Croton ruizianus: Platelet Proaggregating Activity of Two New Pregnane Glycosides

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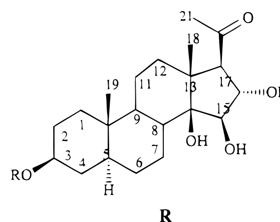
The MeOH extract of the aerial parts of *Croton ruizianus* afforded two new pregnane glycosides **1** and **2**, together with the morphinandienone alkaloids flavinantine (**3**) and *O*-methylflavinantine (**4**). Their structures were elucidated by NMR experiments including $^1H-^1H$ (1D TOCSY and 2D DQF−COSY) and $^1H-^{13}C$ (HSQC, HMBC) spectroscopy. The proaggregating activity of the MeOH extract and the isolates were evaluated. Although the MeOH extract and pregnane glycosides (at different doses) were found to promote platelet aggregation, flavinantine (**3**) and *O*-methylflavinantine (**4**) showed only slight activity. The ability of the MeOH extract and the four compounds to act synergistically with thrombin was also evaluated. All the tested compounds were successful in augmenting the aggregating effect of thrombin, although to different degrees.

The genus *Croton* comprises about 700 species, among which *C. lechleri* L., *C. draconoides*, and *C. erythrochilus* are the most widely used in Amazonian traditional medicine.[1] In particular, the red latex of the above species, known as "*sangre de drago*", is used as a folk remedy for the treatment of wounds, inflammation, and cancer.[1] Among the compounds identified as responsible for the vulnerary effects elicited by the latex are lignans and polyphenols.[2,3]

Continuing our studies on new potentially bioactive compounds from South American medicinal plants, we investigated the MeOH extract of the aerial parts of *Croton ruizianus* Muell.−Arg. (Euphorbiaceae), a small shrub growing in the Central Sierra of Peru, known by the vernacular names of "cabra-cabra", "upalu" and "matarracra". The infusion of the aerial part of this plant is used as vulnerary and antispasmodic in traditional Peruvian medicine.[1] In this paper we report the isolation and structure elucidation of two novel pregnane glycosides (**1** and **2**) by a combination of 1D and 2D NMR techniques, together with two known morphinandienone alkaloids, flavinantine (**3**) and *O*-methylflavinantine (**4**) and the evaluation of the proaggregating activity of the MeOH extract and **1**−**4**.



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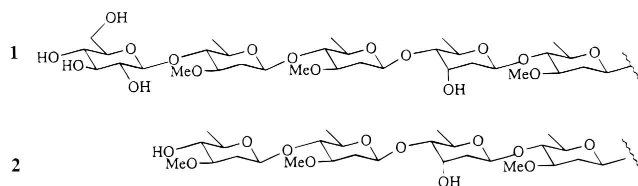

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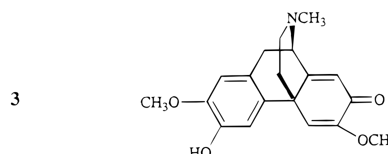

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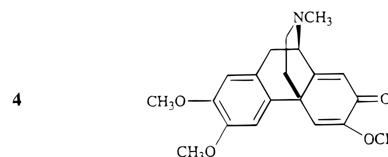

Results and Discussion

Compound **1** had a molecular formula $C_{54}H_{90}O_{22}$, as determined from ^{13}C and ^{13}C DEPT NMR and negative-ion mode FABMS. The FABMS spectrum of **1** showed the $[M - H]^-$ ion at *m*/*z* 1089 and prominent fragments at *m*/*z* 911 $[(M - H) - 178]^-$, *m*/*z* 927 $[(M - H) - 162]^-$ due to the cleavage of a hexose unit with or without the glycosidic oxygen. Also evident were peaks at *m*/*z* 783 and 639, arising from the sequential losses of one (144 mass units) and two (144 × 2 mass units) 3-*O*-methyl-2,6-dideoxyhexopyranose units from the ion at *m*/*z* 927.

The ^{13}C- and DEPT ^{13}C-NMR spectra showed 54 carbon signals, of which 21 were assigned to a pregnane aglycon and 33 to a sugar portion. The ^1H-NMR spectrum of **1** (Table 1) showed three tertiary methyl proton signals of the aglycon moiety at δ 0.84, 0.94, and 2.29 (each s); a proton signal at δ 2.66 (1H, $J = 4.0$ Hz) ascribable to H-17, and three methine proton signals at δ 3.66 (1H, m), 4.18 (1H, d, $J = 5.0$ Hz), and 4.22 (1H, dd, $J = 4.0, 5.0$ Hz). From the DQF−COSY

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Table 1. ^{1}H- and ^{13}C-NMR Data of the Aglycon Moiety of 1 in CD_3OD[a,b]

position	δ_C	δ_H(J_{HH} in Hz)
1	38.2	1.03 m; 1.78 m
2	30.1	1.44 m; 1.85 m
3	78.6	3.66, m
4	35.4	1.24 m; 1.65 m
5	45.4	1.11, m
6	30.0	1.21 m; 1.31 m
7	27.8	1.57 m; 1.95 m
8	41.8	1.63, m
9	50.2	1.07, m
10	36.8	
11	21.7	1.34 m; 1.57 m
12	40.3	1.34 m; 1.68 m
13	47.3	
14	87.5	
15	81.5	4.18, d (5.0)
16	81.9	4.22, dd (4.0, 5.0)
17	70.1	2.66, d (4.0)
18	12.3	0.84, s
19	16.0	0.94, s
20	216.1	
21	32.3	2.29, s

[a] Assignments confirmed by DQF−COSY and HSQC experiments. [b] ^{1}H- and ^{13}C-NMR data of the aglycon moiety of **2** were superimposable to those reported for **1**.

spectrum the sequence −CH(2.66)−CHOH(4.22)CHOH-(4.18)− was deduced. The ^{13}C-NMR spectrum of **1** also suggested that **1** is a pregnane with three secondary alcoholic functions (δ 78.6, 81.5, and 81.9) and one tertiary alcoholic function (δ 87.5) (Table 1). The HSQC correlation of H-3 to C-3 confirmed that a 3β-OH was linked to C-3.[4] Further correlations were observed between C-17, C-15, and C-16 and H-17, H-15, and H-16, respectively. The HMBC (8 Hz) spectrum showed clear long-range correlations between H-15 to C-16 and C-14; H-16 to C-17, C-15, and C-20; and H-17 to C-18, C-21, and C-16. On the basis of these evidences the occurrence of a 14,15,16-trihydroxylated ring D was established.[5] The β-OH configuration of position 15 and the α-OH configuration of position 16 were deduced from the J values of the protons ascribable to C-15 at δ 4.18 (d, J = 5.0 Hz) and to C-16 at δ 4.22 (dd, J = 4.0, 5.0) and on the basis of ^{13}C-NMR data in comparison with literature data.[5] Thus, the structure of the aglycon of **1** was established as 3β,14β,15β,16α-tetrahydroxypregnan-20-one, previously isolated from the plant *Cynanchum boerharifolium* as free aglycone.[6] The glycosidation shifts of aglycon carbon signals were observed at C-2 (ca. −1.2 ppm), C-3 (ca. +8.0 ppm), and C-4 (ca. −4.6 ppm); therefore, the sugar moiety is linked at the C-3 hydroxyl group of **1**. The ^{13}C-NMR spectrum of **1** showed, in addition to the aglycon signals, 33 signals ascribable to sugar portion made up of three 3-*O*-methyl-2,6-dideoxyhexopyranose units, one 2,6-dideoxyhexopyranose unit, and one hexopyranose unit. Five signals were assigned to anomeric carbons (δ 98.4, 99.1, 100.8, 101.8, and 103.9), three to methoxyl groups (δ 57.2 × 2 and 57.9), and four to methyl groups (δ 18.3 and 18.4 × 3). In the ^{1}H-NMR spectra three methoxyl (δ 3.45, 3.46, and 3.49), four secondary methyl (δ 1.27, 1.29, 1.31, and 1.42), and five anomeric proton (δ 4.46, 4.66, 4.68, 4.73, and 5.00) signals also supported the above results. The structure elucidation of the sugar portion was achieved by 1D TOCSY,[7] DQF−COSY,[8] HSQC,[9] and HMBC[10] experiments (Table 2). Because of the selectivity of the multistep coherence transfer, the 1D TOCSY experiment allowed the subspectrum of each monosaccharide unit to be extracted from the crowded overlapped region. The isolated anomeric proton signals resonating at the uncrowded region of the spectrum (between δ 4.46 and 5.00) were the starting point for the 1D TOCSY experiments. Selected 1D TOCSY obtained by irradiating each anomeric proton signal yielded subspectra of each sugar residue with high digital resolution. Each subspectrum contained the scalar-coupled protons within each sugar residue. The 1D TOCSY subspectrum obtained by irradiating the anomeric proton signal at δ 4.46 allowed us to establish this proton as belonging to a glucose unit. In the case of the 2,6-dideoxyhexoses, an easier identification of all the proton signals was obtained by recording the 1D TOCSY experiments while irradiating the methyl doublets.

The identity of each proton signal in 1D TOCSY spectra was deduced by a DQF−COSY experiment that allowed the sequential assignments of all proton resonances within each sugar residue, starting from the well-isolated anomeric proton signals (Table 2). The β-configurations of the five sugar units were shown by the large (J = 7.5 for the glucose unit and J between 9 and 9.5 for the 2,6-dideoxyhexoses) coupling constants of the anomeric proton signals. A HSQC experiment that correlated each hydrogen signal to the corresponding carbon signals allowed the assignment of all the carbon resonances and, therefore, the identification of the sugars, as a terminal β-D-glucopyranosyl, three β-D-oleandropyranosyl, and one β-D-digitoxopyranosyl units. All the 2,6-dideoxyhexoses were glycosidated at C-4. The sugar sequence was deduced from the HMBC spectrum, which showed long-range correlations between C-3_{agl} (δ 78.6) and H-1_{oleI} (δ 4.68), C-4_{oleI}(δ 84.2) and H-1_{dig} (δ 5.00), C-4_{dig}(δ 83.4) and H-1_{oleII} (δ 4.66), C-4_{oleII} (δ 83.9) and H-1_{oleIII} (δ 4.73), and C-4_{oleIII} (δ 83.6) and H-1_{glu} (δ 4.46). Thus, compound **1** was defined as the new 3-*O*-β-D-glucopyranosyl-(1→4)-β-D-oleandropyranosyl-(1→4)-β-D-oleandropyranosyl-(1→4)-β-D-digitoxopyranosyl-(1→4)-β-D-oleandropyranosyl-3β,14β,15β,16α-tetrahydroxy-pregnan-4-one. Compound **2** ($C_{48}H_{80}O_{17}$) showed the $[M - H]^-$ ion at *m/z* 927, 162 mass units lower than that observed for **1**, and fragments at *m/z* 639, due to the loss of two (144 × 2 mass units) 3-O-methyl-2,6-dideoxy-hexopyranose units, and *m/z* 509 arising from the subsequent loss of one (130 mass units) 2,6-dideoxy-hexopyranose unit. The analysis of the NMR data of **2** revealed the same aglycon as in **1** and a sugar chain made up of four monosaccharides (Table 2). Sugar carbon resonances were almost superimposable on those observed for the β-D-digitoxopyranosyl and the first two β-D-oleandropyranosyl units in **1**. Differences were observed for the chemical shifts of C-3 (δ 81.5 in **2** vs δ 79.5 in **1**), C-4 (δ 76.8 in **2** vs δ 83.6 in **1**), and C-5 (δ 73.1 in **2** vs δ 72.5 in **1**) of the third β-D-oleandropyranosyl unit, which suggested this sugar to be terminal.[11] Comparison of the ^{1}H-NMR spectrum of **2** to that of **1** showed the absence of the β-D-glucopyranose anomeric proton signal at δ 4.46 and the highfield shift of the signal ascribable to Me-6_{oleIII} (δ 1.34 in **2** vs 1.42 in **1**) (Table 3). On the basis of the above evidences compound **2** was established to be the new 3-*O*-β-D-oleandropyranosyl-(1→4)-β-D-oleandropyranosyl-(1→4)-β-D-

Table 2. ^{1}H- and ^{13}C-NMR Data of the Sugar Portion of **1** and **2** in CD_3OD[a,b]

		1		**2**	
		δ_C	δ_H, J(Hz)	δ_C	δ_H, J(Hz)
oleI	1	98.4	4.68 dd, 9.1, 1.5	98.5	4.68 dd, 9.1, 1.5
	2	37.9	2.23 ddd, 13.0, 4.0, 1.5 1.38 ddd, 13.0, 9.1, 9.0	37.9	2.22 ddd, 12.9, 4.0, 1.5 1.38 ddd, 12.9, 9.1, 9.0
	3	79.9	3.38 ddd, 9.5, 9.0, 4.0	79.9	3.38 ddd, 9.5, 9.0, 4.0
	4	84.2	3.14 t, 9.5	84.0	3.14 t, 9.5
	5	72.1	3.32 dq, 9.5, 6.2	72.1	3.32 dq, 9.5, 6.2
	6	18.4	1.29, d, 6.2	18.5	1.29, d, 6.2
	OMe	57.2	3.45 s	57.2	3.45 s
dig	1	99.1	5.00 dd, 9.0, 2.0	99.3	4.99 dd, 9.0, 2.0
	2	38.6	2.03 m 1.65 m	38.6	2.03 m 1.62 m
	3	68.2	4.23 br s	68.2	4.23 br s
	4	83.4	3.28 dd 9.5, 3.0	83.4	3.28 dd 9.5, 3.0
	5	69.4	3.83 dq, 9.5, 6.4	69.3	3.83 dq, 9.5, 6.4
	6	18.4	1.27 d, 6.4	18.5	1.27 d, 6.4
oleII	1	101.8	4.66 dd, 9.4, 1.5	101.9	4.66 dd, 9.1, 1.5
	2	37.4	2.32 ddd, 12.5, 4.0, 1.5 1.49 ddd, 12.5, 9.4, 9.0	37.3	2.30 ddd, 12.5, 4.0, 1.5 1.50 ddd, 12.5, 9.5, 9.1
	3	79.9	3.39 ddd, 9.5, 9.0, 4.0	79.9	3.39 ddd, 9.5, 9.5, 4.0
	4	83.9	3.20 t, 9.5	83.7	3.20 t, 9.5
	5	72.1	3.36 dq, 9.5, 6.2	72.1	3.36 dq, 9.5, 6.2
	6	18.4	1.31, d, 6.2	18.3	1.31, d, 6.2
	OMe	57.2	3.46 s	57.2	3.46 s
oleIII	1	100.8	4.73 dd, 9.5, 1.5	101.1	4.72 dd, 9.5, 1.5
	2	37.9	2.34 ddd, 12.5, 3.9, 1.5 1.39 ddd, 12.5, 9.5, 9.0	37.4	2.34 ddd, 13.0, 3.9, 1.5 1.39 ddd, 13.0, 9.5, 9.0
	3	79.5	3.41 ddd, 9.5, 9.0, 3.9	81.5	3.31 ddd, 9.5, 9.0, 3.9
	4	83.6	3.27 t, 9.5	76.8	3.16 t, 9.5
	5	72.5	3.41 dq, 9.5, 6.2	73.1	3.27 dq, 9.5, 6.2
	6	18.3	1.42, d, 6.2	18.2	1.34, d, 6.2
	OMe	57.9	3.49 s	57.9	3.49 s
glu	1	103.9	4.46, d, 7.5		
	2	75.3	3.18, dd, 7.5, 9.0		
	3	77.8	3.36, t, 9.0		
	4	71.6	3.24, t, 9.0		
	5	78.2	3.26, m		
	6	62.8	3.65 dd, 12.0, 5.0 3.88 dd 12.0, 3.5		

[a] Assignments based on DQF− COSY, 1D TOCSY, and HSQC experiments. [b] ^{1}H−^{1}H coupling constants in the sugar spin system were measured from DQF−COSY and 1D TOCSY spectra and are reported in Hertz.

Table 3. Extent of Platelet Aggregation Measured in the Presence of Thrombin or in the Presence of Different Amounts of Tested Compounds[a]

sample	μg/tube	% light transmission 1 min	% light transmission 4 min
platelets alone		0.0	0.0
thrombin	0.5 U	33.9 ± 3.62 (8)[b]	84.5 ± 3.85 (8)
crude MeOH extract	20 μg	8.4 ± 1.71 (4)	52.0 ± 6.59 (4)
1	20 μg	5.3 ± 0.90 (4)	9.4 ± 1.27 (4)
	273 μg[c]	14.1 ± 1.47 (4)	42.3 ± 2.48 (4)
2	20 μg	12.5 ± 2.11 (4)	12.5 ± 1.92 (4)
	232 μg[c]	18.3 ± 2.45 (4)	54.5 ± 4.29 (4)
3	20 μg	2.1 ± 0.83 (4)	3.5 ± 0.91 (4)
	82 μg[c]	6.3 ± 0.83 (4)	17.5 ± 1.58 (4)
4	20 μg	0.0 (4)	0.0 (4)
	85 μg[c]	3.1 ± 1.29 (4)	8.8 ± 1.15 (4)

[a] Data were from aggregation tracings recorded up to 4 min. [b] Mean values ± SEM (n). [c] These amounts correspond to equimolar concentrations (1 mM) of the pure compounds.

digitoxopyranosyl-(1→4)-β-D-oleandropyranosyl-3β,-14β,15β,16α-tetrahydroxypregnan-4-one.

Compounds **3** and **4**, obtained by subjecting the final Sephadex LH-20 fractions to reversed-phase HPLC, were identified, respectively, as the morphinandienones flavinantine and *O*-methylflavinantine, by comparing their NMR data with those reported in the literature.[12]

The occurrence of pregnane glycosides, which are common in the Asclepiadaceae family and are reported to possess antitumor activity,[11,13] in a *Croton* sp. (Euphorbiaceae) is a very unusual finding. Flavinantine (**3**) was previously isolated from *C. balsaminifera*, *C. flavens*, *C. linearis*, and *C. plumieri*[12]. *O*-Methylflavinantine (**4**), previously isolated from *C. menthodorus*, was demonstrated to inhibit electrically induced contractions of isolated guinea-pig ileum,[14] and this finding is in good agreement with the antispasmodic properties attributed to *C. ruizianus* in Peruvian traditional medicine.

On the basis of the vulnerary properties attributed to *C. ruizianus* in Peruvian traditional medicine,[1] the effect of different doses of the MeOH extract and **1**−**4** on platelet functions was evaluated. As shown in Table 3, the crude extract was more effective in stimulating platelet aggregation than **1**−**4**. In fact, at a dose of 20 μg/test tube, the crude extract induced platelet aggregation by more than 50%, whereas the same amounts of **1**−**4** caused only a very weak increase of light transmission. By increasing the doses of pure compounds in the test (1 mM in the assay), pregnane glycosides **1** and **2** also showed a pronounced proaggregating activity, whereas alkaloids **3** and **4** were only slightly active (Table 3). The effect of **3** and **4** at doses higher than 1.5 mM was not evaluable, as solubilization in the platelet suspension of these amounts of dried compounds was not complete.

The ability of tested compounds to act sinergistically with thrombin in inducing platelet aggregation was also evaluated. For this aim we used doses of thrombin lower than 2 U/mL in order to obtain a reduced platelet aggregation (about 50% of light transmission). Experimentally, platelets prewarmed at 37 °C were added to assay tubes containing the dried samples, without stirring. After the agonist addition, the reaction mixture was stirred to let platelet aggregation occurr. All the samples were able to increase the response of platelet to thrombin. In particular, the crude extract (2 μg/test tube) and **1** and **2** (1 mM in the assay) were able to completely restore the level of aggregation (about 83−95%) obtained with 2 U/mL of thrombin. Also, alkaloid samples **3** and **4** (1 mM) acted sinergistically with thrombin, increasing by 20% the extent of platelet aggregation induced by 0.5 U/mL thrombin (data not shown).

The effect of the crude extract and pure samples on platelet aggregation was not due to a cytotoxic action. In fact, lactate dehydrogenase (LDH) released after washed platelet exposure to tested doses never exceeded 3.8% of total LDH activity.

Experimental Section

General Experimental Procedures. A Bruker DRX-600 spectrometer operating at 599.19 MHz for ^{1}H and 150.858 for ^{13}C using the UXNMR software package was used for NMR measurements in CD_3OD solutions. ^{1}H−^{1}H DQF−COSY[8], inverse-detected ^{1}H−^{13}C HSQC[9] (heteronuclear single quantum coherence), and HMBC[10] (heteronuclear multiple bond connectivity) were performed as described previously.[15] 1D TOCSY[7] were acquired using waveform generator-based GAUSS-shaped pulses, mixing times ranging from 100 to 120 ms and a MLEV-17 spin-lock field of 10 kHz preceded by a 2.5 ms trim pulse. Optical rotations were measured on a Perkin−Elmer 141 polarimeter using a sodium lamp operating at 589 nm. FABMS were recorded in a glycerol matrix in the negative ion mode on a VG ZAB instrument (Xe atoms of energy of 2−6 KV). HPLC separation was performed with a Waters model 6000A pump equipped with a U6K injector and a model 401 refractive index detector.

Plant Material. *C. ruizianus* leaves were collected in May 1994, in Aija Province, Ancash Department, Peru. The plant was identified by one of us (H. D. C.) by using the *Flora of Peru*.[16] A voucher specimen (DF 93/177) is stored in the herbarium of the Pharmacy School, University of Salerno.

Extraction and Isolation. The air-dried and powdered leaves (500 g) were defatted with petroleum ether and $CHCl_3$ and then extracted with MeOH to give 12.5 g of residue. Part of the MeOH extract (4 g) was partitioned between *n*-BuOH and H_2O to afford a *n*-BuOH-soluble portion (1.8 g) that was chromatographed on a Sephadex LH-20 column (100 × 5 cm), with MeOH as eluent.

Fractions (8 mL each) were collected and checked by TLC [Si gel plates, $CHCl_3$−MeOH−H_2O (40:9:1) and *n*-BuOH−AcOH−H_2O (12:3:5)]. Fractions 9−14 (750 mg), containing the crude glycosidic mixture, were chromatographed on a μ-Bondapak C-18 column (30 cm × 7.8 mm i.d.) eluting with MeOH−H_2O (7:3), flow 2.5 mL/min to yield pure **1** (34 mg, t_R = 14.0 min) and **2** (16.0 mg, t_R = 24.8 min). Fractions 25−37 (mg 560) afforded **3** (10 mg, t_R = 19.5 min) and **4** (47.0 mg, t_R = 24 min) using as eluent MeOH−H_2O (1:1), flow 2 mL/min.

Compound 1: obtained as an amorphous solid $[\alpha]^{25}_D$ −329° (*c* 0.01, MeOH); ^{1}H and ^{13}C NMR for aglycon, Table 1; ^{1}H and ^{13}C NMR for the sugar portion, Table 2; FABMS *m/z* 1089 $[M - H]^-$, 911 $[(M - H) - 178]^-$, 927 $[(M - H) - 162]^-$, 783 $[(M - H) - (162 + 144)]^-$, 639 $[(M - H) - (162 + 144 + 144)]^-$.

Compound 2: obtained as an amorphous solid $[\alpha]^{25}_D$ −352° (*c* 0.01, MeOH); ^{1}H and ^{13}C NMR for aglycon, Table 1; ^{1}H and ^{13}C NMR for the sugar portion, Table 3; FABMS *m/z* 927 $[M - H]^-$, 639 $[(M - H) - (144 + 144)]^-$, 509 $[(M - H) - (144 + 144 + 130)]^-$.

Preparation of Washed Platelets. Peripheral venous blood, immediately mixed with 3.8% (w/v) sodium citrate (ratio 9:1), was collected from volunteers who, for at least 15 days, had not taken any drugs known to interfere with platelet function. Blood samples were centrifuged at 200 × *g* for 10 min to obtain platelet-rich plasma (PRP). PRP, supplemented with 1 μM prostaglandin E_1, was centrifuged at 800 × *g* for 15 min. The platelet pellet was washed twice with the platelet-washing buffer (tris-HCl 10 mM, pH 7.4, containing 0.15 mM NaCl, 5 mM glucose, 1 mM EDTA) containing 1 μM prostaglandin E_1 and 1 U/mL apyrase (added just before centrifugation). Then platelets were gently resuspended in Hepes−Tyrode's buffer (10 mM glucose, 1mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.138 M NaCl, 2.8 mM KCl, 12 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, and 10 mM Hepes, pH 7.4). Platelet count was adjusted to 5 × 10^8/mL. All procedures were carried out at room temperature.

Platelet Aggregation. The mixture (250 μL final volume) for platelet aggregation experiments contained 125 μL of washed-platelet suspension (2.5 × 10^8/mL, final concentration in the assay), 25 μL of a 10 mg/mL fibrinogen solution, and 100 μL of suspending buffer. Platelet aggregation was monitored on a single channel aggregometer (Chrono-Log aggregometer model 500−CA, Haverton, PA) with continuous stirring at 900 rpm. Washed platelets were incubated for 1 min at 37 °C before being added to the agonist or mixing with tested compounds. Platelet functionality was tested by monitoring thrombin-induced platelet aggregation at the beginning and at the end of each experiment. The extent of platelet aggregation was expressed as a percentage of the light transmission, taking as 100% the light transmission of the reference cuvette containing the suspending medium. The light transmission was recorded for at least 4 min after the addition of the agonist or of the tested compounds. The crude MeOH extract and compounds **1**−**4** were dissolved in MeOH. To avoid interferences of the organic solvent with the aggregatory process, an aliquot of each solution containing the desired amounts of tested samples was dried directly in the test tube by fluxing nitrogen and then was mixed with prewarmed washed platelets.

Cytotoxicity Studies. LDH, which is a parameter of cytoplasmic leakage, was measured in control platelets and in platelet-exposed to samples. Enzyme activity was determined at 37 °C by measuring the rate of

oxidation of NADH (125 μM) in the presence of sodium pyruvate (0.6 mM), dissolved in 50 mM phosphate buffer (pH 7.5) as substrate.[17] The change in the rate of absorbance at 340 nm in samples exposed to tested samples for 5 min was compared to that obtained with control platelets. Total LDH platelet content was measured after platelet lysis by sonication.

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