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Antileishmanial Activities of Dihydrochalcones from *Piper elongatum* and Synthetic Related Compounds. Structural Requirements for Activity

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Abstract—Two dihydrochalcones (**1** and **2**) were isolated from *Piper elongatum* Vahl by activity-guided fractionation against extracellular promastigotes of *Leishmania braziliensis* in vitro. Their structures were elucidated by spectral analysis, including homonuclear and heteronuclear correlation NMR experiments. Derivatives **3**–**7** and 20 synthetic related compounds (**8**–**27**) were also assayed to establish the structural requirements for antileishmanial activity. Compounds **1**–**11** that proved to be more active that ketoconazol, used as positive control, were further assayed against promastigotes of *Leishmania tropica* and *Leishmania infantum*. Compounds **7** and **11**, with a C_6–C_3–C_6 system, proved to be the most promising compounds, with IC_{50} values of 2.98 and 3.65 μg/mL, respectively, and exhibited no toxic effect on macrophages (around 90% viability). Correlation between the molecular structures and antileishmanial activity is discussed in detail.

Introduction

Leishmaniasis is a group of prevalent diseases caused by protozoan parasites belonging to the genus *Leishmania*. This ailment affects some 12 million people in 88 countries with an annual incidence of about 2–3 million; it is also considered that presently some 350 million of people are at risk of infection.[1] Recently, a dramatic increase in the rate of *Leishmania* infections in human immunodeficiency virus patients,[2] together with the development of drug resistance by the parasites,[3] has worsened this problem.

Current therapies for the disease are still inadequate. The recommended standard drugs for treatment are still the pentavalent antimonial drugs Pestostam and Glucantime, despite the requirement of long courses of parenteral administration[4] and increasing levels of resistance.[3] Although alternative drugs or drug formulations have been proved to be effective (e.g., amphotericin B liposomes and paramomycin ointment), they present several drawbacks, such as their very high cost and their scant availability.[4] Therefore, the search for novel, effective and safe drugs for the treatment of the diseases has become a priority.[5]

Recently, a series of synthetic[6] and naturally occurring[7] chalcone derivatives were reported to be potential agents against *Leishmania* in a number of in vitro and in vivo assays. Though a large number of synthetic compounds has been tested, licochalcone A[8] still remains one of the few naturally occurring chalcones under investigation. Thus, oxygenated chalcones, such as licochalcone A, exhibit a strong antileishmanial activity both in vitro and in vivo by interfering with the function of the parasite mitochondria.[9] The parasite fumarate reductase has recently been proposed as the specific target for the action of chalcones.[10] Although the use of chalcones for the treatment of leishmaniasis may result in the suppression of the immune system as an undesirable

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side effect, a study of the structure–activity relationship[6] revealed that it is possible to separate the antileishmanial and antilymphocyte properties of the chalcones. 2′,6′-Dihydroxy-4′-methoxychalcone (DMC) isolated from *Piper aduncum* inflorescences is reported to show a significant activity in vitro against promastigotes and amastigotes of *Leishmania amazonensis*, which was improved by encapsulation in polymeric nanoparticles in vitro and in vivo.[11] However, in contrast to synthetic, semisynthetic or plant-derived chalcones, only a few dihydrochalcones have been investigated.

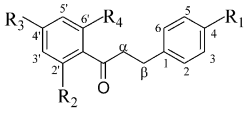
Due to the limited availability of effective pharmaceutical products, most people in areas where leishmaniasis is endemic depend largely on traditional medicine. Besides, traditionally, medicinal plants have already provided valuable leads for potential antiparasitic compounds.[12] In Peru, two forms of leishmaniasis exist, the cutaneous, 'Uta', which predominates in the Andes, and the mucocutaneous form, 'Espundia', which predominates in the jungle, and both are attributed to *Leishmania braziliensis*. The leaves of *Piper elongatum* (Piperaceae), referred to as 'matico' or 'mocco-mocco', are used by the inhabitants of Cusco, Perú, as a powder to heal ulcers produced by the 'Uta'.[13]

To provide a scientific reason for the ethnomedicinal use of *P. elongatum,* and as a part of a program in the search for new antiprotozoal drugs, we carried out an activity-guided fractionation of *P. elongatum* against extracellular promastigotes of *L. braziliensis* in vitro. Two dihydrochalcones (**1** and **2**)[14] were isolated as the active components. A primary screening for in vitro antileishmanial activity of their derivatives **3**–**7** (Fig. 1) and of 20 synthetic related compounds (**8**–**27**) (Fig. 2) were also performed in order to establish the structural requirements for antileishmanial activity. The most active compounds, **1**–**11**, were further assayed against promastigotes of *Leishmania tropica* and *Leishmania infantum*. The structure–activity relationships for antileishmanial activity is discussed in detail.

Results and Discussion

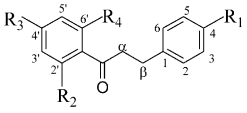
Repeated chromatography on Si gel and Sephadex LH-20 of the EtOH extract of the aerial part of *P. elongatum*, directed by activity-guided fractionation against promastigote forms of *L. braziliensis*, gave the known compounds **1**, 2′,6′-dihydroxy-4′-methoxy-dihydrochalcone,[14] and **2**, 2′,6′,4-trihydroxy-4′-methoxy-dihydrochalcone (asebogenin),[14] as the active compounds, displaying IC_{50} values of 27.04 and 28.47 μg/mL, respectively, slightly higher than that of ketoconazol (IC_{50} 34.89 μg/mL), used as positive control. Although molluscicidal and antimicrobial activities have been previously reported for compounds **1** and **2**,[14] no antiprotozoal activity has been described. Their partial and total acetylated derivatives (**3**–**7**) (Fig. 1) were prepared in order to study the effect of the acetate groups on the activity. The structure of derivatives **3**–**7**, which were not previously described, were determined by spectroscopic data (Tables 1 and 2), including homonuclear and heteronuclear correlation NMR experiments (COSY, ROESY, HSQC and HMBC).

Furthermore, to investigate the relevance of the aromatic rings, and the substituents for the expression of antileishmanial activity, we carried out a primary screening for in vitro activity of a series of synthetic related compounds (**8**–**27**, Fig. 2) against promastigotes of *L. braziliensis*: compounds **8**–**15** having a C_6–C_3–C_6 system (aryl-C_3-aryl) with different substituents; compounds **16**–**18** and **19**–**23**, corresponding to related



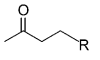
Compound	R_1	R_2	R_3	R_4
1	H	OH	OCH_3	OH
2	OH	OH	OCH_3	OH
3	H	OH	OCH_3	OAc
4	H	OAc	OCH_3	OAc
5	OAc	OH	OCH_3	OH
6	OAc	OH	OCH_3	OAc
7	OAc	OAc	OCH_3	OAc

Figure 1. Naturally dihydrochalcones (**1** and **2**) and its derivatives (**3**–**7**) used in this study.

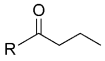


Compound	R_1	R_2	R_3	R_4
8	OAc	OH	OAc	OAc
9	OAc	OAc	OAc	OAc
10	OAc	*O*-tetAcO-β-D-Glu	OAc	OAc
11	H	OAc	*O*-tetAcO-β-D-Glu	OAc
12	OH	*O*-β-D-Glu	OH	OH
13	H	OH	*O*-β-D-Glu	OH
14	OH	OH	OH	OH
15	H	H	H	H

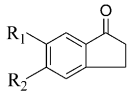
[a] tetra-acetyl-β-D-glucosyl group
[b] β-D-glucosyl group



Compound	R
16	C_6H_5
17	*p*-$OCH_3C_6H_4$
18	*p*-OHC_6H_4



Compound	R
19	C_6H_5
20	2,4-dihydroxy-C_6H_3
21	2,5-dihydroxy-C_6H_3
22	*p*-$OCH_3C_6H_4$
23	3,5-bistrifluoro-methyl-C_6H_3



Compound	R_1	R_2
24	H	H
25	OCH_3	H
26	H	OCH_3
27	OCH_3	OCH_3

Figure 2. Chemical structures of synthetic compounds used in this study.

Table 1. ^{1}H NMR (400 MHz) data (δ, $CDCl_3$, *J* are given in Hz in parentheses) of **1–7**

Proton	1	2	3	4	5	6	7
H-2, H-6	7.19–7.31 m[a]	7.00 d (8.3)	7.21–7.32 m[a]	7.20–7.28 m[a]	7.24 d (8.5)	7.21 d (11.0)	7.25 d (10.0)
H-3, H-5	7.19–7.31 m[a]	6.72 d (8.3)	7.21–7.32 m[a]	7.20–7.28 m[a]	7.00 d (8.5)	7.02 d (11.0)	7.01 d (10.0)
H-4	7.19–7.31 m[a]		7.21–7.32 m[a]	7.20–7.28 m[a]			
H-α	3.39 t (5.6)	3.36 t (5.7)	3.21 t (7.4)	3.05 t (5.3)	3.35 t (7.3)	3.18 t (7.1)	3.78 t (7.6)
H-β	3.02 t (5.6)	2.86 t (5.7)	3.02 t (7.4)	2.97 t (5.3)	3.00 t (7.3)	3.00 t (7.1)	3.39 t (7.6)
H-3′	5.93 s[a]	5.87 s[a]	6.36 d (2.5)	6.57 s[a]	5.92 s[a]	6.35 d (2.5)	6.52 s[a]
H-5′	5.93 s[a]	5.87 s[a]	6.17 d (2.5)	6.57 s[a]	5.92 s[a]	6.16 d (2.5)	6.52 s[a]
OCH_3	3.79 s	3.58 s	3.83 s	3.80 s	3.78 s	3.82 s	3.77 s
Ac-4					2.30 s	2.29 s	2.29 s
Ac-2′				2.16 s[a]			2.15 s[a]
Ac-6′			2.20 s	2.16 s[a]		2.21 s	2.15 s[a]

[a]Overlapping signals.

Table 2. ^{13}C NMR (100 MHz) data (δ, $CDCl_3$) of **1–7**[a]

Position	1	2	3	4	5	6	7
C-1	141.5 s	132.6 s	140.9 s	141.4 s	139.3 s	138.5 s	136.9 s
C-2	128.3 d[b]	129.4 d[b]	128.1 d[b]	128.4 d[b]	129.5 d[b]	129.2 d[b]	129.5 d[b]
C-3	128.5 d[c]	115.5 d[c]	128.5 d[c]	128.5 d[c]	121.4 d[c]	121.6 d[c]	121.7 d[c]
C-4	125.9 d	155.8 s	126.1 d	126.1 d	148.8 s	149.1 s	149.2 s
C-5	128.5 d[c]	115.5 d[c]	128.5 d[c]	128.5 d[c]	121.4 d[c]	121.6 d[c]	121.7 d[c]
C-6	128.3 d[b]	129.4 d[b]	128.1 d[b]	128.4 d[b]	129.5 d[b]	129.2 d[b]	129.5 d[b]
C-α	45.5 t	46.1 t	44.7 t	45.4 t	45.5 t	44.6 t	32.0 t
C-β	30.5 t	30.0 t	29.8 t	29.7 t	29.9 t	29.7 t	29.7 t
C=O	204.5 s	205.4 s	202.6 s	199.2 s	204.3 s	202.4 s	201.0 s
C-1′	104.7 s	105.5 s	108.2 s	120.6 s	104.8 s	108.3 s	121.4 s
C-2′	165.5 s[d]	165.6 s[d]	166.5 s	149.4 s[d]	165.6 s[d]	166.6 s	150.2 s[d]
C-3′	94.3 d[e]	93.3 d[e]	99.2 d	106.7 d[e]	94.4 d[e]	103.2 d	107.0 d[e]
C-4′	165.5 s[d]	164.9 s	164.8 s	161.2 s	163.3 s	165.5 s	160.3 s
C-5′	94.3 d[e]	93.3 d[e]	103.1 d	106.7 d[e]	94.4 d[e]	99.3 d	107.0 d[e]
C-6′	165.5 s[d]	165.6 s[d]	152.4 s	149.4 s[d]	165.6 s[d]	152.4 s	150.2 s[d]
OCH_3	55.4 c	55.1 c	55.7 c	55.7 c	55.5 c	55.7 c	55.7 c
Ac-4					21.1 c	21.1 c	21.1 c
					169.9 s	168.5 s	168.2 s
Ac-2′				20.8 c[f]			20.8 c
				168.8 s[g]			169.3 s
Ac-6′			21.4 c	20.8c[f]		21.5 c	20.4 c
			168.5 s	168.8 s[g]		169.6 s	169.5 s

[a]Values are based on DEPT, HSQC and HMBC experiments.
[b–g]Overlapping signals.

compounds with $CH_3COCH_2CH_2R$ and $RCOCH_2CH_3$ moieties, respectively, and a series of synthetic compounds with a benzocyclopentanone system (**24–27**) and different substituents on the aromatic ring.

In comparison to ketoconazol as reference drug, compounds **1–11**, which are all C_6–C_3–C_6, revealed pronounced effects against promastigotes of *L. braziliensis* (Table 3), while compounds **12–27** were inactive ($IC_{50} > 100$ μg/mL). Analysis of the in vitro antileishmanial activity of **1–7** indicates that the B-ring substitution did not play a major role for the antileishmanial activity, since compounds **1** and **2** showed similar activity, although the toxicity on macrophages is 5-fold higher for **2**. Substitution of hydroxyl groups for acetate groups not only increased the antileishmanial activity but also decreased the cytotoxicity on macrophages. Thus, activity of the monoacetylated, diacetylated, and triacetylated derivatives, **5**, **6** and **7**, are around 2-, 7- and 8-fold, respectively, higher than that of the parent **2**, but also the cytotoxity was strongly reduced. Besides, compounds **8–10** showed a high antileishmanial activity ($IC_{50} < 1$ μg/mL), but also correlated with a high toxicity on macrophages. On the other hand, compound **11** with an IC_{50} of 3.65 μg/mL showed no toxic effect on macrophages (91% viability at 3.6 μg/mL).

Analysis of their structures allowed us to conclude that the substitution of the methoxy group at C-4′ by an acetoxy group increased both the activity and toxicity (**6** and **7** vs **8** and **9**). However, replacement of the methoxy group at C-4′ by an *O*-tetra-acetyl-β-D-glucosyl group increased the activity almost 3-fold without increasing cytotoxity (**4** vs **11**). These results confirm the 2D-QSAR analysis from Liljefors et al.,[6] which suggests that the C-4′ position is highly relevant for the biological activity. On the other hand, the antileishmanial activity and the cytotoxicity remained unaffected by replacement of the hydroxy or acetoxy groups at C-2′ by an *O*-tetra-acetyl-β-D-glucosyl group (**8** and **9** vs **10**).

To compare the differences in compound susceptibility among parasite species, we assayed compounds **1–11** on *L. tropica* and *L. infantum* (Table 3). Among the three parasite species, *L. braziliensis* proved to be the most susceptible line to the compounds assayed, with **7** exhibiting the highest relative activity. Compounds **6** and **7** were the most potent against *L. infantum* (IC_{50} 9.11 μg/mL) and showed low toxicity (94 and 90% viability at 5.0 and 2.4 μg/mL, respectively), while **4** was the most interesting compound against *L. tropica* with an IC_{50} of 6.69 μg/mL and 91% viability at 10.0 μg/mL.

Regarding molecular structure and function, these in vitro studies showed that the antileishmanial activity of the potentially active compounds (**1–11**) appears to have structural features in common since all contain a C_6–C_3–C_6 system, which seems to be a requirement for antileishmanial activity. In addition, these results, in accordance with modelling studies and the effects of chalcones on promastigotes of *Leishmania major* reported by Christensen et al.,[15] support the fact that the pharmacophore is the two aromatic rings, and the propanone chain just functions as a spacer, and that the ability to inhibit parasite growth apparently depends on the presence and ratio of lipophilic/hydrophilic substituents at both aromatic rings, as concluded by Kayser

Table 3. Antileishmanial activity of natural compounds **1** and **2**, derivatives **3–7**, and synthetic related compounds **8–11**

Compd	IC_{50} (μg/mL)			Cytotoxicity[a]	
	L. braziliensis	*L. tropica*	*L. infantum*	(μg/mL)	% Viability
1	27.04	21.29	15.30	20.0	40
2	28.47	3.82	6.35	20.0	8
3	13.97	12.10	9.13	15.0	84
4	11.89	6.69	20.36	10.0	91
5	12.53	13.67	10.03	10.0	35
6	4.08	11.17	9.11	5.0	94
7	2.98	9.29	9.11	2.4	90
8	0.66	9.30	9.11	1.0	65
9	0.44	8.02	12.62	0.5	58
10	0.79	9.30	14.10	0.8	50
11	3.65	11.60	11.60	3.6	91
Ketoconazol	34.89	41.17	21.32		

[a]Cytotoxicity to murine macrophages J774.

et al.[7] On the other hand, the relevance of the acetate groups to the antileishmanial activity of dihydrochalcones, which markedly altered activity but also reduced the inherent toxicity, represents an advance in the search for novel antiprotozoal agents from natural sources.

Earlier work and the results that we report in this paper reinforce the interest for C_6–C_3–C_6 compounds, chalcones, dihydrochalcones, and related compounds as potential drugs against the leishmaniasis diseases at a time when there is an urgent need for leads on new innovative drugs. This study also provides a rational explanation for the use of *P. elongatum* in antiparasitic ethnomedicine.

Experimental

General

Optical rotations were measured on a Perkin-Elmer 241 automatic polarimeter and the $[\alpha]_D$ are given in 10^{-1} deg cm^2 g^{-1}. IR spectra were recorded in $CHCl_3$ on a Bruker IFS 55 spectrophotometer and UV spectra were collected in absolute EtOH on a Jasco V-560. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance 400 NMR spectrometer at 400 MHz and 100 MHz, respectively. EI–MS and HR-EI–MS were recorded on a Micromass Autospec spectrometer. TLC 1500/LS 25 Schleicher and Schüell foils were used for thin-layer chromatography. Silica gel (particle size 40–63 μM, Merck) and Sephadex LH-20 (Pharmacia), were used for column chromatography.

Plant material

P. elongatum was collected in Valle de Urubamba, Microcuenca de Cusichaca, Cusco, Peru, in February 1992. A voucher specimen (no. CUZ-028801 A. Tupayachi 2103) is deposited in the Vargas Herbarium, Faculty of Biology, Universidad Nacional de San Antonio Abad del Cusco, Peru.

Bioassays

Parasite strain. *L. braziliensis* strain MHOM/PE195/LQ7 was originally isolated at La Convención Province, Cusco, Peru, and identified by isoenzyme analysis. *L. tropica* (MON 58/LEM 2578) and *L. infantum* (MON 183/LEM 2592) strains were also used in this study.

In vitro effect on the promastigote forms of *Leishmania* spp. For the in vitro studies samples were dissolved in dimethyl sulfoxide (DMSO) and further dilutions were made with RPMI 1640 medium. Promastigotes were adapted for growth at 22 °C in RPMI 1640 modified medium (Gibco) and supplemented with 20% heat-inactivated fetal bovine serum.[16] Logarithmic phase cultures were used for experimental purposes, and the in vitro susceptibility assay was performed in sterilized 24-well microtiter plates (Corning™). To these wells were added 2.5×10^4/well parasites, and the drug concentration to be tested. The final volume was 500 μL in each well.

Growth of promastigotes was monitored after 48 h by counting the number of motile promastigotes microscopically in a Neubauer camera. Percentage of inhibition and 50% inhibitory concentrations (IC_{50}) were calculated by linear regression analysis with 95% confidence limits. Tests were performed at least in triplicate on three different days in order to verify the results. Ketoconazol was used as positive control (IC_{50} 34.8 μg/mL).

Cytotoxic assays. Murine macrophages J-774 (ATCC TIB-67) cell line were grown in RPMI 1640 (GIBCO BRL) medium supplemented with 10% SBFI, and maintained at 37 °C in 5% CO_2 and 90% humidity. Macrophages were resuspended in growth medium at a final concentration of 1×16^6 cells/mL, placed in a 96-well culture microtiter plate (100 μL/well), and incubated with the test drug for 48 h. Cytotoxicity was assessed using the colorimetric MTT [3-(3,4-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] reduction assay,[17] and cytotoxic effects were expressed as a percentage in cell viability at a concentration of the

compound tested close to the IC_{50} value obtained against *L. braziliensis*.

Extraction and isolation

The aerial part of *P. elongatum* (460 g) was extracted with ethanol in a Soxhlet apparatus, yielding 88 g of residue which was chromatographed by dry flash chromatography on Si gel, using *n*-hexane–EtOAc mixtures of increasing polarity to afford 35×100 mL frs, which were reduced to 5 frs by TLC: A (0–5%, *n*-hexane–EtOAc), B (5–15%), C (15–25%), D (25–45%), and E (45–100%). The *n*-hexane–EtOAc (1:1) eluting fraction was then chromatographed on Sephadex LH-20 (*n*-hexane–$CHCl_3$–MeOH, 2:1:1) and silica gel (*n*-hexane–1,4-dioxan, 3:2) to yield compounds **1** (38.0 mg) and **2** (16.4 mg).[14]

Acetylation of 1. Acetic anhydride (10 drops) was added to compound **1** (24.0 mg) dissolved in pyridine (five drops), and the mixture left at room temperature for 16 h. EtOH (3×2 mL) was added and carried almost to dryness in a rotavapor, and this process was repeated with $CHCl_3$ (3×2.0 mL), and purified by preparative TLC with a mixture of *n*-hexane–AcOEt (1:1), to give derivatives **3** (8.5 mg) and **4** (12.7 mg).

Compound 3. Lacquer; UV λ_{max} nm: 340, 276; IR ν_{max} cm^{-1}: 3545, 2928, 2853, 1769, 1735, 1610, 1369, 1211, 1154, 837; ^1H NMR δ: see Table 1; ^{13}C NMR δ: see Table 2; EI–MS *m*/*z*%: 314 (M^+, 25), 272 (22), 255 (14), 209 (3), 167 (100), 140 (28), 91 (10); HR-EI–MS: *m*/*z* 314.11505 (calcd for $C_{18}H_{18}O_5$ 314.11542).

Compound 4. Lacquer; UV λ_{max} nm: 340, 269; IR ν_{max} cm^{-1}: 2923, 2853, 1774, 1686, 1617, 1459, 1367, 1182, 1038, 877; ^1H NMR δ: see Table 1; ^{13}C NMR δ: see Table 2; EI–MS *m*/*z*%: 356 (M^+, 6), 314 (21), 296 (12), 272 (50), 255 (27), 209 (29), 167 (100), 140 (56), 91 (28); HR-EI/MS: *m*/*z* 356.12668 (calcd for $C_{20}H_{20}O_6$ 356.12599).

Acetylation of 2. Compound **2** (9.0 mg) was treated under the same conditions as described above, to give derivatives **5** (3.5 mg), **6** (1.9 mg) and **7** (1.5 mg).

Compound 5. Lacquer; UV λ_{max} nm: 338, 285; IR ν_{max} cm^{-1}: 3370, 3310, 2920, 2851, 1754, 1720, 1626, 1593, 1428, 1366, 1216, 1191, 836, 807; ^1H NMR δ: see Table 1; ^{13}C NMR δ: see Table 2; EI–MS *m*/*z*%: 330 (M^+, 5), 285 (50), 242 (8), 209 (4), 167 (100), 150 (14), 140 (2), 135 (11); HR-EI–MS: *m*/*z* 330.10697 (calcd for $C_{18}H_{18}O_6$ 330.11034).

Compound 6. Amorphous solid; UV λ_{max} nm: 339, 277; IR ν_{max} cm^{-1}: 3545, 2924, 2854, 1761, 1623, 1507, 1369, 1187, 910, 832; ^1H NMR δ: see Table 1; ^{13}C NMR δ: see Table 2; EI/MS *m*/*z*%: 372 (M^+, 5), 330 (13), 312 (5), 288 (10), 279 (6), 167 (58), 149 (100), 140 (10), 57 (62); HR-EI–MS: *m*/*z* 372.11883 (calcd for $C_{20}H_{20}O_7$ 372.12090).

Compound 7. Lacquer; UV λ_{max} nm: 339, 279; IR ν_{max} cm^{-1}: 2924, 2853, 1759, 1620, 1507, 1369, 1189, 886, 834; ^1H NMR δ: see Table 1; ^{13}C NMR δ: see Table 2; EI–MS *m*/*z*%: 414 (M^+, 23), 396 (19), 372 (23), 354 (37), 330 (45), 312 (57), 288 (44), 270 (64), 209 (17), 167 (100), 140 (20), 120 (44), 107 (37); HR-EI–MS: *m*/*z* 414.13223 (calcd for $C_{22}H_{22}O_8$ 414.13147).

Synthetic compounds

Compounds **12**–**27** were purchased from Sigma and used without further purification. The derivatives **8**–**11** were synthesized as follows, purified and authenticated by analytical and spectroscopic methods.

Compounds 8 and 9. Compound **14** (23.0 mg) was treated under the conditions already described for the synthesis of compounds **3** and **4**, to give derivatives **8** (2.7 mg) and **9** (17.0 mg).

Compound 10. Acetic anhydride (8 drops) and dimethylaminepyridine (2.0 mg) was added to compound **12** (6.0 mg) dissolved in pyridine (15 drops), and the mixture was treated under the same conditions described above for the acetylation of **1**, to give derivative **10** (4.1 mg).

Compound 11. Compound **13** (8.0 mg) was treated under the conditions already described for the synthesis of **10**, to give derivative **11** (6.8 mg).

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