Physocalycoside, a New Phenylethanoid Glycoside from 
*Phlomis physocalyx* Hub.-Mor.

Tayfun Ersöz*a, Kalina Iv. Alipieva*b, Funda Nuray Yalçın*a, Pınar Akbay*a, 
Nedjalka Handjievab, Ali A. Dönmez*c, Simeon Popovb, and İhsan Çalış*a

*a Department of Pharmacognosy, Faculty of Pharmacy, Hacettepe University TR 06100, 
Ankara, Turkey. Fax: +90-3 12-3 11 47 77; E-mail: tersoz@hacettepe.edu.tr

*b Institute of Organic Chemistry with Center of Phytochemistry, Bulgarian Academy of 
Sciences, 1113 Sofia, Bulgaria

*c Department of Biology, Faculty of Science, Hacettepe University TR 06532, Ankara, Turkey

* Author for correspondence and reprint requests


A new phenylethanoid tetracylgoside, physocalycoside (2), was isolated from the aerial 
parts of *Phlomis physocalyx*. Its structure was identified as 3-hydroxy-4-methoxy-β-
phenylethoxy-α-1-rhamnopyranosyl-(1→2)-α-1-rhamnopyranosyl-(1→3)-4-O-feruloyl-β-
glucopyranosyl-(1→6)-β-1-d-glucopyranoside, on the basis of spectroscopic evidence. In addition, one known iridoid glucoside, lamiide (1) and five known phenylethanoid glycosides, 
wiedemannioside C (3), verbascoside (= acteoside) (4), leucosceptoside A (5), martynoside 
(6), and forsythoside B (7) were also characterized. Compounds 2–7 demonstrated radical 
scavenging properties towards the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical.

**Key words:** *Phlomis physocalyx*, Iridoid and Phenylethanoid Glycosides, Radical Scavenging 
Activity

**Introduction**

The genus *Phlomis* (Lamiaceae) is represented by 34 species in the Flora of Turkey (Huber-Morath, 1982). Some members of the genus possess medicinal properties (Saracoğlu et al., 1995) and are used as tonics and stimulants in the Anatolian 
folk medicine (Baytop, 1999). As part of our ongoing search on secondary metabolites of Turkish 
*Phlomis* species (Başaran et al., 1991; Çalış et al., 1990a,b, 1991; Ersöz et al., 2001a–c, 2002a–c; Harput et al., 1998, 1999; Saracoğlu et al., 1995, 1997, 1998, 2002), we studied the Turkish endemic, 
*Phlomis physocalyx* Hub.-Mor. This plant is an eglandular herb to 30 cm, growing on steppes and 
calcireous hills at elevations of 950–1730 m in Inner Anatolia (Huber-Morath, 1982). It was found 
that the methanolic extract of aerial parts of the title plant exhibits antioxidant effects, based on 
the scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical. The present paper 
deals with the isolation and structure elucidation of the new phenylethanoid glycoside, physocalycoside (2), as well as of six known compounds, the iridoid glucoside, lamiide (1) and the phenylethanaoid glycosides, wiedemannioside C (3), verbascoside (= acteoside) (4), leucosceptoside A (5), martynoside (6), and forsythoside B (7). Antioxidant 
activity of the phenylethanoid glycosides (2–7) is also presented.

**Material and Methods**

**General experimental procedures**

Optical rotations were measured on a Rudolph 
autopol IV Polarimeter using a sodium lamp operating at 589 nm. UV (MeOH) spectra were re-
corded on a Shimadzu UV-160A spectrophotometer. IR spectra (KBr) were determined on a 
Perkin-Elmer 2000 FTIR spectrometer. NMR measurements in CD3OD were performed on a 
Bruker AMX 300 (1H: 300.13 and 13C: 75.5 MHz) and Varian Unity 500 (1H: 500 and 13C: 125 MHz) 
spectrometers. Positive mode HR-MALDI MS data were taken on a Ionspec.-Ultima-FTMS in-
strument with DBH as matrix substance. ESIMS were recorded in the positive ion modes on a Fin-
nigan TSQ 7000 spectrometer. For open-column chromatography (CC), Polyamide (Polyamid-MN-
Polyamid SC-6, Machery-Nagel, Düren), Kiesel 
gel 60 (0.063–0.200 mm, Merck) and Sephadex 
LH-20 were used. Low-pressure liquid chromatog-
raphy (RP-8 LPLC) was performed on Lobar pre-
packed columns (310–25 and 240–10), LiChro-
prep RP-8 (40–63 µm, Merck). Analytical and 
preparative-TLC were carried on pre-coated Kie-
selgel 60 F254 aluminum sheets (Merck). Com-
ounds were detected by UV and 1% vanillin/
H₂SO₄ followed by heating at 105 °C for 1–2 min. 
For radical scavenging assay, DPPH (= 2,2-diphe-
nyl-1-picrylhydrazyl, Fluka) was used. Absorbance 
at 517 nm was measured with an automated micro-
plate reader (Bio-Tek Instruments Inc.) spectrophotometer.

**Plant material**

*Phlomis physocalyx* Hub.-Mor. (Lamiaceae) 
was collected in July 2001 at Sivas (1550 m) near 
Gürün-Kangal-Kocakurt crossing, Inner Anatolia, 
Turkey. Voucher specimens have been deposited in 
The Herbarium of the Biology Department, Fac-
ulty of Science, Hacettepe University, Ankara, 
Turkey (AAD 9555).

**Extraction and isolation**

The air-dried and powdered aerial parts of 
*P. physocalyx* (600 g) were extracted with MeOH 
(3×2500 ml) at 40 °C. After evaporation of 
the combined extracts in vacuo 51.6 g of crude MeOH 
extract was obtained. The crude extract was 
dissolved in water (250 ml) and the water-insoluble 
material was removed by filtration. The filtrate 
was then extracted successively with n-BuOH 
(3×150 ml) to obtain the n-BuOH fraction 
(29.4 g). An aliquot of the n-BuOH fraction (10 g) 
was separated on a polyamide column (100 g). 
Elution with H₂O (500 ml) and gradient MeOH–
H₂O (25 to 100%, each 500 ml) mixtures afforded 
8 main fractions (A–H). Fraction A (930 mg) was 
purified on a Lobar RP-8 column (310–25) using 
increasing amounts of MeOH in H₂O (0 to 65%) 
to afford fractions A₁ and A₂. Fraction A₂ (99 mg) 
was separated on a Lobar RP-8 column (240–10) 
using 5 to 25% MeOH in H₂O as eluent to give 
lamide (I, 75 mg). Fr. B (420 mg) was fractionated 
over a Si gel (30 g) column eluting with CHCl₃–
MeOH–H₂O (80:20:0 to 80:20:3 v/v/v) mixture 
to yield six fractions (frs. B₁–B₆). Fraction B₅ 
(74.4 mg) was purified on a Lobar column (240–10) 
employing a 5% stepwise gradient elution of 
MeOH in H₂O (0 to 60%) to get a mixture of 
compounds 2 and 3 (25 mg). Further fractionation 
of this mixture by LPLC with a 5% stepwise gradi-
ent mixture of MeOH in H₂O (5 to 45%) afforded 
physocalyoside (2, 10 mg) and wiedemannioside C 
(3, 5 mg). Fr. D (117 mg) yielded fractions D₁–D₃ 
after separation on a Si gel (20 g) column using 
CHCl₃–MeOH–H₂O (80:20:1 to 70:30:3 v/v/v) 
mixture. Fr. D₂ (33.6 mg) was rechromatographed 
on Si gel (10 g) with CHCl₃–MeOH (98:2 to 90:10 
v/v) mixture to yield martynoside (6, 3 mg). 
An aliquot (340 mg) of fraction E (1.52 g) was sub-
jected to Si gel CC (30 g) with CHCl₃–MeOH–
H₂O (80:20:1 to 60:40:4 v/v/v) mixture to obtain 
nine fractions E₁–E₉. Fraction E₇ (64.4 mg) was 
purified by Sephadex CC (MeOH) to give leuco-
sceptoside A (5, 10 mg). 540 mg of fraction F 
(1.52 g) was subjected to Si gel CC (30 g) employ-
ning CHCl₃–MeOH–H₂O (80:20:3 v/v/v) mixtures 
to give six fractions (frs. F₁–F₆). Fr. F₄ 
yielded verbascoside (4, 28 mg) after purification 
on a Lobar column (240–10) employing 5% step-
wise gradient elution of MeOH in H₂O (25 to 
60%). Fraction F₅ was likely separated on a Lobar 
column (240–10) with a 5% stepwise gradient elu-
tion of MeOH in H₂O (15 to 60%) to give a mix-
ture (40 mg) of verbascoside (4) and forsythoside 
B (7). This mixture was then separated by PTLC 
with a CHCl₃–MeOH–H₂O (61:32:7 v/v/v) 
solvent system to yield verbascoside (4, 13.2 mg) 
and forsythoside B (7, 23.9 mg).

**Physocalyoside (2):** Amorphous yellowish 
powder; [α]₀ᵢ²⁰ = 43.9° (c = 0.1, MeOH); positive-ion 
HR-MALDI-MS m/z: calcd. for C₄₃H₆₀O₂₄Na: 983.3473. 
Found: 983.3358; UV λ max (MeOH, nm) 219, 237, 287 (sh), 328; 
v max (KBr, cm⁻¹) 3400 (OH), 1699 (C=O), 1630 (olefinic C=C), 1605 
and 1508 (arom. ring); ¹H NMR (CD₃OD, 500 MHz): Table I; 
¹³C NMR (CD₃OD, 125 MHz): Table I.

**Reduction of DPPH radical:** Methanolic solutions 
(0.1%) of the phenylethanoid glycosides (2–7) were 
chromatographed on a Si gel TLC plate using 
CHCl₃–MeOH–H₂O (61:32:7) mixture as solvent 
system. After drying, TLC plates were sprayed with 
a 0.2% DPPH (Fluka) solution in MeOH. Com-
ounds showing yellow-on-purple spot were re-
garded as antioxidant (Cuendet et al., 1997).

**DPPH assay in vitro:** The radical scavenging ac-
tivity of the phenylethanoid glycosides was exam-
ined with the DPPH radical, as described by Cuen-
det et al. (2001). Ascorbic acid was used as control. 
50 µl 0.02% DPPH in MeOH, 200 µl MeOH and
30 µl of sample solution in MeOH were mixed in a 96-well plate. After incubation of the plate for 30 min at room temperature, optical density was measured at 517 nm and the inhibition percentage (%) of the radical scavenging activity was calculated using the following equation:

\[
\text{Inhibition (\%)} = \frac{A_0 - A_s}{A_0} \times 100
\]

where \(A_0\) is the absorbance of the control and \(A_s\) is absorbance of the sample at 517 nm. IC$_{50}$ was determined as the amount of the sample (µM) reducing the absorbance by 50%.

**Results and Discussion**

The methanolic extract of aeriale parts of *P. physocalyx* was suspended in water and partitioned with *n*-BuOH. Chromatographic separations of the *n*-BuOH extract by polyamide column chromatography followed by low-pressure liquid chromatography and column chromatography on Si gel and Sephadex LH-20 resulted in the isolation of compounds 1–7 (Fig. 1).

The structures of compounds 1 and 3–7 were established as the known lamiide (Bianco et al., 1977; Assaad et al., 1992), wiedemannioside C (Abou Gazar et al., 2003), verbascoside (= acteoside; Sticher and Lahloub, 1982), leucosceptoside A (Miyase et al., 1982), martynoside (Çalıs et al., 1984), and forsythoside B (Endo et al., 1982), respectively, by comparison of their 1H and 13C NMR spectroscopic properties and ESI-MS data with those reported in the literature.

Physocalycoside (2) was obtained as a yellowish amorphous powder, \([\alpha]_D^{20} = -43.9^\circ\) (c = 0.1, MeOH). The positive-ion HR-MALDI mass spectrum exhibited molecular ion peaks [M+Na]$^+$ and [M+K]$^+$ at \(m/z\) 983 and 999, respectively, consistent with the molecular formula C$_{43}$H$_{60}$O$_{24}$. UV absorption bands at \(\lambda_{max}\) 219(sh), 237, 287(sh), and 237 indicated the polyphenolic nature of 2. The IR spectrum showed absorption bands due to hydroxyl (3400 cm$^{-1}$), \(\alpha,\beta\)-unsaturated ester carbonyl (1699 cm$^{-1}$), olefinic double bond (1630 cm$^{-1}$), and aromatic rings (1605, 1508 cm$^{-1}$). The 13C NMR and DEPT data (see Table I) exhibited 43 carbon resonances, in which 4 methyl, 4 methylene, 28 methine, and 7 quaternary carbon resonances could be assigned for 2. The 1H NMR spectrum of compound 2 (see Table I) exhibited characteristic signals arising from \(E\)-ferulic acid and 3-hydroxy-4-methoxyphenyl-ethanol moieties: six aromatic proton signals (2 × ABX systems, in the region of \(\delta_H\) 7.20–6.76), two \(trans\)-olefinic proton signals (AB system, \(\delta_H\) 7.67, \(d, J_{AB} = 15.8 \text{ Hz and } 6.38, d, J_{AB} = 15.8 \text{ Hz}\)), and \(\beta\)-methylene (\(\delta_H\) 2.88, 2H, \(t, J = 7.6 \text{ Hz}\)) proton signals together with two non-equivalent proton signals (\(\delta_H\) 4.08, \(m\) and 3.77, overlapped) attributed to the side-chain of the phenethyl alcohol moiety. Additionally, four anemic proton resonances at \(\delta_H\) 5.36 (\(d, J = 1.7 \text{ Hz, } H-1''\) of an \(\alpha\)-rhamnose), 4.90 (overlapped, \(H-1'''\) of an \(\alpha\)-rhamnose), 4.41 (\(d, J = 7.8 \text{ Hz, } H-1'\) of a \(\beta\)-glucose), and 4.28 (\(d, J = 7.8 \text{ Hz, } H-1'''\) of a \(\beta\)-glucose) indicated the tetracygosidic nature of 2. The secondary methyl signals appeared at \(\delta_H\) 1.07 (\(d, J = 6.3 \text{ Hz, } H-6''\)) and 1.28 (\(d, J = 6.3 \text{ Hz, } H-6'''\)) supported the presence of two rhamnose moieties in 2. Moreover, the 13C NMR data confirmed the tetracygosidic sugar chain, exhibiting four anemic carbon resonances at \(\delta_C\) 104.7 (C-1$'''$ of a \(\beta\)-glucose), 104.2 (C-1$'\)$ of a \(\beta\)-glucose), 103.8 (C-1$'''$ of an \(\alpha\)-rhamnose) and 101.7 (C-1$'\)$ of an \(\alpha\)-rhamnose), which showed correlations with the anemic protons of the related sugar units. The complete assignments of all proton and carbon resonances were based on the results of 1H-1H COSY, 1H-13C HSQC and HMBC experiments. The feru-
Table I. $^{13}$C and $^1$H NMR (CD$_3$OD, $^{13}$C: 125 MHz and $^1$H: 500 MHz) data and HMBC correlations for 2*.

<table>
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<tr>
<th>C/H Atom</th>
<th>dC ppm**</th>
<th>DEPT</th>
<th>dH ppm, J [Hz]</th>
<th>HMBC (C→H)</th>
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</tr>
<tr>
<td>1</td>
<td>131.6 s</td>
<td>C</td>
<td></td>
<td>H-2, H-6</td>
</tr>
<tr>
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<td>117.1 d</td>
<td>CH</td>
<td>6.76 d (2.2)</td>
<td>H-6</td>
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<td>3</td>
<td>147.6 s</td>
<td>C</td>
<td></td>
<td>H-5</td>
</tr>
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<td>4</td>
<td>147.8 s</td>
<td>C</td>
<td></td>
<td>H-2, H-6, 4-OMe</td>
</tr>
<tr>
<td>5</td>
<td>113.7 d</td>
<td>CH</td>
<td>6.81 d (8.2)</td>
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<tr>
<td>6</td>
<td>121.2 d</td>
<td>CH</td>
<td>6.69 dd (8.2/2.2)</td>
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<tr>
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<td>4.08 m/3.77</td>
<td>H-1', H-β</td>
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<tr>
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<td>72.3 t</td>
<td>CH$_2$</td>
<td>2.88 t (7.6)</td>
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<tr>
<td>4-OMe</td>
<td>56.4 q</td>
<td>CH$_3$</td>
<td>3.85 s</td>
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<td>Glucose</td>
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</tr>
<tr>
<td>1'</td>
<td>104.2 d</td>
<td>CH</td>
<td>4.41 d (7.8)</td>
<td>H-2'</td>
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<td>2'</td>
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<td>3.39 dd (7.8/9.0)</td>
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<td>3.78 t</td>
<td>H-1', H-2', H-4'</td>
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<tr>
<td>6'</td>
<td>69.4 t</td>
<td>CH$_2$</td>
<td>3.95'/3.64'</td>
<td>H-1''</td>
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<tr>
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<td></td>
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<tr>
<td>1''</td>
<td>101.7 d</td>
<td>CH</td>
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<td>H-1''</td>
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<tr>
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<td>CH</td>
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<td>3.32 t</td>
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<td>62.6 t</td>
<td>CH$_2$</td>
<td>3.84'/3.63'</td>
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<td>Rhamnose (→C-2')</td>
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<td>1''''''</td>
<td>127.6 s</td>
<td>C</td>
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<td>H-β', H-5'''</td>
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<tr>
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<tr>
<td>3''''''</td>
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<td>C</td>
<td></td>
<td>H-2'''', H-5'''', 3'''', 4'''', OMe</td>
</tr>
<tr>
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<td>3'''', OMe</td>
<td>56.4 q</td>
<td>CH$_3$</td>
<td>3.89 s</td>
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</table>

* All $^1$H and $^{13}$C assignments are based on 2D NMR (COSY, HSQC and HMBC) experiments.
** Multiplicities are based on DEPT-135 experiment.
† Signal patterns are unclear due to overlapping.

The feruloyl group was supposed to be positioned at C-4' of the core glucose due to the strong deshielding of the H-4' resonance of the glucose unit (δ$_H$ 5.01, $t$, $J$ = 9.0 Hz). This assumption was supported by the heteronuclear long-range copuling observed between the carbonyl carbon resonance (δ$_C$ 168.4) of the acyl moiety and H-4' (δ$_H$ 5.01). On the other hand, a HMBC cross-peak observed from C-α carbon reso-
nance ($\delta_C$ 72.3) of the phenethyl alcohol unit to the anomeric proton of the core glucose ($\delta_H$ 4.41, H-1’) indicated the attachment of the core glucose to be C-α carbon atom of the aglycone. The structure of the tetrascarharide unit was elucidated by means of DQF-COSY and HSQC experiments. Although, the highly deshielded carbon signals arising from the core glucose and the first rhamnose units suggested that the glucose unit to be glycosylied at C-3’ ($\delta_C$ 81.6) and C-6’ ($\delta_C$ 69.4), whereas the rhamnose unit at C-2” ($\delta_C$ 80.2), however, a prominent HMBC experiment allowed us to assign unambiguously all the interglycosidic connectivities of the sugar sequence. Thus, the correlations were observed between C-3’ ($\delta_C$ 81.6) of the core glucose and H-1” ($\delta_H$ 5.36) of the first rhamnose, C-6’ ($\delta_C$ 69.4) of the core glucose and H-1” ($\delta_H$ 4.28) of the second glucose, as well as C-2” ($\delta_C$ 80.2) of the first rhamnose and H-1” ($\delta_H$ 4.90) of the second rhamnose moiety. Some significant long-range correlations confirming the proposed structure were given in Fig. 2. Thus, on the basis of its NMR data, the structure of compound 2 was established as 3-hydroxy-4-methoxy-β-phenylethoxy-O-[α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→3)]-4-O-feruloyl-[β-D-glucopyranosyl-(1→6)]-β-D-glucopyranoside for which the trivial name physocalycoside is proposed.

In recent years papers on the isolation and characterization of numerous mono-, di-, and trisaccharide phenylethanoids have been published. As far as we know, till now only four tetraglycosidic phenylethanoids, magnolioside C (Hasegawa et al., 1988), ballotetroside (Seidel et al., 1997), trichosan-thoside B (Çalış et al., 1999), and marrubioside (Sahpaz et al., 2002) have been described. Therefore, physocalycoside (2) appears as the fifth representative of this class. On the other hand, all previously isolated tetrasaccharidic glycosides are caffeic acid esters and contain a 3,4-dihydroxyphenethyl alcohol moiety, while, physocalycoside (2) is a ferulic acid ester and contains a 3-hydroxy-4-methoxyphenethyl alcohol unit as the aglycone.

Phenylethanoid glycosides (2–7) were screened for antioxidant activities by a TLC autographic assay with 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (Cuendet et al., 1997; Takao et al., 1994) and showed antioxidant properties based on their ability to scavenge free radicals. Then the free-radical scavenging effects of the phenylethanoid glycosides (2–7), corresponding to the intensity of quenching the DPPH radical were evaluated (Cuendet et al., 2001). Compounds 2–7 exhibited a dose-dependent reduction on DPPH. Results are given in Table II.

Table II. Free radical scavenging effects of the phenylethanoid glycosides 2–7 on DPPH.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration [µM]</th>
<th>IC₅₀ [µM]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>Ascorbic acida</td>
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</tr>
<tr>
<td>2</td>
<td>2.30</td>
<td>16.09</td>
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<td>17.53</td>
</tr>
<tr>
<td>7</td>
<td>17.53</td>
<td>28.16</td>
</tr>
</tbody>
</table>

a Reference compound.
b % inhibition.

Acknowledgments

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