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# Chemical constituents from *Piper marginatum* Jacq. (Piperaceae)—antifungal activities and kinetic resolution of (*RS*)-marginatumol by *Candida antarctica* lipase (Novozym 435)

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**Abstract**—The leaves of *Piper marginatum* contain the antifungal compounds 3,4-methylenedioxypropiophenone **1**, 2-methoxy-4,5-methylenedioxypropiophenone **2**, 1-(3,4-methylenedioxyphenyl)propan-1-ol **3** (marginatumol), 5,4′-dihydroxy-7-methoxyflavanone **4** and 5,7-dihydroxy-4′-methoxyflavanone **5**. The absolute configuration of natural marginatumol was determined as (+)-(*R*)-**3** (ee 48%) by comparison of its optical properties with the chiral forms obtained by kinetic resolution of racemic **3** using *Candida antarctica* lipase (Novozym 435).
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## 1. Introduction

The Piperaceae family comprises 14 genera and ca. 1950 species,[1] and among these the genus *Piper* is the most abundant with approximately 700 species.[2] Phytochemical analysis from the *Piper* species showed the occurrence of several secondary metabolites, many of which exhibit a variety of biological activities, mainly as antifungal activity.[3]

*Piper marginatum* Jacq. (Piperaceae) is a common shrub of the Amazon region, popularly known as 'malvaísco'.[4] The extract of its leaves has been used in popular medicine to treat liver and vesicle diseases and also as a tonic with carminative and antispasmodic action.[5] Previous chemical studies carried out on *P. marginatum* have described the occurrence of propiophenones,[6–8] amides,[9] flavonoids,[10] phenylalkanoids[11] and aristolactams.[12]

Over the course of our search aimed at unraveling new antifungal agents from *Piper* species, the crude MeOH extract from leaves of *P. marginatum* was selected for bioactivity-guided phytochemical investigation, due to its potent activity observed against *Cladosporium cladosporioides* and *Cladosporium sphaerospermum*. Thus, the major bioactive compounds isolated were 3,4-methylenedioxypropiophenone **1**, 2-methoxy-4,5-methylenedioxypropiophenone **2**, 1-(3,4-methylenedioxyphenyl)propan-1-ol **3** (marginatumol), 5,4′-dihydroxy-7-methoxyflavanone **4** and 5,7-dihydroxy-4′-methoxyflavanone **5**.

Most natural products are frequently accumulated in plant species as one major stereoisomer.[13] Such an aspect has recently been investigated by means of chiral resolution of enantiomers using specific stationary phases. Surprisingly, chiral analysis has shown that many natural products can be found as enantiomeric mixtures of variable proportions. The chiral synthesis involved in the biocatalytic process has been proven as an effective tool in the preparation of enantiomerically pure compounds. Herein we report the resolution of the racemic (*RS*)-marginatumol **3**, prepared by a Grignard reaction using piperonal and ethylbromide, in order to determine the configuration of the natural marginatumol isolated from *P. marginatum*. The asymmetric synthesis of the natural products was carried out through a chemoenzymatic resolution with lipase of *Candida antarctica* (Novozym 435).

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## 2. Results and discussion

### 2.1. Characterization of *P. marginatum* constituents

The MeOH extract from the leaves of *P. marginatum* was submitted to bioactivity-guided fractionation using column chromatography on silica gel and Sephadex LH-20 followed by prep. TLC to yield compounds **1** (3,4-methylenedioxypropiophenone), **2** (2-methoxy-4,5-methylenedioxypropiophenone), **3** [1-(3,4-methylenedioxyphenyl)propan-1-ol], **4** (5,4′-dihydroxy-7-methoxyflavanone) and **5** (5,7-dihydroxy-4′-methoxyflavanone), as shown in Figure 1. Compounds **1**, **2**, **4** and **5** were identified by comparison of their spectral data with those previously reported.[3b,6]

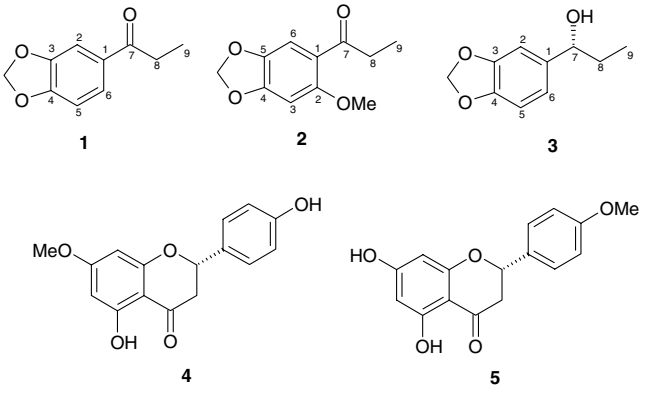



**Figure 1.** Compounds **1**–**5** isolated from the leaves of *P. marginatum*.

The $^{1}$H NMR spectrum of **3** indicated the presence of a 1,3,4-trisubstituted aromatic ring since three hydrogen resonances were observed at $\delta$ 6.55 (dd, $J = 8.8$ and 1.8 Hz), 6.82 (d, $J = 1.8$ Hz) and 6.63 (d, $J = 8.8$ Hz). In addition, the spectrum showed the presence of an ethyl group due to the signals at $\delta$ 0.79 (t, $J = 7.5$ Hz, 3H) and $\delta$ 1.55 (m, 2H) and a methylenedioxy group due to the singlet at $\delta$ 5.30 (2H), which was positioned at C-3/C-4 similarly to compound **1**. The signal at $\delta$ 4.15 (t, $J = 6.6$ Hz, H-7) indicated an oxybenzyl hydrogen, which was confirmed by the presence of an oxymethine carbon at $\delta$ 75.9 in the $^{13}$C NMR spectra (Table 1). The presence of the hydroxyl group in **3** was confirmed by IR spectroscopy due to the typical broad band at 3383 cm$^{-1}$. Finally, in the LREIMS of **3** was observed the molecular ion peak at *m*/*z* 180 Da, in agreement with the molecular formula $C_{10}H_{12}O_3$. Therefore, the structure of **3** was elucidated as 1-(3,4-methylenedioxyphenyl)propan-1-ol.

The natural product **3** was submitted to chiral GC analysis using a β-cyclodextrin column, which indicated the presence of two compounds with $t_R = 49.3$ and 49.8 min (48% excess of the compound with $t_R = 49.3$ min). Attempts to determine the configuration of natural product **3**, further NMR analysis and chiral GC analysis were preceded by its fast intermolecular dehydration. Therefore, a study involving the preparation of racemic **3** on a large scale by Grignard synthesis (Section 3.5) followed by enzymatic resolution was carried out. The comparison of the retention times of the resolved synthetic enantiomers and the natural **3** by GC analysis allowed the determination of its configuration as (*R*), and the enantiomeric excess in comparison to the proportion of the (*S*)-derivative.

### 2.2. Enzymatic resolution

The evaluation of the chemoenzymatic esterification of **3** was performed with a lipase from *Candida antarctica* (Novozym 435, CALB) and vinyl acetate as the acetate donor, using previous conditions as described by our group.[14,15] As can be seen in Table 2, the small scale (50 mg) resolution was very efficient and yielded the (*S*)-alcohol **3** (ee 92%) and (*R*)-acetate **3a** in high enantiomeric excess (>99%) and good conversion (*c* 50%) at 24 h (Table 2, entry 6). The kinetic resolution of **3** by CALB on a preparative scale (500 mg) yielded the (*S*)-alcohol **3** and (*R*)-acetate **3a** with high enantiomeric excess (ee >99%) and with good isolated yields after 48 h (alcohol **3**: 43%; acetate **3a**: 45%; Table 2, entry 7).

### 2.3. Determination of the enantiomeric excesses and absolute configuration

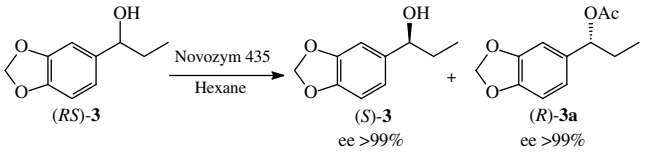
The enantiomeric excesses of alcohol **3** and acetate **3a** were calculated from the peak areas observed in the chiral GC chromatograms and by comparison with those observed in the racemic samples.[16] (*R*)-Acetate **3a** was hydrolyzed to the (*R*)-alcohol **3** ($t_R = 49.2$ min), which showed

**Table 1.** $^{1}$H and $^{13}$C NMR (300 and 75 MHz, $\delta$ ppm, $CDCl_3$) spectral data for compounds **1**–**3** isolated from *P. marginatum*

| Position | 1 | | 2 | | 3 | |
|---|---|---|---|---|---|---|
| | $\delta_H$ (m, *J*/Hz) | $\delta_C$ | $\delta_H$ (m, *J*/Hz) | $\delta_C$ | $\delta_H$ (m, *J*/Hz)[a] | $\delta_C$[a] |
| 1 | — | 131.8 | — | 120.6 | — | 138.7 |
| 2 | 7.51 (d, 1.5) | 107.7 | — | 156.6 | 6.82 (d, 1.8) | 106.4 |
| 3 | — | 148.1 | 6.46 (d, 1.2) | 94.2 | — | 146.9 |
| 4 | — | 151.5 | — | 152.0 | — | 147.8 |
| 5 | 6.92 (d, 8.5) | 107.8 | — | 141.6 | 6.63 (d, 8.8) | 108.0 |
| 6 | 7.65 (dd, 8.5, 1.5) | 124.0 | 7.24 (d, 1.2) | 109.1 | 6.55 (dd, 8.8, 1.8) | 119.4 |
| 7 | — | 198.8 | — | 200.6 | 4.15 (t, 6.6) | 75.9 |
| 8 | 3.00 (q, 7.6) | 31.5 | 2.87 (q, 7.5) | 36.8 | 1.55 (m) | 31.8 |
| 9 | 1.30 (t, 7.6) | 8.4 | 1.46 (t, 7.5) | 8.5 | 0.79 (t, 7.5) | 10.1 |
| $OCH_2O$ | 6.10 (s) | 101.7 | 6.10 (s) | 101.8 | 5.30 (s) | 100.9 |
| $OCH_3$ | — | — | 3.78 (s) | 56.2 | — | — |

[a] Spectra recorded in $C_6D_6$.

**Table 2.** Kinetic resolution of (*RS*)-**3** catalyzed by Novozym 435[a]




| Entry | *t* | *c* **3a** | ee (AC) **3a** | ee (AC) **3** | *E* |
|---|---|---|---|---|---|
| 1 | 1 | Nc | >99 (*R*) | 9 (*S*) | — |
| 2 | 3 | Nc | >99 (*R*) | 16 (*S*) | — |
| 3 | 5 | 22 | >99 (*R*) | 28 (*S*) | — |
| 4 | 7 | 30 | >99 (*R*) | 42 (*S*) | — |
| 5 | 9 | 35 | >99 (*R*) | 53 (*S*) | — |
| 6 | 24 | 48 | >99 (*R*) | 92 (*S*) | >200 |
| 7[b] | 48 | 50 | >99 (*R*) | >99 (*S*) | >200 |
| 8 | — | — | — | 48[c] (*R*) | — |

*t*: time (h); *c* (%): conversion, calculated from the ee's of the substrate ($ee_s$) and the product ($ee_p$); *c*: $ee_s/(ee_p + ee_s)$[16]; ee (%): enantiomeric excess; AC: absolute configuration; *E*: enantiomeric ratio; Nc: not calculated.

[a] The small scale reaction was carried out at 32 °C (160 rpm) using alcohol **3** (50 mg), vinyl acetate (1 mL), hexane (10 mL) and Novozyme 435 (100 mg).

[b] Preparative scale enzymatic reaction (see Section 3.6.2: 500 mg of **3**).

[c] Natural product isolated from *P. marginatum*.

$[\alpha]_D = +31.6$ (*c* 3.20, $CHCl_3$). The absolute configuration of the (*S*)-alcohol **3** and (*R*)-acetate **3a** were suggested by stereochemical preference by CALB based on Kazlauskas rule.[17] In addition, the chemoenzymatic resolution of the (*RS*)-1-phenylpropanol **6** produced (*S*)-alcohol **6** and (*R*)-1-phenylpropane acetate **6a** in accordance with Kazlauskas rule (Fig. 2).[18]

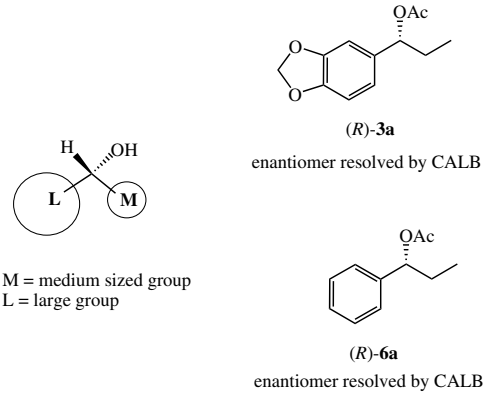



**Figure 2.** Kazlauskas rule for the esterifications of **3** and phenyl-propanol **6** using CALB (Novozyme 435).[17,18]

Finally, the natural compound **3** isolated from *P. marginatum* was determined as having an (*R*)-configuration (Fig. 3) based on the well established configurations and retention times of the (*R*)-**3** and (*S*)-**3** derivatives and an enantiomeric excess of 48% (Table 2, entry 8 and Fig. 2).

## 2.4. Antifungal activity

The antifungal activity of compounds **1**–**5** was determined by means of a direct bioautography on TLC plate.[19] As can

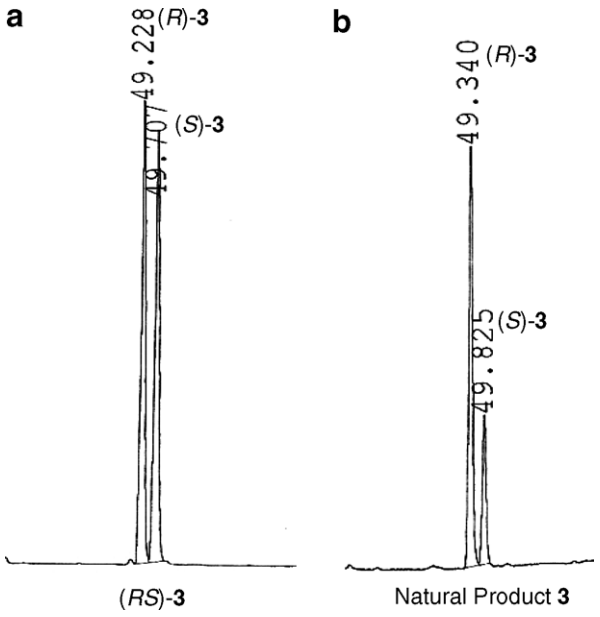



**Figure 3.** (a) GC chiral chromatogram of (*RS*)-**3**; (b) GC chiral chromatogram of natural product **3** isolated from *P. marginatum*.

**Table 3.** Minimum amount of compounds **1**–**5** isolated from *P. marginatum* required for the inhibition of fungal growth on thin-layer chromatographic plates (TLC)

| Compounds | Antifungal activity (μg) | |
|---|---|---|
| | *C. cladosporioides* | *C. sphaerospermum* |
| **1** | 5.0 | 5.0 |
| **2** | 5.0 | 5.0 |
| **3** | 10.0 | 10.0 |
| **4** | 1.0 | 1.0 |
| **5** | 1.0 | 1.0 |
| Nystatin | 1.0 | 1.0 |
| Miconazole | 1.0 | 1.0 |

be seen by the detection limit of these compounds (Table 3), all the substances described displayed antifungal activity when submitted to bioautographic assays. Flavanones **4** and **5** were the most potent with activities comparable to the controls, and compounds **1** and **2** showed higher activity than **3**. Therefore, the carbonyl group appeared to be important to the antifungal activity, since compounds **1** and **2** have their potential increased by the presence of this group. There has been no previous report describing the antifungal activity of compounds **1**–**3**.

# 3. Experimental

## 3.1. General

Silica gel (Merck 230–400 mesh) and Sephadex LH-20 (Pharmacia) were used for column chromatography separations and silica gel 60 PF254 (Merck) for preparative TLC purifications (1.0 mm). $^1H$ NMR (300 MHz) and $^{13}C$ NMR (75 MHz) spectra were measured in $CDCl_3$ and $C_6D_6$ on Bruker DPX-300 instrument with 1% of

TMS as internal standard; LREIMS were measured at 70 eV on a HP 5990/5988A spectrometer; IR spectra were obtained on an FT-IR 510 Nicolet spectrometer and UV spectra were recorded on a UV/Visible Shimadzu 1650-PC spectrophotometer.

The conversions and enantiomeric excesses of the enzyme-catalyzed reactions were determined using a Shimadzu GC-17A gas chromatograph equipped with a chiral capillary column Chirasil-Dex CB β-cyclodextrin (25 m × 0.25 mm × 0.25 μm). The carrier gas was hydrogen with a pressure of 100 kPa. Optical rotation values were measured in a Jasco DIP-378 polarimeter and the reported data refer to the Na-line value using a 1 dm cuvette. Novozym 435 immobilized lipase from *Candida antarctica* was obtained as a gift from Novozymes Latin America Ltda (Paraná-Brazil).[20]

### 3.2. Plant material

Leaves of *P. marginatum* Jacq. were collected at Manaus (Amazonia State, Brazil) in May 2002 and identified by Dr. Elsie F. Guimarães. A voucher specimen (Kato-0223) was deposited in the Herbarium of Instituto de Botânica, São Paulo, SP, Brazil.

### 3.3. Antifungal assay

The microorganisms used in the antifungal assays *C. cladosporioides* (Fresen) de Vries SPC 140 and *C. sphaerospermum* (Perzig) SPC 491 have been maintained at the Instituto de Botânica, São Paulo, SP, Brazil. For the antifungal assays, 10.0 μL of the solutions of the crude extract, fractions and pure compounds were prepared, under different concentrations, corresponding to 100.0 μg of crude extract and 10.0, 5.0 and 1.0 μg of fractions and pure compounds. The samples were applied to TLC plates, which were eluted with $CHCl_3$–MeOH (9:1) and dried for complete removal of solvents. The chromatograms were sprayed with a spore suspension of *C. cladosporioides* or *C. sphaerospermum* in a nutritive medium[19] and incubated for 48 h at 25 °C. After incubation, clear inhibition zones appeared against a dark background chromatogram. Nystatin and miconazole were used as positive controls whereas ampicillin and chloramphenicol were used as negative controls.

### 3.4. Extraction and isolation of constituents

The dried and powdered leaves of *P. marginatum* (82.2 g) were exhaustively extracted with MeOH at room temperature. The resulting MeOH extract was filtered and concentrated in vacuum to yield 3.43 g of crude extract, which was dissolved in MeOH–$H_2O$ (1:1) and extracted with EtOAc. The bioactive EtOAc phase (1.22 g) was subjected to column chromatography over silica gel (gradient of hexane to EtOAc) to yield nine fractions, in which bioactivity was detected in three of them (fractions 1–3).

Fraction 1 (200 mg) was applied to a silica gel column and eluted with gradient mixtures of EtOAc in hexane giving three sub-fractions. Bioactive sub-fraction 2 (100 mg) was composed of pure **1**.

Fraction 2 (100 mg) was submitted to prep. TLC (hexane–$CH_2Cl_2$ 8:2) to afford three sub-fractions. Compound **2** (39 mg) was isolated in pure form from bioactive sub-fraction 2.

Fraction 3 (59 mg) was chromatographed on a Sephadex LH-20 column eluted with hexane–$CH_2Cl_2$ (1:4) followed by $CH_2Cl_2$–$Me_2CO$ 3:2 and $CH_2Cl_2$–$Me_2CO$ 1:4 to yield ten sub-fractions. Compound **3** (5 mg) was isolated from sub-fraction 6, and compounds **4** (4 mg) and **5** (4 mg) were isolated from sub-fractions 9 and 10, respectively.

**3.4.1. 1-(3,4-Methylenedioxyphenyl)propan-1-ol 3 [(+)-(*R*)-marginatumol].** Yellow oil. IR (film) ($cm^{-1}$): 3383, 2965, 2877, 1503, 1487, 1441, 1248, 1040, 930, 811; UV $\lambda_{max}$ ($CHCl_3$) nm (log ε): 286 (3.55), 240 (3.56); LREIMS *m*/*z* (relative intensity): 180 [$M^+$] (25), 151 (89), 93 (100), 77 (76); $^1H$ NMR and $^{13}C$ NMR (Table 1).

### 3.5. Synthesis of (*RS*)-1-(3,4-methylenedioxyphenyl)propan-1-ol 3 [(*RS*)-marginatumol]

To a 125 mL three-necked flask equipped with an additional funnel, one-neck stoppered and one fitted with a condenser, were added 486 mg (20 mmoles) of dry magnesium turnings and the whole system was dried by heating. Then 30 mL of anhydrous ether with a crystal of iodine was added to the flask. Next, a solution of 2.21 g (20 mmoles) of dry ethylbromine in 10 mL of anhydrous ether was added using a separatory funnel. After preparation, the Grignard reagent was cooled (0 °C) and had the piperonal (3 g, 20 mmoles) slowly added. The mixture was stirred until all of the piperonal had been consumed after which the mixture was kept stirring at room temperature for 60 min. Then, the mixture was extracted between aqueous $NH_4Cl$ (20 mL) and EtOAc (3 × 50 mL). The organic phases were combined, dried over $MgSO_4$, concentrated under reduced pressure and the product chromatographed over silica gel eluted with hexane–EtOAc (9:1) to give **3** as a yellow oil (2.98 g, 17 mmoles, 84% yield).

### 3.6. Enzymatic reaction

**3.6.1. Small scale enzymatic reaction.** To a 50 mL Erlenmeyer flask containing 10 mL of hexane (HPLC grade), 1 mL of vinyl acetate and 100 mg Novozym 435 was added **3** (50 mg). The reaction mixture was stirred on a rotatory shaker (32 °C, 160 rpm) and analyzed until the starting material had been totally consumed (Table 2).

**3.6.2. Preparative scale enzymatic reaction.** To a 250 mL Erlenmeyer flask containing 100 mL of hexane (HPLC grade), 1 mL of vinyl acetate and 1 g Novozym 435 was added 500 mg of **3**. The reaction mixture was stirred on a rotatory shaker (32 °C, 160 rpm) and analyzed for consumption of the starting material (48 h, Table 2). The mixture was then filtered and the solvent evaporated. The residue was purified by silica gel column chromatography

using hexane–EtOAc (9:1) as eluent to afford (*S*)-**3** (43%) and (*R*)-**3a** (45%).

**3.6.3. Control of the chemoenzymatic resolution of 3 by Novozym 435.** The reaction progress was monitored by collecting 0.1 mL samples every 1 h until 48 h (Table 2). These samples were previously analyzed by GC/FID in a chiral capillary column. Alcohol **3** and acetate **3a** were compared with the racemic mixtures previously analyzed.

General GC conditions: Injector: 200 °C; Detector: 220 °C. Pressure: 100 kPa. Oven 100–150 °C; rate 1 °C; 150–180 °C; rate 50 °C; retention time for alcohol **3** [(*R*)-isomer = 49.2 min; (*S*)-isomer = 49.7 min] and acetate **3a** [(*S*)-isomer = 30.2 min; (*R*)-isomer = 31.2 min].

### 3.7. Hydrolyses of (+)-(*R*)-1-(3,4-methylenedioxyphenyl)propyl acetate 3a

In a one-neck flask, a mixture of (*R*)-acetate **3a** (150 mg, 0.7 mmoles), MeOH (60 mL), $H_2O$ (30 mL), $K_2CO_3$ (301 mg) were stirred at room temperature for 24 h. After completion of the reaction the MeOH was evaporated under vacuum. The aqueous phase was extracted with EtOAc ($3 \times 30$ mL), dried over $MgSO_4$ and concentrated to give crude hydrolyzed product. The purification by column chromatography over silica gel using hexane–EtOAc (4:1) afforded (*R*)-1-(3,4-methylenedioxyphenyl)propan-1-ol **3** (116 mg, 0.6 mmoles, 77% yield).

### 3.8. Assignment of the absolute configuration of compounds 3 and 3a

The absolute configurations of compounds **3** and **3a** were assigned using the Kazlauskas rule. The specific rotation data of 1-phenylpropanol **6** and 1-phenylpropane acetate **6a** have already been described in the literature.[18]

(−)-(*S*)-1-(3,4-Methylenedioxyphenyl)propan-1-ol **3**: $[\alpha]_D^{25} = -34.1$ (*c* 3.26, $CHCl_3$), ee 99%.

(+)-(*R*)-1-(3,4-Methylenedioxyphenyl)propan-1-ol **3**: $[\alpha]_D^{25} = +31.6$ (*c* 3.20, $CHCl_3$), ee 99% alcohol obtained by hydrolysis of acetate **3a**.

(+)-(*R*)-1-(3,4-Methylenedioxyphenyl)propyl acetate **3a**: $[\alpha]_D^{25} = +101.3$ (*c* 3.10, $CHCl_3$), ee 99%.

(−)-(*S*)-1-Phenylpropan-1-ol **6**: $[\alpha]_D^{25} = -50.7$ (*c* 3.73, $CHCl_3$), ee 99%.

(−)-(*S*)-1-Phenylpropan-1-ol **6**: $[\alpha]_D^{25} = -28.0$ (*c* 1.0, MeOH).[17]

(+)-(*R*)-1-Phenylpropyl acetate **6a**: $[\alpha]_D^{25} = +80.4$ (*c* 3.77, $CHCl_3$), ee 99%.

(+)-(*R*)-1-Phenylpropyl acetate **6a**: $[\alpha]_D^{25} = +100.0$ (*c* 1.0, $CHCl_3$).[18]

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