Antiviral Saponins from *Tieghemella heckelii*

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Arganine C (**1**) and a new saponin, tieghemelin (**2**), were isolated from *Tieghemella heckelii* fruits. Arganine C (**1**) strongly inhibited HIV entry into cells in a cell fusion assay. The less potent tieghemelin (**2**) was converted into arganine C (**1**) by reduction of its ethyl ester with sodium borohydride. The removal of the four-sugar chains from arganine C (**1**) and tieghemelin (**2**) to give 16α-hydroxyprotobassic acid 3-*O*-*β*-D-glucopyranoside (**3**) and 16α-hydroxyprotobassic acid 3-*O*-*β*-D-glucuronopyranoside (**4**), respectively, caused total loss of activity in both cases. Arganine C (**1**) was not significantly cytotoxic to HeLa-CD4$^+$ cells at the level required to reduce the syncytium count to zero, suggesting it to be a promising candidate for further study as an antiviral drug.

Tieghemella heckelii Pierre ex A. Chev. (Sapotaceae) is a large tree found in the West African rain forest. Many parts of the tree are used in traditional medicine.[1] In January and February, the plant blossoms and develops large ovoid fruits. Fatty acids and oily substances have been reported from the dry seeds of the fruit.[2] Upon finding extracts of the fruit to strongly inhibit the growth of HIV,[3] we sought to isolate the active ingredient(s).

Repeated reversed-phase silica gel chromatography of the crude saponin mixture gave two saponins (**1** and **2**) active in the HIV assay and two inactive saponins. The most active compound was shown by negative ion FABMS and ^{1}H, ^{1}H−^{1}H COSY, HSQC, and HMBC NMR spectra to be arganine C (**1**, $C_{58}H_{94}O_{28}$, 0.5% yield w/w from the dried seeds), previously found in the roots of *Crossopteryx febrifuga* (Rubiaceae), a medicinal shrub of South Africa,[4] and in the fruits of *Argania spinosa* (Sapotaceae), a tree endemic to Morocco.[5] The NMR assignments in DMSO-d_6 are given in Table 1. Since NMR parameters of arganine C (**1**) had been previously recorded in CD_3OD,[5] we ran a spectrum of **1** from *T. heckelii* in CD_3OD, which confirmed the identification. From the coupling constants and chemical shifts, the triterpene in DMSO appears to prefer the chair, chair, twist-boat, chair, chair conformation of rings A−E, respectively, usually favored for olean-12-enes.[6]

Adjacent inactive fractions were identified as arganines A (0.2% yield) and D (0.9%),[5] while the other active saponin, which we have named tieghemelin (**2**, $C_{58}H_{92}O_{29}$, 0.5%), was new. Comparison of the spectral data of tieghemelin with the spectra of arganine C (**1**) made it clear that this more polar, more water-soluble new compound was **2**, the analogue of arganine C (**1**) with a glucuronic acid (GlcA) unit replacing glucose (Glc) at C-3. The negative ion FABMS showed peaks that contained the sugar on C-3 of the aglycon at 14 mass units higher in **2**: *m*/*z* 1251 [M − H]$^-$, 1105 [1251 − Rha]$^-$, 973 [1105 − Xyl]$^-$, 827 [973 − Rha]$^-$, and 695 [827 − Ara]$^-$. Significant chemical shift differences were noted between **1** and **2** in the NMR peaks of C-3 and the attached sugar unit, and a ^{13}C NMR peak for GlcA-6 in **2** at δ 172.5 replaced a peak for Glc-6 in **1** at δ 60.8. The large (7.5 Hz) coupling constant between the protons on GlcA-1 and GlcA-2 showed the glucuronic acid unit to be *β*-linked to the aglycon, as in **1**. The large (10 Hz) coupling constant between the protons on GlcA-4 and GlcA-5 confirmed that these protons are both axial.

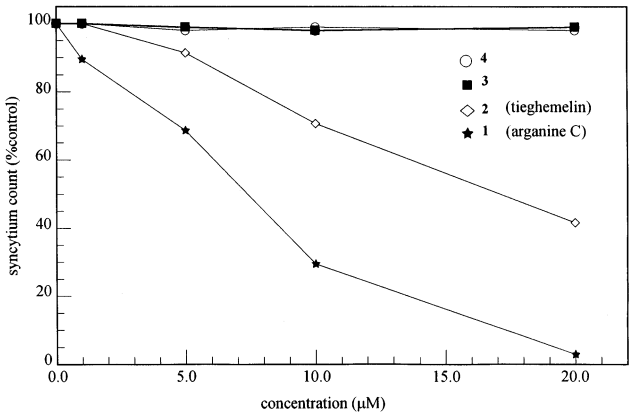

Figure 1. Activity of saponins **1**−**4** in the HIV-based cell−cell fusion assay.

To increase the yield of the more active compound **1** and to avoid the need to separate **1** from **2**, the reduction of **2** to **1** was carried out on the saponin fraction of the extract. This was effected by making the ethyl ester with 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ)[7] and then reducing it with sodium borohydride.

The four-sugar side chains in **1** and **2** were removed with potassium carbonate in methanol to see if the corresponding acids retained biological activity. In the case of arganine C (**1**), the product was the known 16α-hydroxyprotobassic acid 3-*O*-*β*-D-glucopyranoside (**3**);[8] 16α-hydroxyprotobassic acid 3-*O*-*β*-D-glucuronopyranoside (**4**), from **2**, is new. A cell−cell fusion assay (Figure 1) was used to measure the anti-HIV activity. At 20 μM, arganine C (**1**) caused 100%

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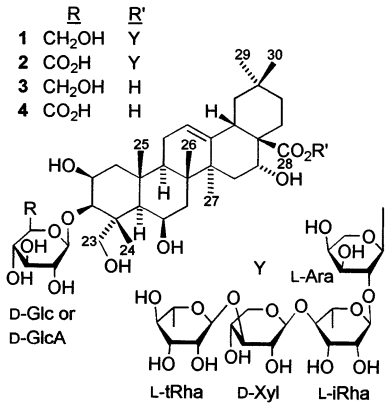
Table 1. NMR Data (δ, DMSO-d_6) of Arganine C (**1**) and Tieghemelin (**2**)[a]

	1			**2**	
position	^{1}H	^{13}C		^{1}H	^{13}C
1	0.99 m, 1.86 m	45.5		0.97 m, 1.84 m	45.5
2	4.14 m	69.0		4.06 m	68.8
3	3.44 m	80.6		3.52 m	77.0
4		42.2			42.0
5	1.16 m	46.8		1.17 m	46.7
6	4.29 m	65.4		4.32 m	65.7
7	1.36 m, 1.55 m	39.8		1.37 m, 1.53 m	39.7
8		37.9			38.0
9	1.48 m	46.7		1.47 m	46.4
10		35.5			35.1
11	1.88 m, 1.91 m	22.7		1.84 m, 1.94 m	22.9
12	5.30 m	122.1		5.30 m	121.8
13		143.0			142.5
14		41.2			41.5
15	1.20 m, 1.71 m	34.1		1.20 m, 1.68 m	34.4
16	4.39 m	71.8		4.39 m	72.0
17		not obs			44.5
18	2.95 dd (13,2)	39.5		2.98 dd (13,2)	39.8
19	2.23 t (13), 0.95 m	45.8		2.23 t (13), 0.96 m	45.8
20		30.1			30.0
21	1.89 m, 1.07 m	34.5		1.87 m, 1.05 m	34.5
22	1.78 m, 1.64 m	30.5		1.76 m, 1.62 m	30.7
23	3.19 m, 3.54 m	63.1		3.11 m, 3.51 m	63.2
24	1.18 s	15.1		1.15 s	15.4
25	1.51 s	17.8		1.50 s	17.6
26	0.90 s	17.2		0.88 s	17.3
27	1.25 s	26.2		1.25 s	26.2
28		not obs			174.5
29	0.83 s	32.5		0.83 s	32.7
30	0.91 s	24.0		0.89 s	24.0
Glc-1	4.28 d (7.2)	103.8	GlcA-1	4.34 d (7.5)	100.9
Glc-2	3.03 m	73.6	GlcA-2	3.03 m	72.6
Glc-3	3.12 m	76.8	GlcA-3	3.11 m	76.4
Glc-4	3.04 m	69.9	GlcA-4	3.02 m	71.7
Glc-5	3.05 m	76.2	GlcA-5	3.22 d (10)	72.9
Glc-6	3.40 m, 3.61 m	60.8	GlcA-6		172.5
Ara-1	5.60 m	91.5		5.59 m	91.2
Ara-2	3.57 m	73.9		3.58 m	73.8
Ara-3	3.78 m	67.0		3.77 m	67.2
Ara-4	3.64 m	63.5		3.65 m	63.7
Ara-5	3.32 m, 3.75 m	60.1		3.32 m, 3.76 m	60.3
iRha-1	4.72 s	99.5		4.74 s	99.1
iRha-2	3.62 m	70.0		3.63 m	70.0
iRha-3	3.64 m	70.4		3.65 m	70.1
iRha-4	3.44 m	80.6		3.43 m	80.4
iRha-5	3.45 m	67.0		3.45 m	66.9
iRha-6	1.12 d (5)	17.1		1.12 d (5)	17.2
Xyl-1	4.44 d (7.5)	104.5		4.44 d (7.5)	104.2
Xyl-2	3.09 m	74.8		3.10 m	74.7
Xyl-3	3.31 m	80.3		3.31 m	80.2
Xyl-4	3.32 m	67.9		3.32 m	67.6
Xyl-5	3.07 m, 3.69 m	65.6		3.07 m, 3.70 m	65.7
tRha-1	4.99 s	100.1		4.99 s	100.0
tRha-2	3.68 m	70.5		3.67 m	70.1
tRha-3	3.48 m	70.5		3.48 m	70.3
tRha-4	3.16 m	71.9		3.16 m	71.7
tRha-5	3.88 dq (9.5,6)	67.5		3.88 dq (9.5,6)	67.6
tRha-6	1.08 d (6)	17.5		1.06 d (6)	17.4

[a] For the aglycon, α protons are listed before β protons.

inhibition of syncytium formation. Tieghemelin (**2**) was about half as effective, whereas the removal of the four-sugar chain gave compounds **3** and **4**, which were devoid of activity. An MTT cytotoxicity assay (data not shown) showed that **1** and **2** were not significantly cytotoxic to HeLa-CD4$^+$ at levels up to 20 μM, the concentration required for arganine C (**1**) to reduce the syncytium count to zero. Together, these bioassays suggest arganine C (**1**) to be a promising candidate for further study as an antiviral drug.



Experimental Section

General Experimental Procedures. Melting points were determined on a Thomas-Hoover capillary apparatus. Optical rotations were measured using a Jasco P-1010 polarimeter. NMR spectra were run on Bruker DRX-500 and DRX-600 instruments in DMSO-d_6. Negative ion FABMS were run on Fisons V.C. ZAP.SPEC., Fisons V.C., and JEOL HX110A spectrometers. The countercurrent chromatography (CCC) apparatus held a pair of nine-layer coil separation columns in series 10 cm from the central axis of a cross-axis coil planet centrifuge (fabricated at the National Institutes of Health). The β value varied from 0.5 at the internal terminal to 0.75 at the external terminal. A Milton Roy Model 196-31 metering pump advanced the solvent. The eluent was monitored at 254 nm with a Model Uvicord S UV detector (LKB Instruments). The revolution speed was adjusted to 650 rpm with a Bodine Electric Company speed controller.

Plant Material. *T. heckelii* Pierre ex A. Chev. (syns. *Dumoria heckelii; Mimusopsis heckelii*) (Sapotaceae), locally called "makoré", was taxonomically authenticated by Prof. Laurent Aké Assi, Centre National Floristique, Université de Cocody, Ivory Coast, with a voucher specimen numbered 9749 deposited at the herbarium of Abidjan Botanic Garden, Université de Cocody. Ripe fruits were collected on August 20, 1999, in the Daloa Forest, Haut Sassandra Region, Ivory Coast.

Extraction and Isolation. The fresh pulp (mesocarp) of the fruits was removed and the hard shell (endocarp) was opened to yield the seed, which was sun-dried and ground in a Wiley mill for extraction. The powdered air-dried seeds (3.4 kg) were macerated with hexane (3 × 4 L) to remove fats. The defatted dry marc was macerated with dichloromethane–methanol (1:1, 3 × 4 L); evaporation gave 134 g of a brown residue, which by TLC was mostly **1** (R_f 0.3) and **2** (R_f 0.08). Purification of a 100 mg sample by countercurrent chromatography (CCC) using the solvent system methyl *tert*-butyl ether–butanol–acetonitrile–0.5% TFA (1:3:1:5) gave 70 mg of a 45:55 mixture of **1** and **2**. This was separated by preparative TLC on silica gel, eluting with chloroform–methanol–0.5% TFA (60:40:5), affording arganine C (**1**, 12 mg, 0.5% yield from the dried seeds),[4,5] tieghemelin (**2**, 12.5 mg, 0.5%), arganine A (4 mg, 0.2%),[5] and arganine D (24 mg, 0.9%).[5]

Arganine C (1): mp 181–183 °C; $[\alpha]^{24}_D$ −37° (*c* 1, MeOH); ^{1}H and ^{13}C NMR, Table 1; negative ion FABMS *m*/*z* 1237 [M − H]$^-$, 1091 [M − Rha − H]$^-$, 681 [M − 2Rha − Xyl − Ara − H]$^-$.

Tieghemelin (2): mp 197–199 °C; $[\alpha]^{24}_D$ −26° (*c* 1, MeOH); ^{1}H and ^{13}C NMR, Table 1; negative ion FABMS *m*/*z* 1251 [M − H]$^-$, 1105 [M − Rha − H]$^-$, 1075 [M − GlcA − H]$^-$, 973 [M − Rha − Xyl − H]$^-$, 827 [M − 2Rha − Xyl − H]$^-$, 695 [M − 2Rha − Xyl − Ara − H]$^-$.

The yield of the more polar compound tieghemelin (**2**) was nearly doubled when the hexane-defatted residue was macerated with water instead of dichloromethane−methanol. Maceration with water (3 × 4 L) and evaporation gave 121 g of a residue. CCC of 100 mg of this residue followed by TLC gave **1** in 0.3% yield and **2** in 0.8% yield.

Conversion of Tieghemelin (2) to Arganine C (1). A solution of the crude saponin fraction from CCC (1.5 g) and EEDQ (150 mg, 1.25 mmol) in DMF (5 mL) was stirred at reflux for 5 h. The solvent was removed using a rotary evaporator, and the brown residue was washed with diethyl ether (5 mL). The residue was dissolved in ethanol (10 mL), and $NaBH_4$ (225 mg) was added with stirring at 0 °C. After 16 h at 25 °C, methanol (10 mL) was added to decompose unreacted $NaBH_4$. The mixture was filtered and the filtrate evaporated to give a brown solid, which was dissolved in 0.2 M ammonium acetate (50 mL) and extracted with butanol (2 × 50 mL). After drying the organic phase over Na_2SO_4, solvent evaporation left 750 mg of white solid in which the **1**:**2** ratio was shown by TLC to be >100:1. CCC as above gave pure arganine C (**1**, 500 mg).

Alkaline Hydrolysis of 1. A solution of **1** (150 mg) and K_2CO_3 (100 mg) in methanol (10 mL) was heated at 80 °C for 24 h. The mixture was neutralized with Dowex 50 × $8H^+$ to pH 7 and filtered. The filtrate was washed with BuOH−H_2O (1:1, 25 mL). The separated BuOH layer (10 mL) was evaporated in a hood, leaving 16α-hydroxyprotobassic acid 3-*O*-*β*-D-glucopyranoside (**3**, 20 mg).[8]

Compound 3: amorphous solid; $[\alpha]^{23}_D$ −0.3° (*c* 0.3, MeOH); ^{1}H NMR *δ* 0.84, 0.88, 0.91, 1.17, 1.26, 1.53 (6 × 3H, s, Me-29, 26, 30, 24, 27, and 25, respectively), 2.25 (1H, t, *J* = 13 Hz, H-19α), 2.93 (1H, dd, *J* = 13, 3 Hz, H-18), 4.15 (1H, m, H-2), 4.30 (1H, d, *J* = 7 Hz, Glc-1), 4.35 (1H, m, H-16), 5.27 (1H, t, *J* = 3 Hz, H-12).

Alkaline Hydrolysis of 2. The above procedure starting with **2** (250 mg) gave 16α-hydroxyprotobassic acid 3-*O*-*β*-D-glucuronopyranoside (**4**, 48 mg).

Compound 4: amorphous solid; $[\alpha]^{23}_D$ −0.1° (*c* 0.25, MeOH); ^{1}H NMR *δ* 0.845, 0.89, 0.92, 1.19, 1.27, 1.54 (6 × 3H, s, Me-29, 26, 30, 24, 27, and 25, respectively), 2.25 (1H, t, *J* = 13.5 Hz, H-19α), 2.94 (1H, dd, *J* = 14, 4 Hz, H-18), 4.15 (1H, m, H-2), 4.30 (1H, d, *J* = 8 Hz, GlcA-1), 4.36 (1H, m, H-16), 5.28 (1H, t, *J* = 3 Hz, H-12); negative ion FABMS *m*/*z* 695 $[M - H]^-$.

Cell−Cell Fusion Assay. The assay evaluating HIV-1 entry involved virus target HeLa-CD4 cells engineered to express the reporter gene, *β*-galactosidase-flanking HIV-LTR promoter, previously described as HeLa-CD4-LTR-*β*-galactosidase cells.[3] The effector cell line used was the highly fusogenic Chinese hamster ovary (CHO-WT) cells expressing wild-type HIV-1 envelope.[9] These cells were grown separately in a 10 cm dish at 37 °C in a 5% CO_2 atmosphere until cells reached 70−80% confluency. CHO-WT cells were seeded at a density of 1.3 × 10^4 cells/well (in triplicate) in a 96-well plate (Costar) with customized-grilled flat bottom wells containing 200 μL of complete GMEM-S medium. Cells were incubated at 37 °C in a 5% CO_2 atmosphere for 1 h, after which various concentrations of test compounds were added, and the plate was incubated for 30−45 min. HeLa-CD4-LTR-*β*-galactosidase cells were then added at a density of 2.6 × 10^4 cells/well. The plate was incubated overnight. X-gal staining of the syncytia was performed as previously described.[3] Cells were gently rinsed and fixed with 0.5% glutaraldehyde made in PBS for 10 min. Cells were then rinsed three times with PBS and stained with X-gal staining solution (0.4% X-gal in DMF, 4 μM potassium ferricyanide, 4 μM potassium ferrocyanide, 2 μM $MgCl_2$). The plate was wrapped in foil and incubated for 1 h, after which cells were rinsed with a solution of 20 mM EDTA made in PBS, and blue syncytia were immediately scored under a light microscope.

MTT Cytotoxicity Assay. HeLa-$CD4^+$ cells were seeded at a density of 3.5 × 10^4 cells/well in a 96-well plate and incubated overnight. The monolayers were washed and incubated with various concentrations of test compounds for 3 days. After this incubation period, 20 μL of 7.5 mg/mL of MTT stock solution was added to each well. The plate was incubated for another 90 min, after which the culture medium was carefully aspirated without disturbing the formazan crystals. DMSO (150 μL) was added to the wells, and the plate was incubated for 30 min on a rotary shaker at 37 °C to dissolve the crystals. Absorbance of the reaction mixtures was read at 570 nm (reference at 690 nm) on a Power Wave 200-microplate scanning spectrophotometer (Bio-Tek Instruments).

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