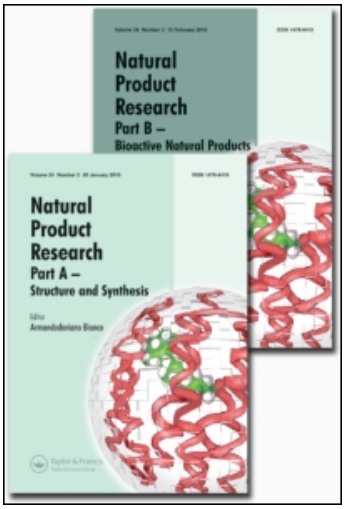

Natural Product Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713398545

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To cite this Article San-Martín, Aurelio , Rovirosa, Juana , Astudillo, Luis , Sepúlveda, Beatriz , Ruiz, Daniela and San-Martín, Carol(2008) 'Biotransformation of the marine sesquiterpene pacifenol by a facultative marine fungus', Natural Product Research, 22: 18, 1627 — 1632
To link to this Article: DOI: 10.1080/14786410701869440
URL: http://dx.doi.org/10.1080/14786410701869440

Natural Product Research
Vol. 22, No. 18, 15 December 2008, 1627–1632

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(*Received 30 July 2007; final version received 9 January 2008*)

Continued investigation of the marine alga *Laurencia claviformis* has led to the isolation of pacifenol, a halogenated sesquiterpene as the major metabolite. The microbial transformation of pacifenol is reported. It was cultivated with a marine strain of *Penicillium brevicompactum* yielding a new compound. The structure was elucidated on the basis of spectroscopic data. The anti-microbial activity of pacifenol derivatives is reported.

Keywords: *Laurencia claviformis*; biotransformation; sesquiterpene; pacifenol

1. Introduction

The microbial transformation of natural products is a suitable methodology to produce more useful substances, as this approach allows the ready functionalisation of inactivated carbon atoms. In a wide sense, biotransformations involve the use of either enzymes or microorganisms to perform functional groups modifications. In these reactions the reactants and products yielded are similar in chemical complexity. Several conversions of terpenoids have been focused to enhance the biological activity of the compounds produced, e.g. insecticidal derivatives from cadinanes by incubation with *Beauveria bassiana* (Buchanan, Williams, & Reese, 2000), or the microbial hydroxylation of patchoulol as a route to fragrant compounds (Suhara et al., 1981). Terrestrial metabolites had been widely used in biotransformations, but a reduced amount of degradation of marine secondary metabolites has been reported. Recently, the microbial and chemical transformation studies of the marine sesquiterpene phenols (S)-(+)-curcuphenol and (S)-(+)-curcudiol, isolated from the Jamaican sponge *Didiscus oxeata*, were reported. In this study, *Kluyveromyces marxianus* var. lactis was used (Khalid El Sayed et al., 2002). Although the bacteria were reported to show ability to degrade chlorinated organic compounds, Haggblom, Janke and Salonen (1989) and Steiert and Crawford (1985, 1986) claimed that white-rot fungal strains gave more promising results in this reaction (Huynh, Chang, Joyce, & Kirk, 1985; Yadav, Tiedje, & Reddy, 1995). Basidiomycetes, which cause white-rot decay, are able to degrade lignin in wood. Broad substrate specificity of the lignolytic enzymes, as phenoloxidase and peroxidase groups secreted by white-rot fungi

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ISSN 1478–6419 print/ISSN 1029–2349 online

DOI: 10.1080/14786410701869440
http://www.informaworld.com

are commonly related with the degradative ability of the organisms. Laccase and tyrosinase are well-known phenoloxidases produced by some white-rot fungal strains. Recently the white-rot fungus *Stereum hirsutum* was used to degrade methoxychlor [2,2,2-trichloro-1,1-bis(4-methoxyphenyl) ethane] in broth cultures (Lee et al., 2006). The dechlorination effectiveness of laccase produced by *Trametes versicolor* on pp'DDT has been investigated in order to reduce its toxicity (Unal & Kolankaya, 2004). Also, a *Penicillium camemberti* strain has been studied as a potential chlorinated pulping waste degrader. It is likely that this waste product is a mixture of many chlorinated polyphenols (Taseli, Gokcay, & Taşeli, 2004).

The marine environment is a very rich source of extremely potent compounds that showed significant activities in anti-tumour, anti-inflammatory, analgesia, immunomodulation, allergy, and anti-viral assays (Newman & Cragg, 2004). Besides, the microbiological biotransformations are potentially useful in chemical modifications for their ability to introduce modifications by oxidation, reduction, epoxidation, etc.; sometimes difficult transformations to make by chemical methods.

According to the actual tendencies to preserve the environment, we consider it important to evaluate the biodegradation of natural bioactive polyhalogenated compounds, especially by marine microbes. *Laurencia claviformis*, collected in Easter Island, yielded a considerable amount of pacifenol **1**, a bioactive polyhalogenated sesquiterpene of chamigrene skeleton (Rovirosa, Astudillo, Sánchez, Palacios, & San-Martín, 1989). Biotransformation of pacifenol **1** was attempted in the present investigation utilising *Penicillium brevicompactum*, a facultative halotolerant fungi isolated from the sponge *Cliona* sp. The fermentation methods, isolation and culture of *P. brevicompactum*, as well as the structural identification of the main secondary metabolite, are reported (Rovirosa, Diaz-Marrero, Darias, Painemal, & San-Martìn, 2006). Pacifenol **1** and its derivatives had been obtained in previous investigations (Argandoña, San-Martín, & Rovirosa, 1993; Rovirosa et al., 1989; San-Martín, J. Rovirosa, Darias, & Astudillo, 1996). Also, the anti-microbial activity of pacifenol **1** and derivatives has been reported. This report includes the assessment of degradation of the halogenated sesquiterpene, the anti-microbial activity of the products, including two semi-synthetic pacifenol derivatives.

2. Experimental section

2.1. *General experimental procedures*

The ^{1}H NMR and ^{13}C NMR, HMQC, HMBC, NOESY, and ^{1}H-^{1}H COSY spectra were measured employing a Bruker AMX 500 instrument operating at 500 MHz with TMS as internal standard for ^{1}H NMR and at 125 MHz for ^{13}C NMR. Two-dimensional NMR spectra were obtained with the standard Bruker software. HPLC was run on a Waters instrument equipped with a differential refractometer. The TLC (Merck, Kieselgel 60F-254) spots were visualised by spraying the chromatograms with H_2SO_4 – methanol (1 : 9) and heating at 110°C for 2 min. Column chromatography was performed over Merck Kieselgel 60, particle size 0.063–0.200 mm. Mass spectra were determined on a Hewlett Packard 5995 and VG Micromass ZAB-2F mass spectrometers.

2.2. *Plant material*

Pacifenol **1** was isolated from the alga *L. claviformis*, as previously reported (Rovirosa et al., 1989). Compound **1** was purified by CC (silica gel, petrol: EtOAc) (Figure 1). The fungus

P. brevicompactum was grown in shake cultures at 200 rpm, 23°C in conical flasks (250 mL), each containing 50 mL of a liquid sterile medium Czapek comprising (per litre of sea water) KNO_3 (2 g), KCl (0.5 g), $FeSO_4$ 7 H_2O (0.01 g), $MgSO_4 \times 7\ H_2O$ (0.5 g), KH_2PO_4 (1 g), yeast extract (5 g), sacarosa (30 g). The pH was adjusted to 5.5 with HCl 0.1N.

Compound **1** (150 mg) dissolved in DMF (4 mL) was equally distributed among five conical flasks, and the fermentation was continued for a further 6 days before the mycelium was filtered and the broth extracted with EtOAc. The microbial transformation was followed by TLC and measuring of the glucose in order to detect the presence of carbon source. The extract was dried over Na_2SO_4 and the solvent was evaporated to give a residue, which was chromatographed on a Sephadex LH-20 column with petrol: CH_2Cl_2 : MeOH (3 : 2 : 1) and silica gel column using a petrol: EtOAc gradient. Fractions collected were purified further by HPLC (normal phase and using a gradient of 35% EtOAc to 55% in *n*-hexane). In this way compound **2** (30 mg) was obtained.

Compound **2**: needles, m.p. 156–157°C (petrol: EtOAc) IR ν_{max}^{KBr} cm^{-1}: 3385 (OH). ^1H NMR ($CDCl_3$, 500 MHz): δ 1.09 (3H, s), 1.32 (3H, s), 1.36 (3H, s), 1.51 (3H, s), 2.20 (1H, dd, $J = 3.4$; 14.7 Hz, H-5β), 2.28 (1H, dd, $J = 14$: 0; 14.0 Hz, H-2α), 2.38 (1H, dd, $J = 7.4$; 14.0 Hz, H-2β), 2.53 (1H, dd, $J = 14.7$; 14.7 Hz, H-5β), 4.44 (1H, dd, $J = 7.4$; 14.0 Hz, H-1), 4.93 (1H, dd, $J = 3.4$; 14.7 Hz, H-4), 5.30 (1H, d, $J = 9.7$ Hz, H-8), 6.04 (1H, d, $J = 9.7$ Hz, H-9). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 23.4 (C-12), 24.8 (C-13), 25.5 (C-14), 29.5 (C-15), 32.5 (C-5), 42.6 (C-2), 52.1 (C-11), 53.7 (C-6), 61.9 (C-4), 69.4 (C-3), 74.4 (C-1), 76.6 (C-7), 100.1 (C-10), 132.7 (C-9), 134.1 (C-8). HREIMS observed m/z 389.9835 (calcd for $C_{15}H_{20}\ Br_2O_2$) EIMS (probe) 70 eV, m/z (rel. int.): 392 $(M\text{-}H_2O)^+$ (20), 313 $(M\text{-}H_2O\text{-}^{79}Br)^+$ (100), 312 $(M\text{-}H_2O)^+$ (20), 311$(M\text{-}H_2O\text{-}^{81}Br)^+$ (90), 295 (10).

2.3. *Anti-bacterial activity assays*

The bacteria used to perform the anti-bacterial activity were grown and maintained in agar Muller–Hilton 3.5%. Samples were prepared with pure compounds diluted in CH_2Cl_2. Each 10 mm sterile filter paper disks (Whatman No. 1) were saturated with 50 µg of each compound. Results were registered as the inhibition zone (millimetre) developed after 24 h of incubation at 37°C.

2.4. *Test organisms*

The American type culture collection names and numbers of the organisms used in this study are as follows: *Staphylococcus aureus* (ATCC-6538-P), *Staphylococcus enteriditis* (Instituto de Salud Publica), *Proteus vulgaris* (Instituto de Salud Publica), *Bacillus cereus* (ATCC-9342), *Escherichia coli* (ATCC-10,536), and *Pseudomonas aeruginosa* (ATCC-23,389). Ampicillin (10 mg/disk) and chloramfenicol (30 mg/disk) were used as control antibiotics in all plates. The determination of the minimum inhibitory concentration (MIC) of the compounds was conducted according to published standard procedures (Dhar, Dhar, Dhawan, Mehrotra, & Ray, 1968; Rasadah & Muhamad, 1988). All tests were performed in triplicate.

3. Results and discussion

Pacifenol **1** was available from a previous study of the chemistry of the red alga *L. claviformis* (Rovirosa et al., 1989), and its structure was conformed by X-ray

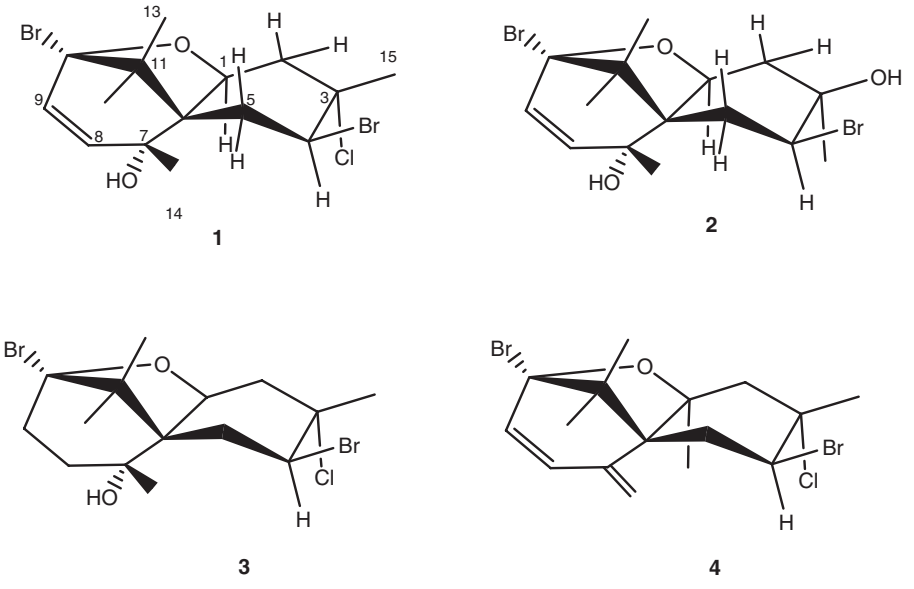

Figure 1. Pacifenol and pacifenol derivatives.

crystallographic analysis (Sims, Fenical, Wing, & Radlick, 1971). The incubation of **1** with *P. brevicompactum* for 6 days afforded one hydroxylated product **2**, a most polar compound. It was isolated as odourless needles in a 7.3% yield. The molecular formula $C_{15}H_{22}Br_2O_3$ of compound **2** was established based on ^{13}C NMR, DEPT. EI mass spectrometry does not show the M^+ peak. The peak cluster of major mass in HREIMS corresponds to M^+-H_2O, and gave the molecular formula $C_{15}H_{20}$ Br_2O_2 (m/z 389.9830 calcd, 389.9835 obs). This formula suggests that new oxygen was introduced in the molecule of **1** instead of the chlorine atom. The ^1H-decoupled ^{13}C NMR spectrum of **2** showed resonances of 15 carbons; DEPT analysis using an angle of 90°, indicated two saturated methines at δ 69.4, and 61.9, and two unsaturated methines at δ 132.7 and 134.1. The DEPT 135° spectrum showed two methylenes and four methyl carbons indicating, after comparison with a decoupled spectrum, that the carbons at δ 53.7, 76.6, 52.1, and 100.1 were not attached to hydrogens. The IR, ^1H NMR and ^{13}C NMR data of **2** suggested the presence of hydroxyl groups (3385 cm^{-1}). The ^1H NMR spectrum of **2** showed signals corresponding to two olefinic protons at 5.30 (1H, d, $J = 9.7$ Hz) and 6.04 (1H, d, $J = 9.7$ Hz). Four methyl groups were observed at 1.09, 1.32, 1.51, and 1.36. The two methine protons double doublet resonating at 4.93 were assigned to H_1 and H_4. Finally, the chemical shift at 2.38, 2.28, 2.53, and 2.20 were assigned to two methylenes. The most significant spectroscopic difference between **1** and **2** were related with the chemical shift of methyl geminal to heteroatom (chlorine in **1** and oxygen in **2**). The relative stereochemistry of **2** was obtained from ROESY NMR experiments. Thus, there was a correlation between the signal at δ_H 1.09 (H-13) and the signals at 2.28 (C-2α). The methyl group which appeared at 1.32 (C-12) was correlated with H-14 (1.36) and H-14 was correlated with H-5β. Finally, the singlet at 1.51 showed correlations with H-1 (4.44), H-2α (2.38), H-4 (4.93) and H-5α (2.53). Hence the new compound has the structure 2 (Figure 1). In compound **2** the hydroxyl group is at the exo face of the molecule, which is

the appropriate position for this hydroxylation reaction. In order to discard a possible nucleophyllic substitution reaction which would be made by the culture medium used to grow the fungus, a control experiment was carefully conducted. As there was no transformation of compound **1** into compound **2** in the control experiment this is the first report of a wild facultative marine microorganism transforming into a marine polyhalogenated sesquiterpene. The anti-microbial activity of four compounds was tested against six microorganisms. Compound **1** presented a partial activity with *P. aeruginosa* and compound **2** presented a moderate anti-bacterial activity against *S. enteriditis*. Compounds **3** and **4** (Figure 1) are two pacifenol derivatives obtained previously (San-Martín et al., 1996) and none of them present any anti-bacterial activity.

According to these results, there was not an inactivation of pacifenol by fungus, although it was biotransformed.

Acknowledgements

This work was subsidised by FONDECYT (Chile, Grant No. 1040895) and Proyecto Anillo ACT-38. The experiments conducted comply with the current laws of Chile.

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