CLSI guidelines.^[17] Microbes were exposed to the compounds at a final concentration of 50 μ M (for the EtOH extract, the concentration was 50 μ g/ml), and the microbial growth was measured photometrically after 24 h (or 48 h for *C. albicans*) using Victor multilabel counter (PerkinElmer, Finland) at a wavelength of 620 nm. Reference antibiotics used as positive controls in the assay are given as supporting information. The results of the primary screening are presented in Table 2 in supporting information. The compounds that showed >90% growth inhibition in the primary screening (compounds **4**, **14** and **15** against *S. aureus* and/or *E. faecalis*) were selected for secondary testing and MIC₉₀ determination with concentrations ranging from 50 to 6.25 μ M in two-fold dilution steps. Minimum inhibitory concentration (MIC₉₀; the lowest concentration that inhibited the growth by > 90% after 24 h) was hence determined.

Supplementary Material

1D and 2D NMR spectra, IR and MS spectra for compound **1**, and antimicrobial assays and computational methods are available as supporting information.

Acknowledgements

This work was funded through the EU FP7 (FP7-KBB-2008-2B-227239) ForestSpecs project.

Author Contribution Statement

M. K. Langat and C. Horner conducted the extraction, isolation, purification and identification of the compounds. M. K. Langat conducted the ECD and DP4+ probability analysis. D. A. Mulholland reviewed the experiments and confirmed the proposed structures. M. K. Langat and D. A. Mulholland prepared, revised and polished the manuscript. A. Helfenstein and P. Tammela conducted the antimicrobial assays and prepared the data for the manuscript. H. Hokkanen and D. Izotov Collected, identified and availed the plant material. All authors reviewed the manuscript.

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