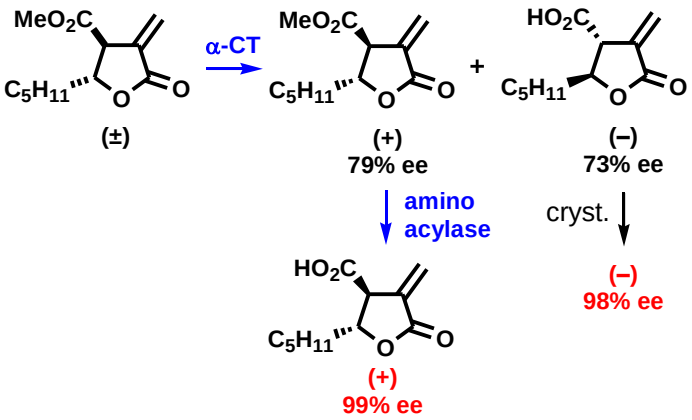
Graphical Abstract



Enzymatic resolution of α-methyleneparaconic acids and evaluation of their biological activity

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ABSTRACT Both enantiomers of three biologically relevant paraconic acids — MB-3, methylenolactocin, and C75 — were obtained with enantioselectivities up to 99% by kinetic enzymatic resolutions. Good enantiomeric excesses were obtained for MB-3 and methylenolactocin, using α-chymotrypsin and aminoacylase as enantiocomplementary enzymes, while C75 was resolved with aminoacylase. They all were evaluated for their antiproliferative, antibacterial, and antifungal activities, showing weak effects and practically no difference between enantiomers in each case. At high concentrations (16–64 μg/mL), (–)-C75 acted as an antimicrobial agent against Gram-positive bacteria.

KEY WORDS: α-methylene-γ-butyrolactones, MB-3, methylenolactocin, C75.

INTRODUCTION

The α-methylene-γ-butyrolactone moiety is part of a variety of natural compounds, most of which belong to the sesquiterpene series and are associated with a wide spectrum of biological activities both in vitro and in vivo.[1] γ-Butyrolactones containing a β-carboxy group are classified as "paraconic acids". They are generally lichen products and many of them were isolated and characterized as antibiotic, antiviral, antifungal, and antitumor agents.[2–4]

Insert Figure 1

A few structures of known α-methyleneparaconic acids are reported in Figure 1 which also shows the correct configuration for the enantiomer which is dextrorotatory in chloroform. It must be underlined that the active diastereomer is that having the substituents in *trans* relationship, namely 2*R**,3*S**. Compound (+)-**1a** (indicated as MB-3 by Sigma-Aldrich in the racemic form) is a toxin known to be responsible for the black spots observed on fruit peel[5] and for its activity as inhibitor of acetyltransferase Gcn5 of human histone.[6,7] Methylenolactocin (–)-**1b**, found in the culture filtrate of *Penicillium sp.*,[8] is active against

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some Gram-positive bacteria and Ehrlich carcinoma.[9] Compound C75 **1c**, although not natural, is a fatty acid synthase (FAS) inhibitor,[10–15] that also shows antitumor,[16–23] antiviral[24,25] and antiinflammatory activity[26] and it is active against mycobacteria of the tuberculosis complex.[27] Nephrosterinic acid (+)-**1d**, isolated from lichen *Nephromopsis endocrocea*,[28] showed antibacterial[29] and antitumor[30] properties. Protolichesterinic acid (+)-**1e**, isolated from various sources of lichens,[31–37] exhibits in vitro and in vivo antiinflammatory activity,[38] in vitro antibacterial activity against *Helicobacter pylori*,[39] inhibited 5-lipoxygenase,[40] and it also showed in vitro antiproliferative effects on malignant cell-lines.[41-43]

The cytotoxic and antitumor activity of these α-methyleneparaconic acid derivatives is generally attributed to the interaction between the external double bond and nucleophilic sites of biologically important molecules.[44–46] This mechanism was proposed for both molecules exhibiting cytotoxic activity and those showing allergenic properties.

These findings stimulated the interest in this class of compounds and new syntheses of a variety of racemic and enantiopure paraconic acids are reported.[47–56]

As a part of our continuing study on enzymatic resolutions of esters of α-methyleneparaconic acids,[57] we have taken into account compounds **1a**–**d** (Figure 1) with a twofold purpose: to achieve their resolutions by means of enzymatic methods and to evaluate the biological activity of both enantiomers.

MATERIALS AND METHODS

IR spectra were recorded on a Thermo Nicolet AVATAR 320 FT/IR spectrophotometer. ^{1}H-NMR and ^{13}C-NMR spectra were run on a Jeol (Tokyo, Japan) EX-400 spectrometer (400 MHz for proton, 100 MHz for carbon), and on a Jeol EX-270 spectrometer (270 MHz for proton, 68 MHz for carbon) using deuteriochloroform as a solvent and tetramethylsilane as the internal standard. Coupling constants are given in Hz. Optical rotations at 589 nm were determined on a Perkin Elmer (Boston, MA) Model 241 polarimeter; optical rotatory power values are given in 10^{-1} deg cm^2 g^{-1}. CD spectra were obtained on Jasco (Tokyo, Japan) J-710 spectropolarimeter (0.1 cm pathlength cell); $\Delta\varepsilon$ values are given in L cm^{-1} mol^{-1}. Capillary gas chromatographic measurements were performed on a Carlo Erba (Milan, Italy) GC 8000 instrument and on a Shimadzu (Kyoto, Japan) GC-14B instrument, equipped with a flame ionization detector, the capillary columns being OV 1701 (25 m x 0.32 mm) (carrier gas He, 40 KPa, split 1:50) and a Chiraldex type G-TA, trifluoroacetyl γ-cyclodextrin (γ-CDX)(40 m x 0.25 mm) (carrier gas He, 180 KPa, split 1:100, isotherm 150 °C) or DiMePe ß-cyclodextrin (25 m x 0.25 mm) (ß-CDX) (carrier gas He, 110 KPa, split 1:50, isotherm 150 °C). High-

performance liquid chromatography (HPLC) measurements were performed on a Hewlett Packard (Corvallis, OR) series 1100 instrument, the column being Lux 5µ Cellulose-2 Phenomenex, detector UV 220 nm, a mixture of hexane-isopropanol in a 9:1 ratio as the eluent, flow 1 ml/min. Melting points were measured with a Büchi apparatus and were not corrected. Enzymatic hydrolyses were performed using a pH-stat Controller PHM290 Radiometer (Copenhagen, Denmark). Mass spectra were recorded on an ion trap instrument Finningan (San Jose, CA) GCQ (70 eV) and on a ESI-MS ion trap Bruker (Karlsruhe, Germany) Esquire 4000 instrument. TLC's were performed on Polygram Sil G/UV_{254} silica gel pre-coated plastic sheets (eluent: light petroleum-ethyl acetate). Flash chromatography was run on silica gel 230-400 mesh ASTM (Kieselgel 60, Merck, Darmstadt, Germany). Light petroleum refers to the fraction with b.p. 40–70 °C and ether to diethyl ether. Anhydrous tetrahydrofuran [THF] was prepared by distillation over sodium benzophenone ketyl.

The following enzymes were used in enzymatic hydrolyses of **5a**–**c**: Acylase I from *Aspergillus* immobilized on Eupergit C (Fluka), 102 U/g: 44 U/mmol substrate in 1 mM $CoCl_2$; Aminoacylase from *Aspergillus melleus* (Amano Acylase from Sigma Aldrich), >30000 U/g: 1:1 w/w; Acylase I porcine kidney (Sigma), 3442 U/mg: 78019 U/mmol; α-Chymotrypsin (α-CT) from bovine pancreas (Fluka), 90 U/mg: 2400 U/mmol; Lipase from *Candida antarctica* "Novozym 435" (Novo Nordisk A/S, Bagsvaerd, Denmark), ≥10000 U/g: 1:1 w/w; Pig pancreatic lipase (PPL type II, Sigma, crude), 46 U/mg of protein 6133 U/mmol; Esterase from hog liver (PLE, Fluka), 250 U/mg: 5000 U/mmol; Liver acetone powder equine (HLAP, Sigma), 1:1 w/w; Porcine liver acetone powder (PLAP, Sigma), 27 U/mg: 6230 U/mmol substrate; SPRIN Lipo CALB, adsorbed immobilised preparation of CALB on polystyrene DVB cross-linked (300 – 800 µm), >2000 U/g dry: 1:1 w/w; Lipase from *Pseudomonas cepacea* immobilized by SPRIN: 1:1 w/w; Lipase from *Pseudomonas fluorescens* (Fluka), 42.5 U/mg: 1:1 w/w; Protease from *Bacillus subtilis*, 11.6 U/mg: 1:1 w/w.

General Procedure for the Synthesis of Racemic Substrates

To a solution of 17.35 ml of lithium bis(trimethylsilyl)amide (LiHMDS) (1.0 M in THF), cooled to –78 ^{o}C, 1.0 g (6.94 mmol) of methyl hemi-ester of itaconic acid in anhydrous THF(10.0 ml) was added dropwise. The mixture was stirred at –78 ^{o}C for 1 h. The suitable aldehyde (9.7 mmol) in 1.0 ml of anhydrous THF was then added and the mixture was stirred for further 5 h at –78 ^{o}C under argon atmosphere. The reaction was quenched with 6N H_2SO_4

(5 ml). It was then extracted with diethyl ether and dried over anhydrous Na_2SO_4. The solvent was removed and the residue was added with a solution of 250.0 µl of trifluoroacetic acid [TFA] in dichloromethane (10.0 ml) and stirred overnight at room temperature. The solvent was removed and traces of TFA were removed by coevaporation with diethyl ether (three times). The residue was a mixture diastereomeric lactones which were separated by flash column chromatography (ethyl acetate-light petroleum, gradient from 2% up to 10%). Before charging the column with the crude reaction mixture, the column was eluted with 1% ethyl acetate in light petroleum with 1 ml of acetic acid added to avoid isomerization of the α-methylene-γ-lactones into their α,ß-butenolide isomers.

Methyl (*2R*,3S)-4-methylene-5-oxo-2-propyltetrahydrofuran-3-carboxylate 5a.**[58] Oil, 18% yield, after purification; IR (neat): ν = 1769, 1742, 1663 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, δ): 6.42 (d, J = 2.9 Hz, 1H; =CH), 5.93 (d, J = 2.9 Hz, 1H; =CH), 4.82 (dt, $J_1 = J_2$ = 5.6 Hz, J_3 = 7.5 Hz, 1H; H2), 3.80 (s, 3H; OCH_3), 3.58 (dt, $J_1 = J_2$ = 2.8 Hz, J_3 = 5.7 Hz, 1H; H3), 1.80–1.63 (m, 2H; CH_2), 1.60–1.40 (m, 2H, CH_2), 0.97 (t, J = 7.3, 3H; CH_3); ^{13}C NMR (68 MHz, $CDCl_3$, δ): 169.7 (s), 168.3 (s), 133.0 (s), 125.15 (t), 78.8 (d), 52.9 (q), 49.8 (d), 37.8 (t), 18.1 (t), 13.6 (q); MS (ESI, *m/z*): 221 (100) $[M + Na]^+$.

Chiral HRGC: ß-CDX: retention time [t_R] = 11.7 min for (+)-(2*R*,3*S*)-**5a**, t_R = 12.1 min for (–)-(2*S*,3*R*)-**5a.**

Chiral HRGC, γ-CDX: t_R = 17.3 min for (+)-(2*R*,3*S*)-**5a**, t_R = 18.1 min for (–)-(2*S*,3*R*)-**5a**.

Methyl (*2R*,3R)-4-methylene-5-oxo-2-propyltetrahydrofuran-3-carboxylate 6a**.[58] Oil, 23% yield, after purification; IR (film): ν = 1770, 1740, 1667 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, δ): 6.42 (d, J = 2.2 Hz, 1H; =CH), 5.83 (d, J = 2.2 Hz, 1H; =CH), 4.64 (m, 1H; H2), 4.00 (dt, $J_1 = J_2$ = 2.2 Hz, J_3 = 7.7 Hz, 1H; H3), 3.76 (s, 3H, OCH_3), 1.7–1.5 (m, 3H), 1.45 (m, 1H), 0.95 (t, J = 7.0 Hz, 3H; CH_3); ^{13}C NMR (68 MHz, $CDCl_3$, δ): 169.5 (s), 169.0 (s), 133.6 (s), 125.0 (t), 77.9 (d), 52.4 (q), 49.1 (d), 33.5 (t), 18.9 (t), 13.7 (q); MS (ESI, *m/z*): 221 (100) $[M + Na]^+$.

Methyl (*2R*,3S)-4-methylene-5-oxo-2-pentyltetrahydrofuran-3-carboxylate 5b.** Yellow oil, 22% yield, after purification. All spectroscopic data are in accordance with those reported in the literature.[58–61]

Chiral HRGC, ß-CDX: t_R = 28.9 min for (+)-(2*R*,3*S*)-**5b**, t_R = 30.2 min for (–)-(2*S*,3*R*)-**5b**.

Chiral HRGC, γ-CDX: t_R = 34.9 min for (+)-(2*R*,3*S*)-**5b**, t_R = 37.0 min for (–)-(2*S*,3*R*)-**5b.**

Methyl (*2R*,3R)-4-methylene-5-oxo-2-pentyltetrahydrofuran-3-carboxylate 6b.** Yellow oil, 32% yield, after purification. Spectroscopic data are in accordance with those reported in the literature.[58–60]

Methyl (*2R*,3S)-4-methylene-5-oxo-2-octyltetrahydrofuran-3-carboxylate 5c**. All spectroscopic data are in accordance with those reported in the literature.[57]
Chiral HRGC, ß-CDX: t_R = 122.5 min for (+)-(2*R*,3*S*)-**5c**, t_R = 126.9 min for (–)-(2*S*,3*R*)-**5c**.
Chiral HRGC, γ-CDX: t_R = 155.6 min for (+)-(2*R*,3*S*)-**5c**, t_R = 164.5 min for (–)-(2*S*,3*R*)-**5c**.

Methyl (*2R*,3R)-4-methylene-5-oxo-2-octyltetrahydrofuran-3-carboxylate 6c.** All spectroscopic data are in accordance with those reported in the literature.[57]

Methyl (*2R*,3S)-4-methylene-5-oxo-2-undecyltetrahydrofuran-3-carboxylate 5d.** Yellow solid, 23% yield, after purification, mp 38–39 °C (lit.[61] mp 38 °C). The other spectroscopic data are also in accordance with those reported in the literature.[61]
Chiral HPLC: t_R = 10.0, 10.7 min for (±)-**5d**.

Methyl (*2R*,3R)-4-methylene-5-oxo-2-undecyltetrahydrofuran-3-carboxylate 6d.** Yellow solid, 33% yield, after purification, mp 43–44 °C; IR (neat): ν = 1760, 1732 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, δ): 6.41 (d, *J* = 2.2 Hz, 1H; $=CH_2$), 5.83 (d, *J* = 2.2 Hz, 1H; $=CH_2$), 4.60–4.64 (m, 1H; H2), 4.01 (dt, J_1 = 2.2 Hz, J_2 = 7.6 Hz, 1H; H3), 3.76 (s, 3H; OCH_3), 1.53–1.63 (m, 2H; CH_2), 1.21–1.39 (m, 18H; 9 CH_2), 0.88 (t, *J* = 7.2, 3H; CH_3); ^{13}C NMR (68 MHz, $CDCl_3$, δ): 169.3 (s), 168.8 (s), 133.6 (s, C4), 125.0 (t, $=CH_2$), 78.2 (d, C2), 52.3 (q, OCH_3), 49.1 (d, C3), 31.9 (t), 31.5 (t), 29.6 (2t), 29.4 (t), 29.34 (t), 29.30 (t), 29.2 (t), 25.5 (t), 22.6 (t), 14.1 (q). EIMS (*m/z* (%)): 310 (24) $[M^{+\bullet}]$, 251 (100) $[M^{+\bullet} - CO_2CH_3]^+$, 233 (46), 205 (36), 187 (49), 179 (39), 165 (35), 155 (35), 149 (42), 137 (39), 121 (44), 119 (45), 109 (36), 95 (45), 93 (45), 79 (52), 67 (56).

Enzymatic Hydrolyses

General Procedure for Small-Scale Enzymatic Hydrolyses

Three slightly different procedures were used owing to the different solubility of the substrates in diethyl ether. For compounds **5a** and **5b**, the enzyme was added to the substrates (0.15 mmol) in phosphate buffer at pH 7.4 (10 ml), the mixture was stirred for the time indicated in Table 1, while maintaining the pH value constant by addition of 1M NaOH, and eventually extracted with ether. From the ethereal solution the unreacted ester **5a** (or **5b**) was recovered after evaporation of the solvent, while the acid **1a** (or **1b)** was isolated from the remaining buffer solution, by acidification with 2M HCl to pH 2 and extraction with ether. In

order to measure the enantiomeric excess of the acids by chiral HRGC, they were esterified with ethanol (2 ml) and Me_3SiCl[62] (20 µl) to give the corresponding derivatives **5'a** and **5'b.**

Ethyl (*2R*,3S)-4-methylene-5-oxo-2-propyl-tetrahydrofuran-3-carboxylate 5'a.**
HRGC, ß-CDX: t_R = 14.4 min for (+)-(2*R*,3*S*)-**5'a**, t_R = 14.8 min for (–)-(2*S*,3*R*)-**5'a**.
HRGC, γ-CDX: t_R = 19.9 min for (+)-(2*R*,3*S*)-**5'a**, t_R = 20.4 min for (–)-(2*S*,3*R*)-**5'a**.

Ethyl (*2R*,3S)-4-methylene-5-oxo-2-pentyltetrahydrofuran-3-carboxylate 5'b.**
HRGC, ß-CDX: t_R = 36.3 min for (+)-(2*R*,3*S*)-**5'b**, t_R = 37.5 min for (–)-(2*S*,3*R*)-**5'b**.
HRGC, γ-CDX: t_R = 44.1 min for (+)-(2*R*,3*S*)-**5'b**, t_R = 47.6 min for (–)-(2*S*,3*R*)-**5'b**.

As to compound **5c**, at the end of the procedure described above for **5a** and **5b**, the unreacted ester could not be separated from its hydrolysis product **1c** since both were soluble in ether. Therefore compound **1c** was separated from its ester **5c** treating the ethereal phase with 5% aqueous solution of $NaHCO_3$. The organic phase containing the ester was dried on anhydrous Na_2SO_4 and, after elimination of the solvent under vacuum, the unreacted ester **5c** was obtained. The basic aqueous phase was acidified with 2M HCl to pH 2, extracted with ether and dried on anhydrous Na_2SO_4. Evaporation of the solvent afforded the acid **1c** which was esterified with ethanol and Me_3SiCl[62] to give **5'c**, in order to measure its ee by chiral HRGC.

Ethyl (*2R*,3S)-4-methylene-5-oxo-2-octyltetrahydrofuran-3-carboxylate 5'c.**
HRGC, ß-CDX: t_R = 153.0 min for (+)-(2*R*,3*S*)-**5'c**, t_R = 159.0 min for (–)-(2*S*,3*R*)-**5'c**.
HRGC, γ-CDX: t_R = 183.6 min for (+)-(2*R*,3*S*)-**5'c**, t_R = 201.7 min for (–)-(2*S*,3*R*)-**5'c**.

Similarly, compound **1d** was recovered in admixture with its ester **5d** but it could not be separated from it following the procedure used for **1c** and **5c** and therefore the two compounds were separated by flash chromatography (eluent: gradient from 7.5% ethyl acetate, 92% light petroleum, 0.5% acetic acid up to 35.5% ethyl acetate, 64% light petroleum, 0.5% acetic acid). The ee's of **1d** and **5d** were determined by chiral HPLC. To note that during HPLC elution both compounds largely isomerized into their α,ß-butenolide isomers.

Large-Scale Enzymatic Hydrolyses

Hydrolysis of 5a with α-CT. To an emulsion of **5a** (565 mg, 2.85 mmol) in phosphate buffer (40 ml) α-CT (74 mg) was added under vigorous stirring. After 10 min, following the above described procedure, the unreacted ester (+)-**5a** with 11% ee was isolated (390 mg, 69% yield), together with (–)-**1a** with 74% ee (76 mg, 14% yield).

(–)-(2*S*,3*R*)-**1a**: white solid, mp 77–80 °C (Lit.[5] mp 77–80 °C); $[\alpha]_D^{25}$ = –2.9 (*c* = 0.25 in chloroform); CD: $\Delta\varepsilon_{263}$= –0.2, $\Delta\varepsilon_{225}$= +6.1 (MeOH).

The unreacted ester (+)-**5a** (390 mg, 1.97 mmol) having 11% ee was hydrolysed in phosphate buffer (36 ml) with α-CT (51 mg) for 73 min. After the usual workup, (+)-**5a** with 68% ee was isolated in admixture with 33% of its α,ß-butenolide isomer (144 mg, 37% yield), together with (–)-**1a** with 39% ee (163 mg, 45% yield).
(+)-(2*R*,3*S*)-**5a**: $[\alpha]_D^{25}$= +15.1 (*c* = 0.24 in methanol) in admixture with 33% of its α,ß-butenolide isomer.

Hydrolysis of 5a with Aminoacylase. The unreacted ester (+)-**5a** (144 mg with 33% of its α,ß-butenolide isomer**)** having 68% ee was hydrolysed with aminoacylase (130 mg) in phosphate buffer (30 ml) for 95 min. After the usual workup, a mixture of 22% of (–)-**5a** with 26% ee and 78% of its α,ß-butenolide isomer with 30% ee was isolated in 55% yield, together with (+)-**1a** with 81% ee (49 mg, 37% yield).
(+)-(2*R*,3*S*)-**1a**: white solid, mp 69–73°C; $[\alpha]_D^{25}$= +3.7 (*c* = 0.35 in chloroform); $[\alpha]_D^{25}$ = –5.3 (*c* = 0.15 in methanol) {Lit.[5] mp 77–80 °C; $[\alpha]_D^{25}$ = +18.2 (*c* = 0.22 in chloroform for the (2*R*,3*S*) enantiomer}; CD: $\Delta\varepsilon_{267}$= +0.1, $\Delta\varepsilon_{225}$= –4.8 (MeOH).
All the other spectroscopic data are in accordance with those found in the literature.[5,6]

Hydrolysis of 5b with α-CT. To an emulsion of **5b** (370 mg, 1.64 mmol) in phosphate buffer (90 ml), α-CT (36 mg) was added under vigorous stirring. After 7 h, following the above described procedure, the unreacted ester (+)-**5b** with 79% ee was isolated (143 mg, 39% yield) together with (–)-methylenolactocin **1b** with 73% ee (63 mg, 18% yield).
After recrystallization from ethyl acetate-light petroleum, the ee of (–)-**1b** increased to 98%.
(–)-(2*S*,3*R*)-**1b**: white solid, mp 81–83 °C; $[\alpha]_D^{25}$= –7.5 (*c* = 0.71 in methanol); {Lit.[9] mp 82.5–83.5 °C; $[\alpha]_D^{26}$= –2.37 (*c* = 3.00 in methanol); Lit.[48] mp 81–83 °C; $[\alpha]_D^{25}$= –7.0 (*c* = 0.12 in methanol); Lit.[50] mp 82–84 °C; $[\alpha]_D^{24}$= –17.13 (*c* = 1.96 in chloroform); Lit.[51] $[\alpha]_D^{23}$= –6.46 (*c* = 0.5 in methanol); Lit.[52] $[\alpha]_D^{20}$= –10 (*c* = 0.5 in methanol); Lit.[53] mp 83–84 °C; $[\alpha]_D^{25}$= –2.23 (*c* = 1.56 in methanol); Lit.[63] mp 82–83 °C; $[\alpha]_D$ = –12.4 (*c* = 0.5 in methanol); Lit.[59] mp 82.5 °C; $[\alpha]_D^{32}$= –18.8 (*c* = 0.31 in chloroform); Lit.[64] mp 82–83 °C; $[\alpha]_D^{32}$ = –8.5 (*c* = 0.31 in methanol), $[\alpha]_D^{29}$ = –11.6 (*c* = 0.31 in chloroform); Lit.[65] $[\alpha]_D^{25}$= –6.77 (*c* = 0.52 in methanol); Lit.[66] mp 82–84 °C; $[\alpha]_D^{26}$= –6.7 (*c* = 0.5 in methanol)}; CD: $\Delta\varepsilon_{261}$= –0.27, $\Delta\varepsilon_{224}$= +11.1 (MeOH).

Hydrolysis of 5b with Aminoacylase. The unreacted ester (+)-**5b** (143 mg, 0.63 mmol) recovered from the previous reaction and having 79% ee was hydrolysed with aminoacylase (143 mg) in phosphate buffer (20 ml) for 30 min. After the usual workup, (+)-methylenolactocin **1b** with 99% ee was isolated (20 mg, 15% yield).

(+)-(2*R*,3*S*)-**1b**: white solid, mp 80–81 °C; $[\alpha]_D^{25}$ = +6.8 (c = 0.28 in methanol) {Lit.[51] $[\alpha]_D^{23}$= +6.5 (c = 1.5 in methanol), Lit.[53] mp 82 °C; $[\alpha]_D^{25}$ = +2.25 (*c* = 1.46 in methanol), Lit.[64] mp 82–83 °C $[\alpha]_D^{32}$= +7.4 (*c* = 0.33 in methanol), $[\alpha]_D^{30}$= +16.6 (*c* = 0.33 in chloroform)}; CD: $\Delta\varepsilon_{262}$= +0.16, $\Delta\varepsilon_{224}$= –8.62 (MeOH).

Hydrolysis of 5b with Aminoacylase IPE*:* To a solution of **5b** (40 mg, 0.18 mmol) in isopropyl ether (2 ml), aminoacylase (40 mg) in phosphate buffer (2 ml) was added under vigorous stirring. After 4 days, following the above described procedure, the unreacted ester (–)-**5b** with 96% ee was isolated (10 mg, 24% yield) together with (+)-methylenolactocin **1b** with 70% ee (5 mg, 12% yield).

(–)-(2*S*,3*R*)-**5b**: oil; $[\alpha]_D^{25}$ = –4.7 (*c* = 0.42 in methanol) [Lit.[63] $[\alpha]_D^{25}$ –5.3 (*c* = 0.956 in chloroform)]; CD: $\Delta\varepsilon_{258}$= –0.27, $\Delta\varepsilon_{223}$= +7.98 (MeOH).

All spectroscopic data are in accordance with those reported in the literature.[63]

Hydrolysis of 5c with Aminoacylase:[57] To an emulsion of **5c** (362 mg, 1.35 mmol) in phosphate buffer (61.2 ml) and acetone (2 ml) aminoacylase (242 mg) was added under vigorous stirring. After 4 h 30 min, following the above described procedure, the unreacted ester (–)-**5c** with 32% ee was isolated (159 mg, 44% yield), together with (+)-C75 **1c** with 91% ee (38 mg, 11% yield). After recrystallization from light petroleum the ee of (+)-**1c** increased to 98%.

(+)-(2*R*,3*S*)-**1c**: white solid, mp 88-90 °C; $[\alpha]_D^{25}$ = +8.4 (*c* = 0.15 in methanol), {Lit.[10] mp 88-89 °C; $[\alpha]_D^{25}$ = +9.5 (*c* = 0.15 in methanol), $[\alpha]_D^{25}$ = +11.4 (*c* = 1.0 in chloroform)}; CD: $\Delta\varepsilon_{258}$= +0.26, $\Delta\varepsilon_{225}$= –10.58 (MeOH).

The unreacted ester (–)-**5c** (158 mg, 0.59 mmol) having 32% ee was hydrolysed with aminoacylase (158 mg), in phosphate buffer (26.5 ml) and acetone (4 ml) added for 24 h. After the usual workup, (–)-**5c** with 94% ee was isolated (87 mg, 55% yield), together with (+)-C75 **1c** with 89% ee (12 mg, 8% yield).

Hydrolysis of 5c with HLAP*:* To a suspension of (–)-**5c** (86 mg, 0.32 mmol) having 94% ee in phosphate buffer (27.3 ml) and acetone (2 ml) HLAP (86 mg) was added under vigorous stirring. After 22 h, following the above described procedure, (–)-C75 **1c** with 94% ee was isolated (24 mg, 29% yield). After recrystallization from light petroleum, the ee of (–)-**1c** increased to 99%.

(–)-(2*S*,3*R*)-**1c**: white solid, mp 88–90 °C; $[\alpha]_D^{25}$ = –9.5 (*c* = 0.49 in methanol) {Lit.[10] mp 88–89 °C; $[\alpha]_D^{25}$ = –11.4 (*c* = 1.0 in chloroform)}.

All spectroscopic data are in accordance with those reported in the literature.[10,14,57,67]

Hydrolysis of 5d with PLAP: To a solution of **5d** (82 mg, 0.26 mmol) in phosphate buffer (8 ml) and acetone (2 ml) PLAP (61 mg) was added under vigorous stirring. After 75 min, following the above described procedure, the unreacted ester **5d** with 8% ee was isolated (22 mg, 27% yield), together with nephrosterinic acid **1d**[47,49,61,64] with 11% ee (4 mg, 5% yield). Chiral HPLC: t_R = 7.7, 8.8 min for (±)-**1d**.

Biological Assays

Cytotoxicity assays. Cytotoxicity assays were carried out against human MCF-7 breast carcinoma and rat C6 glioma cells. MCF-7 cell lines were maintained at 37 °C in a humidified incubator containing 5% CO_2 in Dulbecco's Modified Eagle's medium (DMEM) (Lonza) nutrient supplemented with 10% heat inactivated Fetal Bovine *Serum* (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. The percentage of DMSO, the organic solvent in which the tested compounds were dissolved, never exceeded 1% (v/v) in the samples. In preliminary experiments, we verified that this amount did not affect cell viability. Cytotoxicity of the tested compounds is expressed as IC_{50} values — the concentrations that cause 50% growth inhibition. A compound was considered inactive when its IC_{50} value was > 100 µM. The results were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Cells were dispensed into 96-well microtiter plates at a density of 5,000 cells/well. Following overnight incubation, cells were treated with a range of compound concentrations (0.5–100 µM). Then, the plates were incubated at 37 °C for 72 h. An amount of 10 µL of 0.5% w/v MTT was further added to each well and the plates were incubated for additional 4h at 37 °C. Finally the cells were lysed by addition of 100 µL of 1:1 v/v DMSO:EtOH solution. The absorbance at 570 nm was determined using a Perkin Elmer 2030 multilabel reader Victor TM X3.

Antimicrobial Studies

Antibacterial studies. The in vitro minimum inhibitory concentrations (MICs, µg/mL) were assessed by the broth microdilution method, using 96-well plates, according to CLSI guidelines.[68,69] Stock solutions of the tested compounds were obtained in DMSO. Then two-fold serial dilutions in the suitable test medium between 512 and 0.03 µg/mL were plated. To be sure that the solvent had no adverse effect on bacterial growth, a control test was carried out by using DMSO at its maximum concentration along with the medium. Bacteria strains available as freeze-dried discs, belonging to the ATCC collection, were used: *S. aureus* 29213, *E. faecalis* 29212 as the Gram-positive strains, and *E. coli* 25922 as the Gram-negative one. To preserve the purity of cultures and to allow reproducibility, a series of criovials of all

microbial strains in glycerolic medium were set up and stored at –80 °C. Pre-cultures of each bacterial strain were prepared in Cation Adjusted Mueller–Hinton broth (CAMHB) and incubated at 37 °C until the growth ceased. The turbidity of bacterial cell suspension was calibrated to 0.5 McFarland Standard by spectrophotometric method (625 nm, range 0.08–0.10), and further the standardized suspension was diluted 1:100 with CAMHB to have 1–2 x 10^6 CFU/mL. All wells were seeded with 100 μL of inoculum. A number of wells containing only inoculated broth as control growth were prepared. The plates were incubated at 37 °C for 24 h, and the MIC values were recorded as the last well containing no bacterial growth. Each assay was repeated twice in triplicates. Norfloxacin was used as the reference drug. A compound was considered inactive when its MIC was > 64 μg/mL.

Antifungal studies. Antifungal studies[68,70] were carried out against *C. albicans* 10231, *C. parapsilosis* 22019, *C. tropicalis* 750, *C. krusei* 6258, belonging to the ATCC collection. Preparation of stock solutions and purity of cultures preservation were obtained as above described for antibacterial studies. Pre-cultures of each yeast strain were prepared in Sabouraud broth 2% glucose (SAB), and incubated at 37 °C until the growth ceased. The turbidity of yeast stock suspension was calibrated to 0.5 McFarland Standard by spectrophotometric method (530 nm, range 0.12–0.15), and further the standardized suspension was diluted first 1:50 with SAB and then 1:20 in the same medium to have 1–5 x 10^6 CFU/mL. All wells were seeded with 100 μL of inoculum. A number of wells containing only inoculated broth as control growth were prepared. The plates were incubated at 37 °C for 24–48 h, and the MIC values were recorded as the last well containing no fungal growth. Each assay was repeated twice in triplicates. Fluconazole was used as the reference drug. A compound was considered inactive when its MIC was > 64 μg/mL.

RESULTS AND DISCUSSION

Synthesis of Racemic Substrates

The methyl esters of the target compounds **1a**–**d** to be subjected to enzymatic kinetic resolutions, namely **5a-d**, were synthesized using a slightly modified version of the procedure reported by Carlson and Oyler[47] (Scheme 1). Methyl hemi-ester of itaconic acid **2**,[71] prepared by nucleophilic ring fission of itaconic anhydride, was converted into its lithium enolate and reacted with the suitable aldehyde (RCHO = butanal, hexanal, nonanal, and dodecanal). Acidification of the resulting reaction mixture carried out at –78 °C gave the corresponding *anti* and *syn* hydroxy hemi-esters **3a–d** and **4a–d** in the ratio of 2:3, identified only by ^{1}H NMR analysis. Their lactonization, carried out with TFA in CH_2Cl_2, furnished the

corresponding (2*R**,3*S**) and (2*R**,3*R**) lactonic esters **5a-d** and **6a-d** respectively, in about 50% total yield. Compounds **5a-d** were separated from **6a-d** by flash chromatography using as stationary phase a silica gel pretreated with acetic acid to avoid the double bond isomerization.

Attempts were made to isomerise compounds **6a-d** into their respective diastereomers **5a-d**, however unsuccessfully. The first strategy involved protection of the double bond *via* a Michael addition of a thiol (ethanethiol and dodecanethiol),[72] equilibration and deprotection *via* a retro-Michael reaction. This reaction sequence was attempted on **6d**, but in the first step already, conversion into its α,ß-butenolide isomer occurred, probably because of the slightly basic conditions required by the procedure. Following a second strategy proposed in the literature for the transformation of **6b**,[58,59,63] compound **6a** was treated with 6M HCl in butanone under reflux for 2h, leading to a complex inseparable mixture of compounds.

Insert Scheme 1

Enzymatic Hydrolyses of Lactonic Esters 5a-d

Prior to enzymatic hydrolysis, it was important to determine whether the lactonic esters **5a-d** underwent chemical hydrolysis under the conditions used. This was tested on compound 5**b**. On standing in phosphate buffer at pH 7.4 for 3.5 h, **5b** was recovered unchanged, except for the presence of less than 5% of its α,ß-butenolide isomer. Incidentally, under the same conditions, the diastereomer **6b** underwent more important modifications not including hydrolysis.

Enzymatic hydrolyses were carried out using a series of commercially available enzymes, namely Acylase I on Eupergit, Aminoacylase, Acylase I from porcine kidney, α-chymotrypsin (α-CT), *Candida antarctica* lipase (Novozym 435), Porcine pancreatic lipase (PPL), Esterase from hog liver (PLE), and Liver acetone powder equine (HLAP). A preliminary evaluation of enzyme selectivity was performed on 30–50 mg of substrates **5a**–**d** in 10 mL of phosphate buffer at pH 7.4. The results obtained for compounds **5a-c** are listed in Table 1. Compounds **5d** is not included in the Table because it was hydrolysed only by porcine liver acetone powder (PLAP, 20% conversion, 2 h, E[73] = 1.3), however with negligible enantioselection, whereas the other enzymes checked, namely Acylase I on Eupergit® C, Aminoacylase, α-CT, Lipase from *Candida antarctica* (CAL-B), Lipase from *Candida antarctica* (Novozym 435), PLE, Lipase from *Pseudomonas cepacea*, Lipase from *Pseudomonas fluorescens*, Proteases from *Bacillus subtilis* and PPL, were uneffective.

Insert Table 1

As it is evident from the *E* values listed in Table 1 for the various hydrolyses, enantioselectivity was good only for **5c**. In fact, at low conversion values the corresponding acid **1c** was isolated with ee's ranging from 90 to 96% using acylases from *Aspergillus sp*.[57] (Entries 1 – 3). Novozym 435 was enantiocomplementary to the previous enzymes, although with poor enantioselectivity (Entry 6), and Acylase I from porcine kidney, α-CT and PPL were uneffective. The best enzymes for lactones **5a** and **5b** were acylases from *Aspergillus* sp. and α-CT (Entries 1 – 3, 5), although with less enantioselectivity, as indicated by the ee's of their respective acids **1a** and **1b** that ranged from 70% to 87%. Interestingly however, acylases and α-CT were enantiocomplementary. On the contrary, Acylase I from porcine kidney, Novozym 435 and PPL were found poorly enantioselective (Entries 4, 6, 7). As it is evident from Table 1 (Entry 8), PLE and HLAP were able to hydrolyse all substrates, however with no enantioselectivity, and therefore they were used as alternatives to chemical hydrolysis[63] of enantiopure methyl α-methyleneparaconates. Attempts to increase the enantioselectivity of these hydrolyses by adding acetone or ethanol as cosolvents were unsuccessful, while addition of isopropyl ether resulted in a slight increase of the *E* values (Entry 3).

In scaling up the enzymatic hydrolyses, both enantiomers of **1a** and **1b** were obtained from the corresponding methyl esters **5a** and **5b** using α-CT and aminoacylase which were enantiocomplementary. However, as their efficiencies were not high, as demonstrated by their low *E* values, a particular procedure was followed to take advantage of both enzymes. For compound **5b**, in a first run, performed with α-CT, the hydrolysis reaction was stopped at about 50% conversion. The acid (–)-**1b** was isolated with 73% ee in 18% yield and the unreacted ester (+)-**5b** was isolated with 79% ee in 39% yield. To improve the optical yield of both compounds, the acid (–)-**1b** was fractionally crystallized thus increasing its ee to 98%, while the ester (+)-**5b** was subjected to a further resolution with aminoacylase that allowed the isolation of (+)-**1b** with 99% ee (Scheme 2). However, since optical yield optimization was detrimental to chemical yield, the biological assays were performed with (+)-**1b** and (–)-**1b** having 92 and 93% ee respectively.

The strategy adopted for the obtainment of (+)-**1b** and (–)-**1b** in pure enantiomeric forms was not so satisfactory when applied to **1a**, as fractional crystallization of enantioenriched acids did not improve their respective ee's significantly; (+)- and (–)-**1a** (optical rotations determined for chloroform solutions) were obtained with 81% and 74% ee respectively.

Insert Scheme 2

As to the lactonic acid **1c**, both enantiomers were obtained with excellent ee [(+)-**1c**, 98% ee, (–)-**1c**, 99% ee], as reported in the literature.[57]

The resolution outcome depends on the rate at which the α-methylene-γ-lactone turns into its α,ß-butenolide isomer and this is a function of the chain length, being higher for C3 than for C8, under the experimental conditions used. In spite of the presence of this competitive reaction, α-methyleneparaconic acids can be obtained in pure state because their α,ß-butenolide isomers do not undergo hydrolysis within the enzymatic reaction times, and thus they can be easily separated from the esters.

The (2*R*,3*S*) absolute configuration was assigned to the enantiomers which in chloroform were dextrorotatory, by comparison of the signs of their optical rotation values with those reported in literature (see Material and Method) and by comparison of their CD spectra with that of (2*R*,3*S*)-(+)-protolichesterinic acid **1e** ($\Delta\varepsilon_{257}$ +0.32, $\Delta\varepsilon_{220}$ –9.62),[75] whose absolute configuration is known (Compound **1a**: $\Delta\varepsilon_{267}$= +0.1, $\Delta\varepsilon_{225}$= –4.8 (MeOH); compound **1b**: $\Delta\varepsilon_{262}$= +0.16, $\Delta\varepsilon_{224}$= –8.62 (MeOH); compound **1c**: $\Delta\varepsilon_{258}$ +0.26, $\Delta\varepsilon_{225}$ –10.58 (MeOH)).

The optical rotation values for the α-methylene paraconic acid derivatives deserve a comment. The values reported in the literature for compounds having approximately the same high enantiomeric excess, at comparable concentrations, are rather variable. If one compares literature data for methylenolactocin (–)-**1b** optical rotation values obtained for methanolic solutions range from –6.46 (23 °C) to –12.4 (temperature not given), while those obtained for chloroform solutions, vary from –11.6 to –18.8. The syntheses reported are multi-step and either the chirality is already present in the parent molecule or it is introduced in a stereochemically controlled step of the synthesis. In no case however the enantiomeric excess of the target molecule is measured because it is assumed to be the same as in the parent molecule. On the contrary, in the present paper the enantiomeric excess is measured on the final esterified products.

Finally, it is interesting to note that **1a** showed a solvent dependency of the optical rotation, which changed its sign replacing methanol for chloroform, differently from what observed for **1b** (see Materials and Methods), **1c**[10] and **1d**.[64]

Biological Assays

Cytotoxicity Assays

Antiproliferative activities of both enantiomers of lactonic acids **1a**–**c** were evaluated in both human breast carcinoma and rat glioma cells (MCF-7 and C6, respectively) by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. All tested compounds were inactive on both cell lines but (+)- and (–)-**1c** displayed moderate cytotoxic effects at the

middle micromolar level. Both enantiomers were about four-fold higher on C6 glioma cells (IC_{50} = 12–18 μM) but still less active than cisplatin (0.6 ± 0.2 μM), taken as a reference compound. The observed antiproliferative activity was characterized by low, significant (p < 0.01) stereoselectivity in the MCF-7 assay, (–)-**1c** resulting more active than its enantiomer (IC_{50} values 68.4 ± 0.3 vs 84.5 ± 2.3 μM). This observation was in agreement with previously reported results.[10] No stereoselectivity was observed when the activity on G6 cells was evaluated.

Antimicrobial Studies

Antibacterial studies. These studies were performed in agreement with a previously reported protocol.[68] According to the Clinical Laboratory Standards Institute (CLSI) guidelines,[69] compounds **1a**–**c** were tested against Gram-positive and Gram-negative bacteria belonging to the ATTC collection (*Staphylococcus aureus*, *S. aureus* 29213; *Enterococcus faecalis*, *E. faecalis* 29212; *Escherichia coli*, *E. coli* 25922) using norfloxacin as a reference compound.

All tested compounds displayed no antibacterial activity against *E. coli*, while moderate activity was observed against Gram-positive bacteria. (–)-**1c** was the most active compound, (MIC: 16 μg/mL; 60 μM) about three times less potent than norfloxacin on *E. faecalis*

Antifungal studies. These studies were performed in agreement with a previously reported protocol.[68] According to the CLSI guidelines,[70] compounds **1a**–**c** were tested against a panel of fungi strains belonging to the ATCC collection (*Candida albicans*, *C. albicans* 10231; *Candida tropicalis*, *C. tropicalis* 750; *Candida parapsilosis*, *C.* 22019; *Candida krusei*, *C. krusei* 6258) using fluconazole as a reference compound.

All tested compounds were inactive as antifungal agents.

CONCLUSIONS

Optically pure paraconic acids can be obtained by enzymatic resolutions of their methyl esters. High enantioselectivity for hydrolyses of **5a** and **5b** could be achieved taking advantage of enantiocomplementary enzymes. Thus the unreacted esters, isolated from the reaction with α-CT could be resolved successfully with aminoacylase.

As to the biological results, in agreement with previously reported results,[10] C75 displayed moderate cytotoxic effects on human breast carcinoma and rat glioma cells. Both C75 enantiomers displayed higher cytotoxic effects on C6 glioma cells with no stereoselectivity.

(–)-C75 also acted as the most potent antimicrobial agent against Gram-positive bacteria. Unfortunately, the antibacterial activity required concentrations as high as the ones displaying cytotoxicity on the tumoral cells. This observation casts doubt on the possibility to develop antibacterials using C75 as the lead compound.

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