Complement-Inhibiting Cucurbitacin Glycosides from *Picria fel-terrae*

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Four cucurbitacin glycosides were isolated from *Picria fel-terrae* and identified by MS and NMR spectroscopy as picfeltarraenin IA (**1**), picfeltarraenin IB (**2**), picfeltarraenin IV (**4**), and a new compound picfeltarraenin VI (**3**) (picfeltarraegenin I 3-*O*-*β*-D-xylopyranoside). All four compounds acted as inhibitors on both the classical and alternative pathways of the complement system, with compound **3** exhibiting the highest inhibitory activity (IC_{50} 29 ± 2 *μ*M and 21 ± 1 *μ*M, respectively). Compounds **1**−**4** showed no antiviral, antibacterial, or antifungal activities. Picfeltarraenin IA and IB were tested in an in vitro human tumor cell line panel, but displayed no cytotoxic activity.

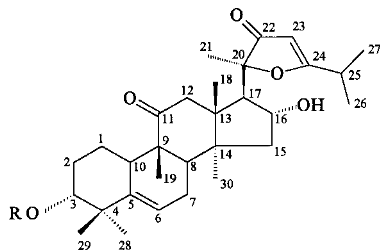
Picria fel-terrae Lour. (Scrophulariaceae) is used in traditional medicine in the southern part of China against fever, herpes infections, cancer, and inflammation.[1,2] Although in a number of publications the binomial *Picria fel-tarrae* has been used,[2−4] in our opinion this is a misspelling, with the correct name being *Picria fel-terrae*.[5] In a preliminary report we have presented the antiviral, antibacterial, and anticomplementary activity of some fractions of a crude extract of this plant. A moderate antibacterial and antiviral activity against selected organisms was observed, as well as an anticomplementary activity on the classical pathway.[1] The anticomplementary activity was evaluated because the complement system is one of the major effector pathways in the process of inflammation. Herein, we report the isolation of four cucurbitacin glycosides (**1**−**4**) with complement-inhibiting properties from *Picria fel-terrae*.

R = α-L-rhamnopyranosyl-(1→2)-β-D-xylopyranosyl

R = α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl

R = β-D-xylopyranosyl

R = α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→3)-β-D-xylopyranosyl



Results and Discussion

In our screening program for complement-modulating activity of medicinal plants, both the aqueous extract of *Picria fel-terrae* and the *n*-BuOH fraction obtained by partition of this aqueous extract showed interesting complement-inhibiting properties. Four cucurbitacin glycosides (**1**−**4**) were isolated from this *n*-BuOH fraction, identified mainly by MS and NMR spectroscopy (including DEPT and, for compound **3**, 2D NMR), and evaluated for their complement activity.

In FABMS compound **1** showed $[M + H]^+$ and $[M + Na]^+$ quasi-molecular ions at *m*/*z* 763 and *m*/*z* 785, respectively, corresponding to a molecular weight of 762 ($C_{41}H_{62}O_{13}$). The protonated aglycon was also revealed by an ion at *m*/*z* 485. The product ion spectrum of the protonated molecule (*m*/*z* 763) showed ions at *m*/*z* 617 (loss of 146 u) and *m*/*z* 485 (loss of 146 + 132 u), consistent with the loss of a rhamnosyl−pentoside moiety. Interpretation of the ^{13}C NMR spectral data of **1** (Table 1) led to the identification of **1** as picfeltarraenin IA (picfeltarraegenin I 3-*O*-α-L-rhamnopyranosyl-(1→2)-*β*-D-xylopyranoside).[3,4] The aglycon part could also be characterized by MS by performing a product analysis on the protonated aglycon (*m*/*z* 485) formed during FAB. Thus, abundant product ions were found at *m*/*z* 467 ($-H_2O$), 449 ($-2 \times H_2O$), 327, 309, 175, and 135, which can be considered characteristic of the aglycon (Figure 1).

The structure of compound **2** was elucidated in a similar manner. It showed $[M + H]^+$ and $[M + Na]^+$ quasi-molecular ions in FABMS at *m*/*z* 793 and 815, respectively, corresponding to a molecular weight of 792 ($C_{42}H_{64}O_{14}$), as well as the protonated aglycon at *m*/*z* 485. The product ion spectrum of the protonated molecule (*m*/*z* 793) revealed ions at *m*/*z* 647 (loss of 146 u) and *m*/*z* 485 (loss of 146 + 162 u), consistent with a rhamnosyl−hexoside moiety. Compound **2** was identified as picfeltarraenin IB (picfeltarraegenin I 3-*O*-α-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranoside) by interpretation of its ^1H and ^{13}C NMR spectral data (Table 1).[3,4]

In FABMS compound **3** showed $[M + H]^+$ and $[M + Na]^+$ quasi-molecular ions at *m*/*z* 617 and 639, respectively, corresponding to a molecular weight of 616 ($C_{35}H_{52}O_9$). The protonated aglycon showed an ion at *m*/*z* 485, suggesting the same aglycon as **1** and **2**, and a pentosyl unit. Indeed, product ion analysis on the protonated aglycon ion (*m*/*z* 485) indicated that the

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Table 1. ^{13}C NMR Assignments for Picfeltarraenins IA (**1**), Ib (**2**), VI (**3**), and IV (**4**)

carbon	multiplicity	**1**[a]	**2**[a]	**3**[b]	**4**[b]
1	t	24.4	24.4	25.9	26.1
2	t	25.6	25.8	27.3	27.7
3	d	81.8	81.8	83.5	84.3
4	s	40.9	40.9	42.6	42.6
5	s	141.8	141.9	143.6	143.7
6	d	117.6	117.5	119.5	119.5
7	t	23.3	23.3	24.8	24.8
8	d	34.4	34.4	36.6	36.6
9	s	47.9	47.9	48.9	48.9
10	d	42.2	42.2	44.4	44.5
11	s	212.5	212.5	215.9	215.8
12	t	47.9	47.9	48.9	c
13	s	47.0	47.0	50.5	50.2
14	s	49.6	49.6	51.5	51.5
15	t	45.5	45.5	46.7	46.8
16	d	70.4[d]	70.4[d]	71.0[d]	69.7[d]
17	d	57.9	57.9	59.3	59.3
18	q	18.1[e]	18.1[e]	19.5[e]	18.5[e]
19	q	18.1[e]	18.1[e]	19.8[e]	19.6[e]
20	s	89.8	89.8	92.2	92.2
21	q	22.7	22.7	22.7	22.9
22	s	205.8	205.7	210.2	210.1
23	d	99.4[f]	99.3[f]	101.5	101.4[f]
24	s	194.5	194.5	198.8	198.7
25	d	29.4	29.4	31.6	31.6
26	q	19.1[e]	19.1[e]	20.0[e]	19.6[e]
27	q	19.3[e]	19.3[e]	20.3[e]	20.3[e]
28	q	24.5	24.5	25.2	25.3
29	q	21.3	21.3	22.2	22.8
30	q	19.4[e]	19.4[e]	20.4[e]	20.4[e]
1’	d	100.2[f]	100.2[f]	102.5	101.6[f]
2’	d	78.3	78.2	75.0	75.9[g]
3’	d	74.6	74.6	78.0	89.3
4’	d	68.2[d]	70.4[d]	71.3[d]	70.3[d]
5’		65.6 (t)	76.5(d)	66.9 (t)	66.3 (t)
6’	t		61.1		
1”	d	99.5[f]	98.8[f]		104.4
2”	d	70.2[d]	70.3[d]		75.2[g]
3”	d	69.8[d]	68.2[d]		78.3
4”	d	72.0	72.0		71.6
5”	d	67.7	67.6		78.3
6”		19.7[e] (q)	19.7[e] (q)		62.6 (t)
1”’	d				101.5[f]
2”’	d				72.2[h]
3”’	d				72.0[h]
4”’	d				74.1
5”’	d				71.1[d]
6”’	q				20.0[e]

[a] Recorded in DMSO-d_6. [b] Recorded in CD_3OD. [c] Overlapped with solvent. [d–h] Assignments bearing the same superscript may be interchanged within the same column.

aglycon part was the same as for **1** and **2**. In the ^{13}C NMR spectrum two carbonyl signals were observed at δ 215.9 (C-11) and 210.2 (C-22). The C-22 carbonyl group was found to be α,β-unsaturated and was substituted at the β-position with an oxygen atom, which explains the unusual chemical shifts observed for C-23 (upfield at δ 101.5, d) and C-24 (downfield at δ 198.8, s). Other typical signals of the aglycon include the C-5/C-6 double bond (δ 143.6, s and δ 119.5, d, respectively) and C-16 (δ 71.0, d), which bears a hydroxyl substituent, with C-3 (δ 83.5, d) being the site of glycosylation. The presence of an isopropyl side chain (C-25/C-26/C-27) was confirmed in the ^1H NMR spectrum by the appearance of a characteristic septet at δ 2.82 (J = 6.9 Hz, H-25) and the corresponding doublets of the methyl groups at δ 1.26 and 1.27. After the assignment of ^1H and ^{13}C NMR signals for the aglycon part, the five remaining peaks were typical for a β-xylopyranosyl moiety. ^{13}C NMR assignments were based on those reported for methyl β-D-xylopyranoside.[6] ^1H NMR assignments for the xylopyranosyl residue were based on a detailed NMR analysis, including DQF–COSY, HSQC, and HMBC carried out in our laboratory for (+)-taxifolin-3-*O*-β-D-xylopyranoside.[7] The β-configuration of xylopyranose in **3** was evident from the coupling constant observed for H-1′ (d, J = 7.6 Hz).[8] These ^1H NMR assignments were confirmed in a ^1H–^1H COSY experiment. Based on the evidence mentioned above and by comparison of NMR assignments for the known picfeltarraenins, compound **3** could be determined as picfeltarraegenin I 3-*O*-β-D-xylopyranoside. This compound has not been reported before. Because six picfeltarraenins have been isolated before from *Picria fel-terrae* (IA, IB, II–V)[2], the name picfeltarraenin VI is proposed for compound **3**. To avoid confusion, we do not recommend changing the earlier assigned names to picfelterraenins IA, IB, and II–V.

Compound **4** showed a $[M + Na]^+$ quasi-molecular ion at *m/z* 947, corresponding to a molecular weight of 924 ($C_{47}H_{72}O_{18}$), which was in agreement with a picfeltarraegenin I aglycon, a hexosyl, a deoxyhexosyl, and a pentosyl moiety. MS, ^1H NMR, and ^{13}C NMR spectral data were in agreement with those reported for picfeltarraenin IV (picfeltarraegenin I 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→3)-β-D-xylopyranoside).[2]

Compounds **1**–**4** were evaluated for their complement-modulating properties. The complement system is a potent mechanism for the initiation and amplification of inflammation, composed of a complex enzyme system, which can be activated in specific cascade reactions upon stimulation. Two possible activation routes, referred to as the classical pathway(CP) and the alternative pathway(AP), converge at complement factor C3, from which activation proceeds through C5 to the lytic pathway. The terminal route is the final phase of the activation of the complement cascade, resulting in the formation of the Membrane Attack Complex. Screening systems for complement-activity are most commonly based on its hemolytic properties, involving a spectrophotometric measurement of the hemoglobin released.[9]

For the cucurbitacin glycosides (**1**–**4**) isolated from *P. fel-terrae* a dose-dependent inhibitory effect on the CP and the AP of the complement system could be observed at certain concentration ranges. IC_{50} values (50% inhibitory concentrations) for compounds **1**–**4** on the CP and the AP are listed in Table 2. In this table, rosmarinic acid is included as a positive control substance.[10] Compound **3** (picfeltarraenin VI) was the most potent inhibitor of the CP. For compound **2**, no IC_{50} value could be calculated, but at the highest concentration tested (420 μM), an inhibition of 48 ± 11% was observed (IC_{50} > 420 μM). Also, in the AP, picfeltarraenin VI (**3**) showed the highest inhibitory value. Compounds **1** and **4** were more than 10 times less active as inhibitors in the AP, and compound **2** showed an intermediate activity. Although no direct relationship could be established between the number of sugar units and the complement-inhibiting activity, remarkably the monoglycosidic compound picfeltarraenin VI (**3**) was the strongest inhibitor in both the CP and the AP. To our knowledge, this is the first report of the complement-modulating properties of triterpene glycosides. Previ-

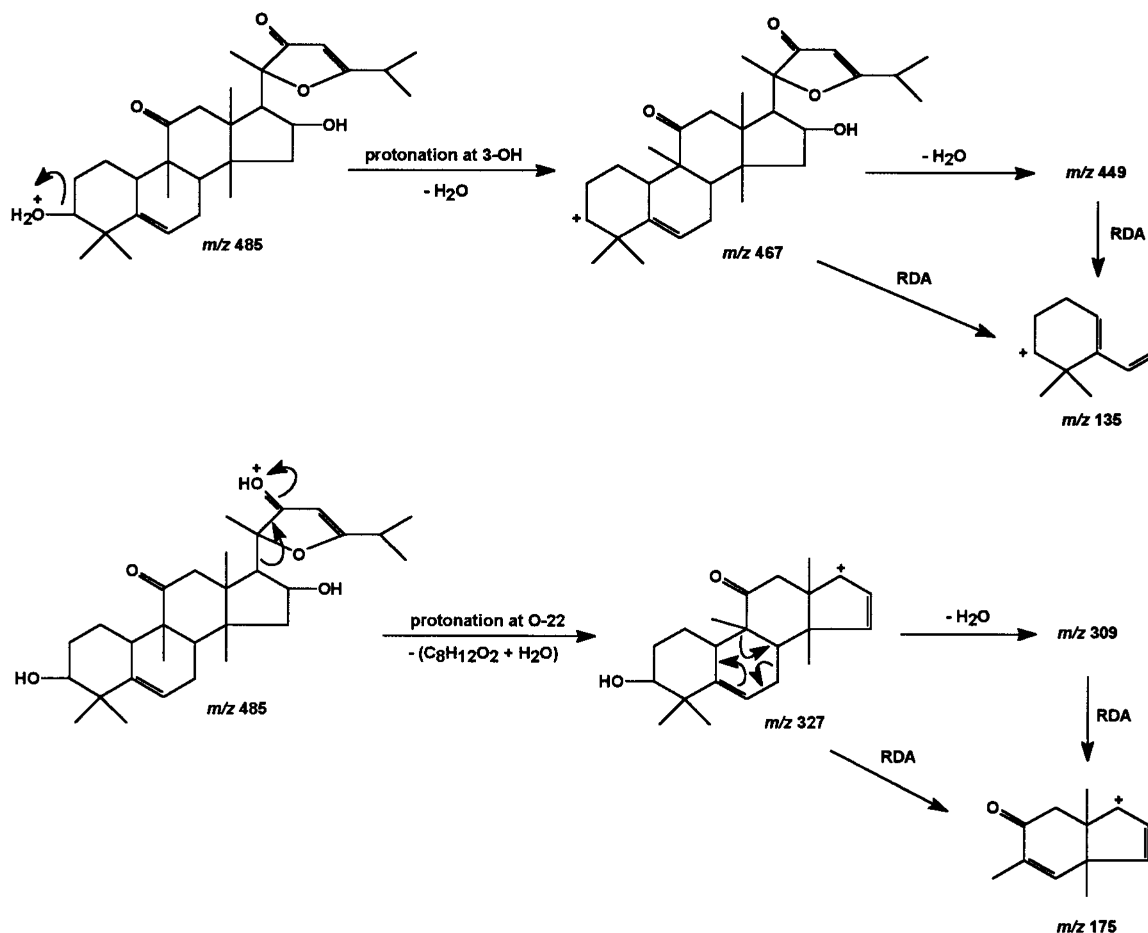

Figure 1. Proposed fragmentation pattern for the protonated aglycon (*m/z* 485) of compounds **1**–**4**, as determined by FABMS–MS (RDA: *retro*-Diels–Alder).

Table 2. Biological Test Data [IC_{50} Values ± SEM (μM)] for Picfeltarraenins IA (**1**), IB (**2**), VI (**3**), and IV (**4**) on the Classical (CP) and the Alternative Pathways (AP) of Complement ($n = 2$)

compound	CP	AP
1	95 ± 8	336 ± 52
2	> 420	68 ± 4
3	29 ±2	21 ± 1
4	131 ± 3	313 ± 45
rosmarinic acid[a]	81 ± 3	408 ± 53

[a] Positive control substance.

ously, the complement-activity of a number of oxygenated triterpenoids such as β-boswellic acid, crategolic acid, cucurbitacin I, glycyrrhetic acid, oleanolic acid, quillaic acid, and ursolic acid was evaluated by Knaus and Wagner.[11,12] β-Boswellic acid was a potent inhibitor of both the CP and the AP of complement. The complement-activity of the aglycon picfeltarraegenin I, prepared by acid hydrolysis of picfeltarraenin IA, could not be determined because of its low solubility.

Although the complement system plays an important role in the host's defense against invading microorganisms, its activation may contribute to or even evoke pathological reactions in a variety of inflammatory or degenerative diseases (rheumatoid arthritis, microbial infections, gout, etc.). Drugs inhibiting the complement system could be valuable therapeutic agents in those cases.[9] The traditional use of *Picria fel-terrae* to treat inflammation may at least partly be explained by the complement-inhibiting properties of the picfeltarraenins present in the plant.

The antimicrobial activity of compounds **1**–**4** was evaluated against a series of fungi and bacteria and against the herpes simplex virus type 1 (HSV-1). The minimum inhibitory concentration (MIC) for all fungi and bacteria tested was >100 μg/mL (highest concentration tested). At 100 μg/mL a reduction factor of the viral titer of 10 was observed for HSV-1. In conclusion, compounds **1**–**4** showed no interesting antimicrobial activity against the test organisms.

Picfeltarraenins IA (**1**) and IB (**2**) were tested in an in vitro disease-oriented primary cytotoxicity screen (a cell panel consisting of 60 human tumor cell lines). GI_{50}, TGI, and LC_{50} values, referring to the concentration where 50% growth inhibition, total growth inhibition, and a lethal effect to 50% of the tumor cells are observed, respectively, were >100 μM (highest concentration tested) for almost all cell lines included in the panel, and both compounds were considered inactive. For the CNS cancer-cell line SF-295, however, the log GI_{50} value of compound **1** was −4.23 (GI_{50} 59 μM). This is not in agreement with the results reported by Cheng et al.,[4] who claimed that picfeltarraenins possess significant antitumor activity.

Experimental Section

General Experimental Procedures. TLC was carried out on precoated Si gel 60 F_{254} plates (Merck), developed with EtOAC–HOAc–H_2O (8:2:1) (solvent

system A), $CHCl_3$−MeOH (3:1) (system B), or other solvent systems. The Liebermann−Burchard spray reagent was used to visualize the spots (after heating). Column chromatography was carried out on Si gel and LiChroprep RP-18. ^{1}H, ^{13}C, DEPT-135, DEPT-90, and 2D NMR spectra (pulsed-field gradient COSY) were recorded in CD_3OD (99.8% D) or DMSO-d_6 (99.8% D) on a Bruker DRX-400 instrument operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. Chemical shifts are reported in parts per million (δ) downfield from tetramethylsilane (0 ppm). FABMS were recorded in the positive ion mode on a VG 70 SEQ instrument using glycerol as the liquid matrix. Product ion spectra (FABMS−MS) were obtained by high-energy (8 keV) collision-induced dissociation, with helium as target gas and employing 50% beam attenuation in the first field-free region and B/E linked scanning.

Plant Material. The whole plant of *Picria fel-terrae* Lour. (Scrophulariaceae) was collected in Lonlin, China, in June 1991, and identified by S.-Y. Liu, Department of Pharmaceutical Sciences, The Traditional Medicine College of Guangxi, Nanning, China, where a voucher specimen is kept.

Extraction and Isolation. Dried and powdered plant material (500 g) was extracted exhaustively with warm H_2O. The filtrate was concentrated under reduced pressure and extracted sequentially with EtOAc and *n*-BuOH. The *n*-BuOH extract was evaporated to dryness under reduced pressure and subjected to column chromatography over LiChroprep RP-18. Elution with H_2O−MeOH mixtures (increasing polarity) yielded nine subfractions (I−IX). Repeated column chromatography on Si gel of subfraction V, eluted with $CHCl_3$−MeOH−H_2O mixtures (increasing polarity), followed by column chromatography on Si gel, eluted with solvent system A, yielded compounds **1** and **2** (344 and 46 mg, respectively). Compound **3** (8.3 mg) was isolated by preparative TLC (Si gel, layer thickness 1 mm) from subfraction IV with 2-pentanol−H_2O (1:1) (upper phase) as the mobile phase (R_f = 0.72). Subfraction II was repeatedly subjected to column chromatography on Si gel. Elution with solvent system A, monitored on TLC, led to the isolation of compound **4** (18 mg).

Picfeltarraenin IA (1): ^{1}H NMR (DMSO-d_6, 400 MHz) δ 5.63 (1H, m, H-6), 5.46 (1H, s, H-23), 5.20 (1H, br s, H-1″), 4.24 (1H, m, H-16), 4.19 (1H, d, J = 7.3 Hz, H-1′), 2.73 (1H, septet, J = 6.9 Hz, H-25), 2.43 (1H, d, J = 6.8 Hz, H-17), 1.29 (3H, s, $-CH_3$), 1.17−1.20 (9H, m, 3 × $-CH_3$), 1.08 (3H, s, $-CH_3$), 1.04 (3H, d, J = 6.1 Hz, H-6″), 0.88 (3H, s, $-CH_3$), 0.83 (3H, s, $-CH_3$), 0.68 (3H, s, $-CH_3$); ^{13}C NMR, see Table 1; FABMS (glycerol) *m*/*z* 785 [M + Na]$^+$, 763 [M + H]$^+$, 617 [M + H − 146]$^+$, 485 [M + H − (146 + 132)]$^+$, 467 [485 − 18]$^+$; FABMS−MS (*m*/*z* 763) *m*/*z* 727, 617, 485, 467; FABMS-MS (*m*/*z* 485), *m*/*z* 467, 449, 327, 309, 175, 135; TLC (Si gel, solvent system A) R_f 0.47.

Picfeltarraenin IB (2): ^{1}H NMR (DMSO-d_6, 400 MHz) δ 5.64 (1H, m, H-6), 5.47 (1H, s, H-23), 5.20 (1H, br s, H-1″), 4.24 (1H, m, H-16), 4.20 (1H, d, J = 7.8 Hz, H-1′), 2.74 (1H, septet, J = 6.9 Hz, H-25), 2.43 (1H, d, J = 6.8 Hz, H-17), 1.29 (3H, s, $-CH_3$), 1.17−1.20 (9H, m, 3 × $-CH_3$), 1.08 (3H, s, $-CH_3$), 1.04 (3H, d, J = 6.1 Hz, H-6″), 0.88 (3H, s, $-CH_3$), 0.83 (3H, s, $-CH_3$), 0.68 (3H, s, $-CH_3$); ^{13}C NMR, see Table 1; FABMS (glycerol) *m*/*z* 815 [M + Na]$^+$, 793 [M + H]$^+$, 685, 647 [M + H − 146]$^+$, 485 [M + H − (146 + 162)]$^+$, 467 [485−18]$^+$; FABMS−MS (*m*/*z* 793) *m*/*z* 647, 485, 467; FABMS−MS (485) *m*/*z* 467, 449, 327, 309, 175, 135; TLC (Si gel, solvent system A) R_f 0.38.

Picfeltarraenin VI (3): ^{1}H NMR (CD_3OD, 400 MHz) δ 5.72 (1H, m, H-6), 5.48 (1H, s, H-23), 4.23 (1H, d, J = 7.6 Hz, H-1′), 4.20 (1H, m, H-16), 3.80 (1H, dd, J = 11.5 Hz, J = 5.1 Hz, H-5′a), 3.48 (1H, m, H-4′), 3.27 (2H, m, H-3′, H-12a) (overlapped with solvent peak), 3.13 (2H, m, H-2′, H-5′b), 2.82 (1H, septet, J = 6.9 Hz, H-25), 2.58 (1H, d, J = 7.0 Hz, H-17), 2.44 (1H, d, J = 14.5 Hz, H-12b), 1.40, 1.30, 1.16, 1.03, 1.00, 0.85 (3H each, s each, H-18, H-19, H-21, H-28, H-29, H-30), 1.27, 1.26 (3H each, d, J = 6.9 Hz, H-26, H-27); ^{13}C NMR, see Table 1; FABMS (glycerol) *m*/*z* 639 [M + Na]$^+$, 617 [M + H]$^+$, 485 [M + H − 132]$^+$, 467 [485 − 18]$^+$; FABMS−MS (*m*/*z* 617), *m*/*z* 485, 467, 449; FABMS−MS (*m*/*z* 485) *m*/*z* 467, 449, 327, 309, 175, 135; TLC (Si gel, solvent system B) R_f 0.72.

Picfeltarraenin IV (4): ^{1}H NMR (CD_3OD, 400 MHz) δ 5.71 (1H, m, H-6), 5.47 (1H, s, H-23), 5.46 (1H, br s, H-1‴), 4.46 (1H, d, J = 7.8 Hz, H-1″), 4.33 (1H, d, J = 7.3 Hz, H-1′), 4.21 (1H, m, H-16), 2.81 (1H, septet, J = 6.9 Hz, H-25), 2.58 (1H, d, J = 7.0 Hz, H-17), 2.42 (1H, d, J = 14.5 Hz, H-12b), 1.42, 1.30, 1.16, 1.02, 0.94, 0.84 (3H each, s each, H-18, H-19, H-21, H-28, H-29, H-30), 1.27, 1.26 (3H each, d, J = 6.9 Hz, H-26, H-27), 1.19 (3H, d, J = 6.2 Hz, H-6‴); ^{13}C NMR, see Table 1; FABMS (glycerol) *m*/*z* 947 [M + Na]$^+$, 817, 485; FABMS−MS (*m*/*z* 485) *m*/*z* 467, 449, 327, 309, 175, 135; TLC (Si gel, solvent system A) R_f 0.29.

Acid Hydrolysis of Compound 1. Compound **1** (80 mg) was dissolved in a mixture of 10 mL EtOH, 18 mL H_2O, and 1.5 mL H_2SO_4 and heated under reflux for 1.5 h. Thereafter, the pH was adjusted to 7 with 10% NaOH, and the solution was extracted with EtOAc (3 × 10 mL). Evaporation of the EtOAc extract yielded the aglycon (32 mg), which was identified as picfeltarraegenin I by MS and NMR spectroscopy. Its purity was checked by TLC ($CHCl_3$−EtOAc, 1:1). Evaporation of the residual aqueous layer yielded the sugar fraction; monosaccharides were identified by paper chromatography (solvent system *n*-BuOH−pyridine−H_2O, 6:3:1) by comparison with authentic samples (Merck, Darmstadt) (detection with β-naphthol−H_2SO_4 reagent).

Bioassays: Modulation of the Complement System. The complement modulation test applied was based on the assay models as described by Mayer[13] and by Platts-Mills and Ishizaka[14] for the CP and the AP, respectively. It was adapted for use on a microscale by Klerx et al.[15]

Buffers and Reagents. Test samples were dissolved with DMSO in the appropriate buffer (maximum effective DMSO concentration is lower than 1%) and incubated in an ultrasonic H_2O bath (Julabo USR 3). Human pooled serum (HPS) from 10 healthy volunteers was used as source of complement. All reagents used were of analytical grade. In the classical hemolytic complement assay, an ascorbic acid solution is added to a five-fold concentrated CP−veronal saline buffer pH 7.35 (CP−VSB^{5x}) serving as a stock solution for the preparation of the CP-buffer, containing 144.8 mM NaCl, 4 mM diethylbarbituric acid, 0.25 mM Ca^{2+}, 0.83

mM Mg^{2+}, 0.02% NaN_3, and 0.142 mM ascorbic acid. In the alternative hemolytic complement assay, an ascorbic acid solution is added to a five-fold concentrated AP−veronal saline buffer pH 7.35 (AP−VSB^{5x}) serving as a stock solution for the preparation of the AP-buffer, containing 95.8 mM NaCl, 4 mM diethylbarbituric acid, 7 mM Mg^{2+}, 10 mM ethyleneglycol-bis(2-aminoethyl)-tetraacetic acid, 0.02% NaN_3, and 0.284 mM ascorbic acid. Ascorbic acid was added to prevent oxidation of test compounds during the assay. Sheep and rabbit erythrocytes were sensitized by incubating 4×10^8 cells/mL with an equal volume of a 1:400 dilution of rabbit anti-sheep serum for 10 min at room temperature. The antibodies were purchased from BioMérieux (no. 72202). Excess of antibodies was removed by centrifugation. Just before use, the suspension of sensitized sheep erythrocytes was adjusted to an absorbance of 0.8 (CP) and the suspension of uncoated rabbit erythrocytes to an absorbance of 1.4 (AP).

Microtiter Hemolytic Complement Assay. Briefly, the assay was performed in U-well microtiter plates (Falcon 3077). All samples were diluted in the microtiter plate (seven consecutive logarithmic dilutions) with the appropriate buffer (CP/AP-buffer) in such a way that the final volume in each well was 50 μL (CP) or 100 μL (AP). Subsequently, 50 μL of a CP−HPS dilution (classical pathway) or 25 μL of an AP−HPS dilution (alternative pathway) were added to each well containing sample or standard. After an incubation of 30 min at 37 °C, 50 μL of a suspension of sensitized sheep erythrocytes (CP) or 25 μL of a suspension of uncoated rabbit erythrocytes (AP) was added. The plates were incubated at 37 °C for 60 min (CP and AP). Subsequently, the plates were centrifuged for 10 min at 800 $\times$ *g* (Hettich centrifuge Rotanta/TR). To quantify hemolysis, 50 μL of the supernatant was mixed with 200 μL H_2O in a flat-bottom microtiter plate (Falcon 3072), and the absorption at 414 nm was measured in a multiscan (Labsystems Multiscan MCC/340). Controls in this assay consisted of erythrocytes incubated both in buffer (0% hemolysis) and with sample (color and toxicity control) as well as the color of HPS dilution. The HPS dilution used in the classical and the alternative standard assay gave rise to approximately 50% hemolysis. For each test compound, the IC_{50} value (50% inhibitory concentration) was calculated. At least two IC_{50} values were determined for each test compound, and results were expressed as the mean ± S.E.M. (standard error of the mean).

Antimicrobial Activity. The antimicrobial activity of compounds **1**−**4** was tested against the fungi *Aspergillus niger* and *Trichophyton rubrum*; the yeast *Candida albicans*; the bacteria *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*; and HSV-1, as previously described.[16,17]

Cytotoxicity Screen. Compounds **1**−**4** were evaluated for potential anticancer activity in the in vitro human disease-oriented tumor cell line screening panel developed by the National Cancer Institute (NCI, Bethesda, MD). This cell line panel consisted of a total of 60 human tumor cell lines arranged in nine subpanels representing diverse histologies (leukemia, nonsmall cell lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, renal cancer, prostate cancer, and breast cancer). Results are evaluated in terms of specificity and potency.[18]

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