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Microbial Transformation of Marine Halogenated Sesquiterpenes

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The sesquiterpene pacifenol is one of the main constituents of the red alga *Laurencia claviformis.* Earlier work on the semisynthetic derivatives of pacifenol afforded a series of halogenated sesquiterpenes. The aim of the present work was to obtain new hydroxylated derivatives of halogenated sesquiterpenes by means of microbial transformation using *Aspergillus níger*, *Gibberella fujikuroi* and *Mucor plumbeus*. The best results were obtained with *M. plumbeus*. The microbiological transformation by *M. plumbeus* of pacifenol, and two semisynthetic derivatives, is described. The structures of the new compounds obtained were determined by spectroscopic means.

Keywords: *Laurencia claviformis*, *Mucor plumbeus*, biotransformation, pacifenol, pacifidiene.

Biotransformation is an important tool in the structural modification of organic compounds, especially natural products, due to its significant regio and stereoselectivies [1-3].

Filamentous fungi have frequently been used to catalyze selective hydroxylation reactions that are usually difficult to achieve by chemical means [2]. This study looks at the microbial hydroxylation of pacifenol and its derivatives **2** and **3**, using the fungal microorganisms *Aspergillus níger*, *Gibberella fujikuroi* and *Mucor plumbeus*. From screening experiments, *M. plumbeus* was selected as the best yielding microorganism and incubations with this (6 days, 27°C, 0.5 g/L) afforded, after extraction, compounds **4** – **9**.

Pacifenol (**1**), from the red alga *Laurencia claviformis* [4], when treated with *p*-toluenesulfonic acid, yielded pacifidiene (**2**). However, when pacifenol was treated with sodium hydride, compound **3** was obtained [5].

A previous study described the microbiological transformation of pacifenol by the fungus *Penicillium brevicompactum* [6]. Continuing these studies, we report here results obtained from incubations with *M. plumbeus* of pacifenol and two semi-synthetic pacifenol derivatives (**2** and **3**).

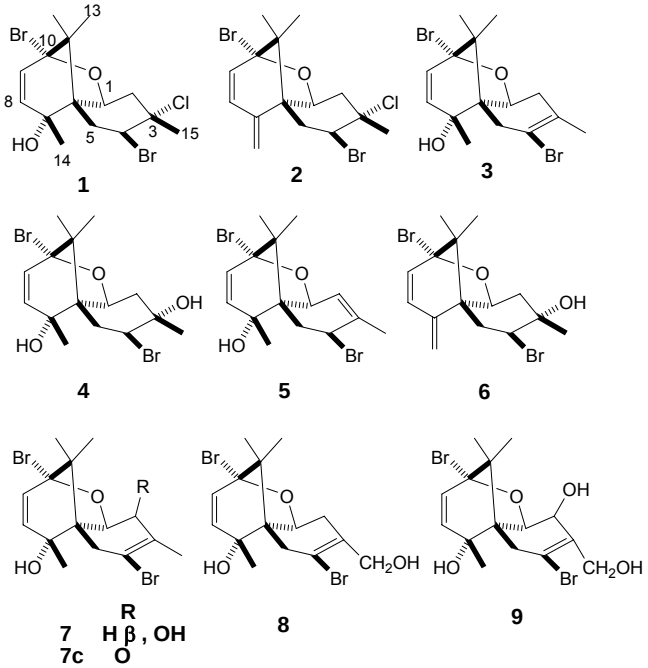

Figure 1: Compounds obtained from the biotransformation from pacifenol **1** and the semisynthetic derivatives **2** and **3**.

The fermentations were carried out for a period of 6 days. The combined broth and mycelium were extracted with ethyl acetate, and then separated into neutral and acid fractions.

In the biotransformation of pacifenol with *M. plumbeus* two metabolites that were not present in a control fermentation were detected by TLC. The ethyl acetate extract of the medium was purified by chromatography to yield the biotransformation products **4** and **5**.

Metabolit**e 4,** obtained in good yield, was identified by comparing the physical and spectroscopic data with those of the product obtained in the biotransformation of pacifenol with *P. brevicompactum* [6].

Comparison of the ^{1}H NMR spectra of compound **5** and the substrate **1** showed the disappearance of a double doublet due to H-2 in compound **1** and the appearance of a broad doublet at δ_H 5.81, corresponding to the presence of a tri-substituted double bond in compound **5**; also, the signal of the methyl (Me-12) geminal to a chlorine atom at δ_H 1.72 in **1** was shifted downfield to δ_H 1.85 in **5**.

The position of the tri-substituted double bond was inferred from the following correlations observed in the 2D experiments and COSY and TOCSY. The HMBC interaction of the H-1 resonance at δ_H 4.76 (C-1, δ_C 75.0) with the olefinic carbon at δ_C 136.3, and also the correlation between Me-12 at δ_H 1.85 with the olefinic carbons at δ_C 136.3 and 126.7 ppm, and with the methine carbon bearing the bromine atom at δ_C 67.9, indicate the presence of the C_2-C_3 double bond. Furthermore, a COSY experiment revealed correlation between a broad doublet at δ_H 5.81 and a methyl at 1.85 ppm. A TOCSY spectrum, when the signal at δ_H 5.81 is irradiated, showed correlations with the signal at δ_H 4.76 and with the methyl at 1.85 ppm. Consequently, structure **5** was assigned to this compound.

The biotransformation of pacifidiene (**2**) with *M. plumbeus* gave the metabolites **1** and **6**. The molecular formula, $C_{15}H_{20}Br_2O_2$, of compound **6** was established based on ^{13}C NMR, DEPT and MS [*m/z*: 392]. This formula suggests that oxygen was introduced in **2** in place of the chlorine atom. Comparison of the ^{1}H NMR spectra of **2** and **6** showed only minor differences in the chemical shift corresponding to the geminal methyl to the heteroatom (chlorine in **2**, δ_H 1.73 and oxygen in **6**, δ_H 1.26).

As in the compound obtained from the biotransformation of pacifenol with *P. brevicompactum*, the hydroxyl group at C-3 is axial (exo face of the molecule) [6]. So, compound **6** is shown to be 3-hydroxydechloropacifidiene.

From the microbiological transformation of compound **3**, two products, **7** and **8**, were obtained, the structures of which were established unambiguously by NMR spectroscopy. Another compound (**9**) was obtained, but only in small quantities such that only a ^{1}H NMR spectrum was recorded.

The ^{1}H NMR spectrum of compound **7** exhibited a downfield CH signal at δ_H 4.23 (brs, H-2). This observation suggested the introduction of an OH group at one of the CH_2 groups, i.e C-2 or C-5. The assignments of all the protons were accomplished by interpretation of the HMQC spectrum. The position for the newly introduced OH group at C-2 was inferred on the basis of HMBC couplings of the protons resonating at δ_H 1.93 (Me-15) and 4.40 (H-C-1) with the newly hydroxylated methine C-atom at δ_C 75.3 (H-C(2)) . The orientation of the OH group at C-2 was inferred from the NOESY correlations of Hβ–C(2) with H β–C(5), Me-15 and with Me-13; Hα–C(1) has correlation only with H α-C(5). Also, there are correlations between the methyl at δ 0.98 (Me-13) with Me–15 (δ 1.93) and Hβ–C(5); between Me-12 with Hα–C(5) and Me-14 (δ 1.34). Other correlations observed are between Me-14 with Hβ–C(5) and Hα–C(5); between H-9 with Me-12 and between H-8 with Me-12 and Me-14; Hα–C(5) with Me-14 and Hα–C(1); and H β–C(5) with Me-13, Hβ –C(2) and Me-14. The stereochemistry of pacifenol was assigned by X-ray-diffraction, and as compound **3** is a synthetic derivative of pacifenol, the stereochemistry of this compound is assumed to be the same as that of pacifenol. Furthermore, the coupling constant between H-1 and H-2 ($J_{1,2}$=1.9Hz) indicates that the H-2 must be β and, therefore, the hydroxyl group must be pseudo-equatorial at the exoface of the molecule, which is appropriate for this hydroxylation.

In order to confirm the spectroscopic data, a DFT/GIAO approach has been used to calculate the ^{1}H and ^{13}C chemical shifts. This methodology has been used for NMR assignments in several natural products [7-9]. The combined approach of extensive spectroscopic analysis and quantum mechanical methods has been used for the reassignment of structures [10], and can be very helpful to either confirm or discard both rigid and flexible molecular structures [11,12]. Recently Bassarello *et al*. [13] have used this methodology to derive the stereo structures of unknown compounds by comparing the experimental NMR spectroscopic data with the corresponding calculated spectra for all the possible stereo isomers. In addition, quantum mechanical calculations of proton-proton and proton-carbon *J* coupling constants have been proposed as useful tools to assign the relative configurations of chiral organic compounds. This approach provides results that were in good agreement with the experimental data [14,15]. When heavy atoms

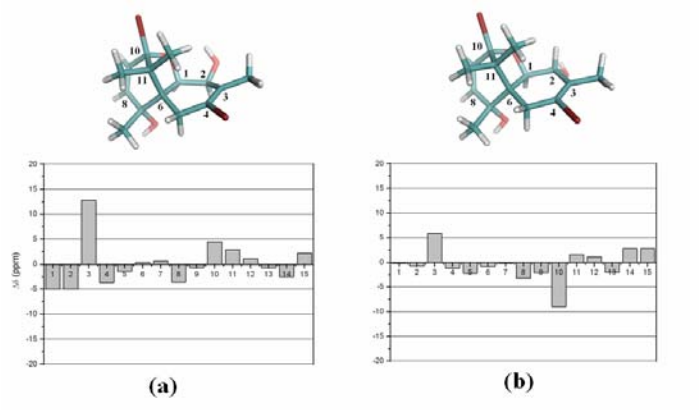

Figure 2: Deviation from calculated and observed ^{13}C NMR chemical shift for both configurations in C2 for configurations (a) and (b) of compound **7**.

are present in the structure, some spin–orbit (SO) coupling may be operative [16]. This effect has been discussed by Braddock *et al.* [10]. These authors report that the use of the extended basis set do not produce a great change in the chemical shift. They recommended that an average correction of ca. -3 ppm for bromine attached to a sp^3 carbon [17] was adequate to empirically reproduce the experimental chemical shift.

Proton-proton $J_{H,H}$ coupling constants were calculated at the mPW1PW91/6-31G(d,p) level of theory for compound **7** and optimized at the mPW1PW91/6-31G(d) level. The resultant structure for compound **7** is shown in Figure 2. The differences between theoretical and experimental values $\Delta\delta = |\delta_{exp}-\delta_{calc}|$ show a measure of the dispersion between the theoretical and experimental chemical shift values for compound **7b**. The coupling constants for protons H-1 and H-2 were calculated for both configurations. The values for $^3J_{H\text{-}H}$ for the protons at C-2 and C-1 are 8.21 Hz for isomer (a) and 1.92 Hz for isomer (b), respectively.

In summary, our results show that GIAO/DFT calculations on the optimized structure at the mPW1PW91/6-31G(d,p) level of theory provide excellent results that are in agreement with experimental values for ^{13}C chemical shifts and in fair agreement with experimental proton–proton $^3J_{H,H}$ coupling constants. The theoretical results confirm and support the experimentally derived assignments of compound **7**.

Compound **7** was treated with Jones' reagent to obtain compound **7c**, the ^1H NMR spectrum of which was very similar to that of compound **7**, with the disappearance of the H-atom geminal to the OH group at δ 4.23, and the appearance in the ^{13}C NMR spectrum of a signal due to an oxo group at δ_C 191.5. The assignments of all the H-C atoms were accomplished by interpretation of the HMQC spectrum. The position of the carbonyl group was inferred on the basis of HMBC couplings, i.e. the H-C-1 proton at δ_H 4.75 correlates with C-6 (δ 54.1), C-7 (δ 75.0) and C-2 (δ 191.5). Me-15 (δ 2.00) correlates with C-3 (δ 135.4), with C-4 (δ 146.5) and with C-2 (δ 191.5)

The least polar compound isolated (**8**) had a molecular formula $C_{15}H_{20}O_3Br_2$, and possessed one more oxygen atom than substrate **3**. Its ^1H NMR spectrum was very similar to that of **3** except that the signal of a methyl group (Me-15) at δ_H 2.01 had been replaced by that of a hydroxymethylene group, two signals, forming an AB system, at δ_H 4.32 and 4.27 (J = 16 Hz). Thus, structure **8** was assigned to this compound.

The most polar substance isolated in the incubation of **3** was the triol **9**. The two novel alcoholic groups introduced in the molecule were located at C-2 and C-15, in accordance with the ^1H NMR spectrum. Thus the resonance of H-2 was similar to that of compound **7** and the resonance for the hydroxymethylene in C-15 was similar to that of compound **8**. The quantity available was insufficient to obtain a ^{13}C NMR spectrum.

Experimental

General experimental procedures: IR spectra were obtained using a Perkin-Elmer Spectrum BX FT-IR spectrometer. ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ at 400.13 and 100 MHz, respectively, with a Bruker AMX2-400 spectrometer. Chemical shifts are given in δ (ppm). Mass spectra were taken at 70 eV (probe) with a Micromass Autospec spectrometer. Semipreparative HPLC was carried out with a Beckman System Gold 125P. Dry column chromatography was performed on Merck 0.02-0.063 mm silica gel.

Computational details: Geometry optimization was performed using the mPW1PW91[18] exchange-correlation function, together with the standard 6-31G(d) basis set [19]. The ^1H and ^{13}C NMR chemical shifts were calculated using the GIAO (Gauge Invariant Atomic Orbitals) method [20] at this level of theory. Relative chemical shift and coupling constants were estimated by using the corresponding TMS shielding calculated at the same level of theory. Spin-spin coupling calculations were performed taking into account the contributions of the following interactions: Fermi contact (FC), paramagnetic spin-orbit (PSO), diamagnetic spin-orbit (DSO), and spin-dipole (SD). All calculations were carried out with the Gaussian 03 suite of programs [21].

Substrate **1** was isolated from *Laurencia claviformis,* an alga that grows on the coast of Easter Island. Substrate

2 was obtained by treating compound **1** with *p*-toluene sulfonic acid and compound **3** was obtained after the reaction of pacifenol with NaH in THF [5].

Incubation and isolation procedures: *Mucor plumbeus* was grown in shaking culture at 25°C for 2 days in 65-75 conical flasks (250 mL), each containing sterile medium (50 mL) [6]. The substrate was dissolved in EtOH (13-15 mL) and distributed equally between the flasks, and the incubation was allowed to continue for 6 days. The broth was separated from the mycelium by filtration, and both were extracted with EtOAc. The extracts were combined and chromatographed on silica gel using as eluent a light petroleum-EtOAc gradient. Some mixtures were resolved by HPLC on an Ultrasphere silica gel 5 μm column (1× 25 cm), eluting with mixtures of isocratic *n*-hexane-EtOAc at 3 mL/min.

Biotransformation of pacifenol (1): The incubation of **1** (300 mg) afforded, from the neutral fraction, starting material **1** (45 mg), **4** (100 mg), and **5** (27 mg).

Compound 5

^{1}H NMR (δ, 400 MHz): 6.09 (1H, d, *J* = 9.9 Hz, H-9), 5.81 (1H, brd, *J* = 2.3 Hz, H-2), 5.48 (1H, d, *J* = 9.9 Hz, H-8), 4.76 (1H, d, *J* = 2.3 Hz, H-1), 4.21 (1H, dd, *J* = 5.0, 4.7 Hz, H-4), 2.07 (1H, dd, *J* = 4.7, 15.3 Hz, H-5), 2.03 (1H, dd, *J* = 5.0, 15.3 Hz, H-5'), 1.85 (3H, brs, Me-15), 1.44 (3H, s, Me-14), 1.11 (3H, s, Me-12), 1.09 (3H, s, Me-13).

^{13}C NMR (δ, 100 MHz, $CDCl_3$): 75.0 (CH_2, C-1), 136.3 (CH_2, C-2), 126.7 (C, C-3), 67.9 (CH, C-4), 29.5 (CH_2, C-5), 55.4 (C, C-6), 77.2 (C, C-7), 134.0 (CH, C-8), 132.5 (CH, C-9), 100.3 (C, C-10), 51.9 (C, C-11), 24.7 (CH_3, C-12), 21.9 (CH_3, C-13), 25.1 (CH_3, C-14), 20.7 (CH_3, C-15).

EIMS (70 eV) *m/z* (%, rel.int): 392 $[M - H_2O]^+$(20), 313 $[M - H_2O - {}^{79}Br]^+$(100), 312 $[M - H_2O - Br]^+$(20), 311 $[M - H_2O - {}^{81}Br]^+$(90), 295(10).

Biotransformation of pacifidiene (2): The incubation of **2** (300 mg) afforded, from the neutral fraction, starting material **2** (60 mg), **6** (86 mg), and **1** (27 mg).

Compound 6

^{1}H NMR (δ, 400 MHz): 5.87 (1H, d, *J* = 9.9 Hz, H-9), 5.58 (1H, d, *J* = 9.9 Hz, H-8), 5.1 and 5.0 (1H each, brs, H-14 and H-14'), 4.48 (1H, d, *J* = 6.1 Hz, H-4), 4.07 (1H, dd, *J* = 4.7, 14.2 Hz, H-1), 2.66 (1H, dd, *J* = 4.7, 14.0 Hz, H-2), 2.20 (1H, dd, *J* = 6.1, 11.8 Hz, H-5a), 1.72 (1H, d, *J* = 11.8 Hz, H-5e), 1.26 (3H, brs, Me-15), 1.15 (3H, s, Me-12), 1.03 (3H, s, Me-13)

^{13}C NMR (δ, 100 MHz, $CDCl_3$): 77.9 (CH, C-1), 43.1 (CH_2, C-2), 75.2 (C, C-3), 61.5 (CH, C-4). 32.8 (CH_2, C-5), 53.8 (C, C-6), 147.0 (C, C-7), 133.0 (CH, C-8), 130.1(CH, C-9), 100.3 (C, C-10), 50.5 (C, C-11), 23.3 (CH_3, C-12), 20.1 (CH_3, C-13), 114.7 (CH_2, C-14), 29.7 (CH_3, C-15).

MS (EI, 70 eV) *m/z* (%, rel.int): 392 $[M - H_2O]^+$ (20), 313 $[M - H_2O - {}^{79}Br]^+$ (100), 312 $[M - H_2O - Br]^+$ (20), 311 $[M - H_2O - {}^{81}Br]^+$ (90), 295 (10).

Biotransformation of compound 3: The incubation of **3** (260 mg) afforded, from the neutral fraction, starting material **3** (50 mg), **7** (120 mg), **8** (35 mg) and **9** (1 mg).

Compound 7

^{1}H NMR (δ, 400 MHz): 6.06 (1H, d, *J* = 9.8 Hz, H-9), 5.42 (1H, d, *J* = 9.8 Hz, H-8), 4.40 (1H, d, *J* = 1.9 Hz, H-1), 4.23 (1H, brs, H-2), 2.93 (1H, d, *J* =18 Hz, H-5), 2.48 (1H, d, *J* = 18 Hz, H-5'), 1.93 (3H, s, Me-15), 1.34 (3H, s, Me-14), 1.08 (3H, s, Me-12), 0.98 (3H, s, Me-13).

^{13}C NMR (δ, 100 MHz, $CDCl_3$): 83.1 (CH, C-1), 75.3 (CH_2, C-2), 121.7 (C, C-3), 132.5 (C, C-4). 35.3 (CH_2, C-5), 54.0 (C, C-6), 75.1 (C, C-7), 133.8 (CH, C-8), 132.8(CH, C-9), 99.4 (C, C-10), 52.4 (C, C-11), 21.3 (CH_3, C-12), 25.1 (CH_3, C-13), 24.3 (CH_3, C-14), 20.1 (CH_3, C-15).

MS (EI, 70 eV): *m/z* (%, rel.int.) = 409.96 $[M^+ + 2]$ (0.45), 407.96 $[M]^+$ (1.2), 405.96 $[M - 2]^+$ (0.7), 391.95 $[M + 2\text{-}H_2O]^+$ (2.1), 389.97 $[M - H_2O]^+$ (3.0), 387.97 $[M^+ - 2\text{-}H_2O]^+$ (1.7), 329.06 $[M - {}^{79}Br]^+$ (18.7), 327.06 $[M - {}^{81}Br]^+$ (18.9), 311.04 [390-^{79}Br] (28.6), 309.05 [390-^{81}Br] (26.5), 283.05 (18.5), 281.05 (12.9), 265.04 (4.7), 229.13 (11.3), 219.14 (11.2), 212.99 (10.1), 211.01 (12.0), 201.13 (19.8), 201.01 (56.0), 200.0 (26.3), 199.01 (61.7), 198.0 (24.7), 196.99 (9.1), 187.11 (13.7), 186.1 (15.0), 184.12 (19.6), 173.13 (13.9), 149.09 (11.7), 149.02 (10.7), 147.07 (14.8), 143.08 (10.5), 141.06 (9.4), 135.08 (17.7), 134.96 (10.3), 120.08 (100), 119.07 (46.6), 105.06 (29.1) 91.05 (65.2), 85.02 (45.1).

Compound 7c: To a solution of compound **7** (30 mg, 0.074 mmol) in acetone (3 mL) cooled to 0ºC was added Jones' reagent (2.67 g CrO_3, 2.3 mL H_2SO_4, dilute to 10 mL with water), dropwise, until disappearance of the starting material, as monitored by TLC. The reaction was quenched by the addition of iso-propanol, followed by saturated $NaHCO_3$. The solution was extracted 3 times with EtOAc and the combined organic layers were washed with water, dried over $MgSO_4$, and filtered. The solvent was removed *in vacuo* and the residue was chromatographed on silica gel using as eluent a light petroleum-EtOAc gradient obtaining **7c**.

^{1}H NMR (δ, 400 MHz): 6.17 (1H, d, *J* = 9.8 Hz, H-9), 5.40 (1H, d, *J* = 9.8 Hz, H-8), 4.75 (1H, s, H-1), 3.33 (1H , ddd, *J* = 2.5, 5.1, 19.6 Hz, H-5), 2.87 (1H,

dd, *J* = 1.7, 19.6 Hz, H-5'), 2.00 (3H, s, Me-15), 1.45 (3H, s, Me-14), 1.14 (3H, s, Me-12), 0.98 (3H, s, Me-13),
^{13}C NMR (δ, 100 MHz, $CDCl_3$): 77.7 (CH, C-1), 191.5 (C, C-2), 135.4 (C, C-3), 146.5 (C, C-4). 36.7 (CH_2, C-5), 54.1 (C, C-6), 75.0 (C, C-7), 134.3 (CH, C-8), 132.8 (CH, C-9), 98.2 (C, C-10), 53.0 (C, C-11), 20.7 (CH_3, C-12), 24.7 (CH_3, C-13), 24.9 (CH_3, C-14), 15.7 (CH_3, C-15).

Compound 8

^{1}H NMR (δ, 400 MHz): 6.11 (1H, d, *J* = 9.8 Hz, H-9), 5.44 (1H, d, *J* = 9.8 Hz, H-8), 4.69 (1H, dd, *J* = 4.0, 8.0 Hz, H-1), 4.32 (1H , d, *J* = 16 Hz, H-15), 4.27 (1H , d, *J* = 16 Hz, H-15'), 2.97 (1H, d, *J* = 18.0 Hz, H-5), 2.95 (2H, dd, *J* = 8.0, 10.0 Hz, H-2), 2.65 (1H, d *J* = 18.0 Hz, H-5'), 2.63 (2H, dd, *J* = 4.0, 10.0 Hz, H-2'), 1.44 (3H, s, Me-14), 1.16 (3H, s, Me-12), 1.15 (3H, s, Me-13).
^{13}C NMR (δ, 100 MHz, $CDCl_3$): 74.7 (CH, C-1), 35.1 (C, C-2), 119.1 (C, C-3), 132.8 (C, C-4). 36.7 (CH_2, C-5), 53.7 (C, C-6), 76.3 (C, C-7), 133.7 (CH, C-8), 133.2 (CH, C-9), 100.2 (C, C-10), 52.8 (C, C-11), 22.3 (CH_3, C-12), 24.8 (CH_3, C-13), 25.1 (CH_3, C-14), 65.8 (CH_2, C-15).

MS (EI, 70 eV): *m/z* (%, rel.int.) = 391.95 $[M + 2\text{-}H_2O]^+$ (8.9), 389.95 $[M\text{-}H_2O]^+$ (17.7), 387.95 $[M^+ - 2\text{-}H_2O]^+$ (9.2), 329.05 $[M\text{-}^{79}Br]^+$ (2.2), 327.06 $[M\text{-}^{81}Br]^+$ (2.9), 311.04 [390-^{79}Br] (41.4), 309.05 [390-^{81}Br] (41.0), 229.03 (16.5), 227.00 (15.6), 214.99 (14.3), 212.99 (19.7), 211.00 (10.0), 201.00 (15.8), 200.00 (53.3), 199.00 (20.9), 198.00 (50.9), 196.99 (10.2), 190.95 (19.8), 188.95 (19.3), 184.12 (22.1), 183.11 (15.5), 171.11 (14.2), 169.10 (16.9), 149.01 (55.1), 147.07 (9.9), 143.08 (13.5), 141.06 (10.4), 135.07 (14.3), 134.06 (22.1), 133.09 (35.4), 132.08 (19.8), 121.09 (19.7), 120.08 (26.1), 119.07 (51.0), 115.05 (23.1), 91.05 (88.3), 57.07 (100).

Compound 9

^{1}H NMR (δ, 400 MHz): 6.02 (1H, d, *J* = 9.8 Hz, H-9), 5.41 (1H, d, *J* = 9.8 Hz, H-8), 4.66 (1H, brs, H-1), 4.53 (1H, d, *J* = 12.7 Hz, H-15), 4.47 (1H, brs, H-2), 4.35 (1H, d, *J* =12.7 Hz, H-15'), 2.97 (1H, brd, *J* = 18.2 Hz, H-5), 2.60 (1H, d, *J* = 18.2 Hz, H-5'), 1.39 (3H, s, Me-14), 1.08 (3H, s, Me-12), 1.02 (3H, s, Me-13).

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