New Compounds from an Extract of *Vernonia colorata* Leaves with Anti-inflammatory Activity

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Bioassay-directed fractionation of an anti-inflammatory $CHCl_3$−MeOH (9:1) extract of leaves of *Vernonia colorata*, using a carrageenan-induced rat paw model, led to the isolation of six new compounds (**1**−**6**).These were assigned as two new androst-8-en glycosides, 3-*O*-{*β*-D-galactopyranosyl-(1−2)-[*β*-D-glucopyranosyl-(1→6)]-*β*-D-glucopyranoside}-5α,14α-androst-8-ene (**1**) and 3-*O*-[*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranoside]-5α,14α-androst-8-ene (**2**), two new stigmastane-type glycosides, 3*β*,21,24-trihydroxy-21,23;22,28;26,28-triepoxy-5α-stigmasta-8(9),14(15)-dien-3-*O*-*β*-D-galactopyranosyl-(1→2)-*β*-D-glucopyranoside (**3**) and 3*β*,21,24-trihydroxy-21,23;22,28;26,28-triepoxy-5α-stigmasta-8(9),14(15)-dien-3-*O*-*β*-D-galactopyranosyl-(1→2)-*β*-D-(6-acetyl)glucopyranoside (**4**), and two new stigmastane-type steroids, 3*β*,25,29-trihydroxy-5α-stigmasta-8(9),14(15),24*Z*(28)-triene (**5**) and 3*β*,23,25-trihydroxy-24,28-epoxy-5α-stigmasta-8(9),14(15)-diene (**6**). The structures of **1**−**6** were elucidated by spectral and chemical studies. Compounds **1**−**6** were tested for the anti-inflammatory activity, but all were inactive or weakly inactive as anti-inflammatory agents.

Vernonia colorata (Willd.) Drake (Asteraceae), known by the name "Kosafunè" in the Bambara language in Mali, has long been used in traditional medicine by the indigenous people for the treatment of cough, fever, hepatitis, gastritis, stomach pain, gastrointestinal disorders, venereal diseases, and skin eruptions. Commonly, the leaves are used as water infusions or decoctions and employed to rinse the mouth, for tonsillitis, to cure earache, and as a febrifuge. The extract of fresh leaves, softened by heating, is applied to wounds for cicatrization.[1−4]

To validate the use of *V. colorata* in the traditional medicine of Mali as an anti-inflammatory remedy, the extracts of the leaves were evaluated in vivo for anti-inflammatory activity using a carrageenan paw edema model in rats. A bioassay-guided fractionation procedure showed that a $CHCl_3$−MeOH (9:1) extract was the most active. Subsequent fractionation and analysis of the $CHCl_3$−MeOH (9:1) extract has led to the isolation and characterization as major constituents of two new androst-8-ene glycosides (**1**, **2**), two new stigmastane glycosides (**3**, **4**), and two new stigmastane-type steroids (**5**, **6**). The structure elucidation of compounds **1**−**6** was accomplished primarily through the extensive use of 1D and 2D NMR techniques.

Results and Discussion

The sequential extraction of *V. colorata* (500 g) leaves using solvents of increasing polarity gave petroleum ether, chloroform, chloroform−methanol (9:1), and methanol extracts. Each extract was tested orally for anti-inflammatory activity using carrageenan-induced edema in the rat paw.[5] All the extracts exhibited some anti-inflammatory activity (Table 1), inducing edema inhibition in the range from 15% to 80%, with the $CHCl_3$−MeOH (9:1) extract being the most active. Therefore, the $CHCl_3$−MeOH (9:1) extract, which induced 80% edema inhibition at the dose of 200 mg/kg po, was further investigated in order to evaluate its contribution to the overall anti-inflammatory activity of the starting plant material. Its effect was compared to that of a total extract prepared by pooling the petroleum ether (7.3%), chloroform (25.3%), $CHCl_3$−MeOH (9:1) (42.1%), and MeOH (25.3%) extracts on the basis of their extraction yields. The total extract (200 mg/kg) or the equivalent dose of the $CHCl_3$−MeOH extract induced similar edema reduction (82 and 71%, respectively), indicating that the $CHCl_3$−MeOH extract gave the highest contribution to the activity of *V. colorata* leaves (Table 1).

Table 1. Anti-inflammatory Activity of *V. colorata* Extracts on Carrageenan-Induced Paw Edema in Rats

test material	dose (mg/kg)	paw volume (mL)[a]	inhibition (%)
control		4.8 ± 0.07	
chloroform extract	200	3.4 ± 0.02[b]	29
chloroform−methanol extract	200	1.0 ± 0.03[b]	80
methanol extract	200	3.1 ± 0.06[b]	35
total extract	200	0.9 ± 0.05[b]	82
chloroform−methanol extract	84.2	1.4 ± 0.06[b]	71
indomethacin	5	0.7 ± 0.07[b]	85

[a] Values, determined 3 h after carrageenan, are mean ± SEM ($n = 8$). [b] $p < 0.05$ vs control. Student's test.

The separation of the crude $CHCl_3$−MeOH (9:1) extract by Sephadex LH-20 column yielded five main fractions, I−V. Fractions I−V were evaluated for their anti-inflammatory activity at the respective doses of 64.8, 14.6, 17.6, 32.4, and 70.6 mg/kg po calculated on the basis of the fractionation yield corresponding to 200 mg of the parent $CHCl_3$−MeOH extract. The most active fractions were II and III, which inhibited edema formation by 43.4% and 51.0% (3 h), respectively, while the inhibitory rates at 3 h of fractions I, IV, and V were 2.56%, 26.8%, and 11%, respectively. By means of reversed-phase HPLC, compounds **1**−**4** were isolated from fraction II, and compounds **4**, **5**, and **6** were isolated from fraction III.

Compounds **1** and **2** displayed the molecular formulas $C_{37}H_{60}O_{16}$ and $C_{31}H_{50}O_{11}$, respectively, as deduced by mass spectrometry and ^{13}C NMR spectroscopy including DEPT

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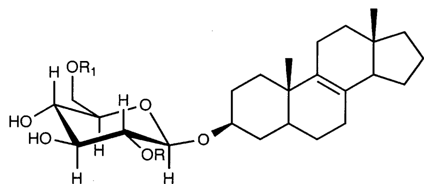

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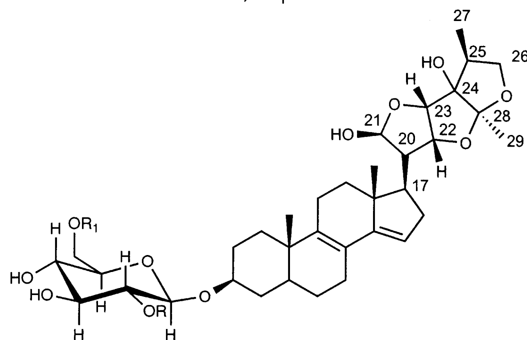

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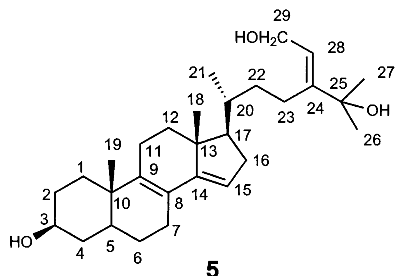

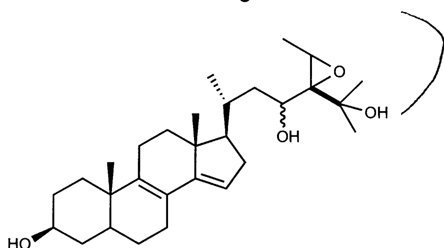

analysis. The FABMS (negative ion) showed the loss of two hexoses (162 mass units) for compound **2** and three hexoses for compound **1** from the respective quasi-molecular anions $[M - H]^-$ at *m/z* 597 and 759. The ^{13}C NMR spectrum of **1** showed the presence of 19 carbon signals for the aglycon moiety ascribable to an androstane nucleus including an oxymethine (81.0 ppm, CH, C-3).[6] The ^1H NMR spectra for the aglycon part of **1** were typical of a sterol structure displaying the angular methyl singlets at δ 1.08 and 0.80 and a characteristic multiplet at δ 3.79 (H-3), which was shifted downfield by glycosylation.[6,7] An unsaturated functionality evident from the ^{13}C NMR spectrum was one tetrasubstituted double bond (125.0 and 133.0 ppm, each a quaternary carbon), situated between C-8 and C-9, as indicated by analysis of the HSQC and HMBC correlations.[8] All the remaining carbon signals of the molecule **1** were assigned from the HSQC and HMBC experiments, by comparison with model steroids and according to substituent effect rules.[9] The NMR data for the sugar moiety (Table 3) linked at C-3 of the aglycon in compound **1** revealed the presence of two β-glucopyranosyl and one

Table 2. ^1H and ^{13}C NMR Data for Aglycons of Compounds **1** and **3**[a] in Methanol-d_4

	1		**3**	
position	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1	36.2	1.69; 1.65	36.2	1.29; 1.85
2	31.8	1.51; 174	30.4	1.31; 2.03
3	81.0	3.79 m	79.5	3.79 m
4	35.5	1.99 (ddd, 11.5, 9.9, 8.7) 1.43	36.4	2.01 (ddd, 11.5, 10.0, 9.0) 1.44
5	42.6	1.35	42.3	1.30
6	22.9	1.47	22.5	1.50
7	37.0	2.29 (ddd, 12.0, 9.0, 4.5) 1.85 (ddd, 12.0, 9.0, 4.5)	37.5	2.23 (ddd, 12.0, 9.0, 4.5) 1.75
8	125.0		125.0	
9	133.0		142.3	
10	38.0		38.2	
11	27.7	2.05; 2.07	28.0	2.03
12	37.0	1.39; 1.81	26.5	1.39; 1.81
13	39.0		45.0	
14	50.0	2.18 (dd, 9.0, 4.0)	151.0	
15	18.8	1.09; 0.93	117.0	5.35 br m
16	36.3	1.70, 1.30	36.5	2.52 (br dd, 11.5, 3.5) 2.35 (br dd, 1.5, 4.5)
17	23.5	1.51, 1.23	50.5	2.07
18	17.9	0.80 (s)	16.9	0.92 (s)
19	18.8	1.08 (s)	18.9	1.08 (s)
20			53.8	2.31 br m
21			105.0	5.27 (d, 6.0)
22			86.2	4.68 (dd, 2.5, 3.5)
23			85.2	4.43 (d, 2.5)
24			89.0	
25			41.5	2.20 br m
26			10.9	1.03 (s)
27			73.3	3.98 (dd, 9.5, 7.0) 3.46 (dd, 9.5, 5.5)
28			119.0	
29			21.5	1.31 (s)

[a] Assignments confirmed by 1D-TOCSY, DQF-COSY, HSQC, and HMBC experiments.

β-galactopyranosyl unit. Their β-linkages were shown by the coupling constant values (J = 7.5, 7.6, and 7.0 Hz) of three anomeric proton signals at δ 4.65, 4.52, and 4.60 and by their chemical shifts in the ^{13}C NMR spectra.[10] DQF-COSY,[11] HOHAHA,[12] and HSQC experiments allowed the assignments of all proton and carbon signals and the identification of one disubstituted β-glucopyranosyl residue. In fact, the expected glycosylation shifts were observed for C-2 and C-6 of one glucose unit (Table 3). The sugar sequence was deduced from the HMBC spectrum, which showed long-range correlations between C-2_{GlcI} (82.9 ppm) and H-1_{Gal} (δ 4.60) and between C-6_{GlcI} (67.8 ppm) and H-1_{GlcII} (δ 4.52). The configuration of the sugar units was assigned after hydrolysis of **1** with 1 N HCl. The hydrolysate was trimethylsilated, and GC retention times of each sugar were compared with those of authentic samples prepared in the same manner. In this way, the sugar units of **1** were determined to be D-glucose and D-galactose. Thus, the new compound **1** was assigned as 3-*O*-{β-D-galactopyranosyl-(1$-$2)-[β-D-glucopyranosyl-(1$\rightarrow$6)]-β-D-glucopyranoside}-5α,14α-androst-8-ene.

The analysis of the NMR data of **2** revealed the same aglycon as in **1** and a sugar chain comprised of two monosaccharides (Tables 2 and 3). Comparison of the ^1H NMR spectrum of **2** with that of **1** showed the absence of the galactopyranose anomeric proton signal at δ 4.60 and the upfield shift of the signal ascribable to H-2_{GlcI} (δ 3.22 in **2** vs δ 3.44 in **1**) (Table 3). Differences were also observed

Table 3. ^{1}H and ^{13}C NMR Data for the Sugar Portions of Compounds **1**–**4**[a] in Methanol-d_4

	1		**2**	**3**		**4**	
position	δ_C	δ_H (J in Hz)	δ_C	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
Glc-1	105.0	4.65 (d, 7.5)	104.8	104.8	4.56 (d, 7.5)	105.0	4.58 (d, 7.5)
2	82.9	3.44 (dd, 7.5, 9.0)	73.4	83.5	3.45 (dd, 7.5, 9.0)	83.5	3.42 (dd, 7.5, 9.0)
3	76.7	3.58 (t, 9.0)	78.0	77.4	3.40 (t, 9.0)	77.4	3.58 (t, 9.0)
4	71.2	3.30 (t, 9.0)	71.4	71.4	3.33 (t, 9.0)	71.2	3.30 (t, 9.0)
5	77.1	3.26 m	77.1	78.1	3.42 m	77.0	3.36 m
6a	67.8	3.80 (dd, 3.5, 12)	68.2	62.5	3.82 (dd, 3.5, 12)	64.2	4.28 (dd, 3.5, 12)
6b		3.95 (dd, 4.5, 12)			3.69 (dd, 4.5, 12)		3.98 (dd, 4.5, 12)
Gal-1	103.1	4.60 (d, 7.0)		102.8	4.62 (d, 7.0)	102.9	4.61 (d, 7.0)
2	73.6	3.35 (dd, 7.0, 9.0)		73.3	3.66 (dd, 7.0, 9.0)	73.5	3.65 (dd, 7.0, 9.0)
3	74.6	3.44 (dd, 3.0, 9.0)		74.8	3.38 (dd, 3.5, 9.0)	74.8	3.38 (dd, 3.0, 9.0)
4	69.6	3.86 m		70.0	3.86 m	69.8	3.88 m
5	76.9	3.60 m		76.8	3.58 m	77.0	3.60 m
6a	61.4	3.73 (dd, 2.5, 12)		61.4	3.74 (dd, 2.5, 12)	61.4	3.73 (dd, 2.5, 12)
6b		3.78 (dd, 4.5, 12)			3.78 (dd, 4.5, 12)		3.78 (dd, 4.5, 12)
Glc-2	103.6	4.52 (d, 7.6)	103.9				
2	73.5	3.28 (dd, 7.5, 9.0)	73.8				
3	78.4	3.46 (t, 9.0)	78.3				
4	71.2	3.30 (t, 9.0)	71.2				
5	77.9	3.42 m	77.9				
6a	62.8	3.80 (dd, 3.5, 12)	62.6				
6b		3.66 (dd, 4.5, 12)					
*CH$_3$*CO						21.8	1.95 (s)
CH$_3$*CO*						172.0	

[a] Assignments confirmed by 1D-TOCSY, DQF-COSY, HSQC, and HMBC experiments.

in the ^{13}C NMR spectra for the chemical shift of C-2 (73.4 ppm) and C-3 (78.0 ppm) of the inner glucopyranosyl unit. The configuration of the β-glucopyranosyl moieties was determined to be D by hydrolysis of **2**, trimethylsilylation, and GC analysis. On the basis of the above evidence, the new compound **2** was established as 3-*O*-[β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside]-5α,14α-androst-8-ene.

Compounds **3** and **4** gave molecular formulas of $C_{41}H_{62}O_{16}$ and $C_{43}H_{64}O_{17}$, respectively, as determined from ^{13}C, ^{13}C DEPT NMR, FABMS, and ESIMS analyses. The FABMS of **3** showed a $[M - H]^-$ ion at *m/z* 809 and prominent fragments at *m/z* 647 $[(M - H) - 162]^-$ and *m/z* 485 $[(M - H) - (162 + 162)]^-$, due to the sequential cleavage of two hexose units. The ^{13}C and ^{13}C DEPT NMR spectra showed 41 carbon signals, of which 29 were assigned to a stigmastane[13,14] aglycon and 12 to the sugar portion. The ESIMS of compound **3** showed a peak at *m/z* 253, consistent with the composition [aglycon-side chain-H_2O]$^+$. This indicated that the steroid-cyclic system of compound **3** had a molecular weight of 270 ($C_{19}H_{26}O$), identical to that in a series of several vernionosides reported previously.[13] This was ascribable to a $\Delta^{8(9),14(15)}$ steroidal nucleus with one hydroxyl group at C-3 by ^{13}C NMR spectroscopy, since the data for C-1 to C-19 were very similar to those reported for vernonioside D_1[13] (Tables 2 and 3). The extensive use of 1D and 2D NMR techniques led to the determination of an unusually oxygenated side chain ($C_{10}H_{15}O_5$), and analysis of these NMR data indicated that the side chain of **3** was the same as vernonioside D_1.[13,14] The structure elucidation of the sugar portion of **3** was achieved by 1D TOCSY,[15] DQF-COSY, HSQC, and HMBC experiments. Analysis of the 1D TOCSY and DQF-COSY spectra allowed the complete assignments for all proton resonances of the glucose and galactose units, starting from the anomeric proton signals. A HSQC experiment correlated all proton signal resonances in **3** with those of the corresponding carbons (Table 3). Data from the above experiments determined the sugar sequence, and the glycosylation shift for C-2 of the glucose unit allowed the structure of **3** to be defined with a glycoside chain linked to C-3. The relative stereochemistry of the side chain of **3** was established by the NOESY spectrum. Key correlations peaks were observed between H-21 and Me-29 and between Me-29 and H-25, suggesting that they were all on the α-face of the side chain. Correlations were also observed between H-20 and H-22, H-23 and between H-23 and Me-27. The ^{1}H and ^{13}C NMR data indicated a β-configuration for the galactopyranosyl and glucopyranosyl units.[10] The configuration of the sugar units was assigned after hydrolysis of **3** with 1 N HCl. The hydrolysate was trimethylsilated, and GC retention times of each sugar were compared with those of authentic samples prepared in the same manner. In this way, the sugar units of **3** were determined to be D-glucose and D-galactose. The structure 3β,21,24-trihydroxy-21,-23;22,28;26,28-triepoxy-5α-stigmasta-8(9),14(15)-dien-3-*O*-β-D-galactopyranosyl-(1→2)-β-D-glucopyranoside was assigned to **3**.

The FABMS of compound **4** gave a quasi-molecular anion peak at *m/z* 851 $[M - H]^-$ and fragments at *m/z* 809 $[(M - H) - 42]^-$, *m/z* 647 $[(M - H) - (42 + 162)]^-$ corresponding to the sequential loss of an acetyl group and an hexose unit. In the ^{1}H NMR spectrum of compound **4**, a signal at δ 1.95 (3H, s) and ^{13}C NMR signals at 21.8 and 172.0 ppm indicated the presence of an acetyl group. In the ^{1}H and ^{13}C NMR spectra (Table 4) on comparing **4** with **3**, the signals due to C-6 and H_2-6 of the glucose unit were displayed downfield and the C-5 signal was upfield, while the other signals remained almost unshifted. These data indicated that the acetyl group was located at C-6 of the glucose unit in **4**. The configuration of the β-glucopyranosyl and β-galactopyranosyl moieties was determined to be D by hydrolysis of **4**, trimethylsilylation, and GC analysis. On the basis of the foregoing data the structure of compound **4** was proposed as 3β,21,24-trihydroxy-21,23;22,-28;26,28-triepoxy-5α-stigmasta-8(9),14(15)-dien-3-*O*-β-D-galactopyranosyl-(1→2)-β-D-(6-*O*-acetyl)glucopyranoside.

The molecular formula of **5** was determined as $C_{29}H_{46}O_3$ by ESIMS at *m/z* 443 $[M + H]^+$ as well as from its ^{13}C NMR DEPT data. Compound **5** could be assigned from its ^{1}H NMR data with a $\Delta^{8(9),14(15)}$ conjugated diene at δ 5.82 (1H, br m), a terminal hydroxypropyl group [δ 1.22 (s) and 1.25 (s)], a secondary methyl [δ 1.02 (d)], two tertiary methyls [δ 0.80 (s) and 1.04 (s)], one hydroxymethylene group [δ 4.16 (d)], and one hydroxymethine group [δ 3.49 (m)]. These

Table 4. ^{1}H and ^{13}C NMR Data for Compounds **5** and **6**[a] in Methanol-d_4

	5		**6**	
position	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	36.4	2.01; 1.55	36.4	2.01; 1.55
2	32.0	1.51; 1.92	32.0	1.51; 1.92
3	71.0	3.49 m	71.3	3.49 m
4	38.0	2.12; 1.48	37.8	2.12; 1.48
5	41.0	1.51	41.0	1.51
6	22.8	1.57 br m	22.8	1.57
7	37.5	2.19; 1.35	37.5	2.19; 1.35
8	125.0		125.0	
9	142.4		142.4	
10	38.6		38.6	
11	28.0	2.05; 2.45	28.0	2.05; 2.45
12	26.5	1.39; 1.81	26.5	1.39; 1.81
13	45.6		45.4	
14	151.5		151.0	2.82
15	117.1	5.82 br m	117.4	1.09; 0.93
16	36.9	2.44 (dd, 11.0 and 3.0)	36.9	2.44 (dd, 11.0 and 3.0)
		2.41 (d, 11.0 and 5.0)		2.42 (dd, 1.0 and 5.0)
17	53.5	1.66 m	53.5	1.69
18	13.4	0.80 (s)	13.3	0.79
19	17.9	1.04 (s)	17.9	1.04
20	31.6	1.97 br m	33.6	1.27
21	17.8	1.02 (d, 6.0)	16.8	1.08 (d, 6.0)
22	36.9	1.87; 1.26	43.9	2.35; 2.00
23	28.2	2.13; 1.95	78.0	4.32 (t, 7.0)
24	147.4		70.0	
25	72.0		71.2	
26	28.0	1.22 (s)	28.4	1.37 (s)
27	28.4	1.25 (s)	28.2	1.40 (s)
28	124.0	5.40 (br t, 6.5)	64.0	3.06 (q, 5.5)
29	59.0	4.16 (d, 6.5)	14.5	1.70 (d, 5.5)

[a] Assignments confirmed by 1D-TOCSY, DQF-COSY, HSQC, and HMBC experiments.

data, in combination with fragment ions at m/z 425 $[M - H_2O]^+$, 253 [loss of side chain $(C_{19}H_{26}O)-H_2O$], suggested that **5** is based on a 3-hydroxystigmastane skeleton, possessing a $\Delta^{8(9),14(15)}$ diene system and a C-10 side chain containing a hydroxypropyl group.[14,15] A combination of 2D NMR experiments delineated for the side chain three main connectivities: the first one comprised C-15, C-16, C-17, C-20, C-22, and C-23, the second was C-21, C-20, and C-17, and the third was C-28 and C-29. The ^{1}H NMR signals of CH_2-29 and CH-28 appeared at δ 4.16 (2H, d, $J = 6.5$ Hz) and 5.40 (1H, br t, $J = 6.5$ Hz), respectively, indicating a $-CH_2OH$ group linked to a sp^2 carbon, while the signal of Me-21 appeared as a doublet at δ 1.02 (3H, d, $J = 6.0$ Hz). The elucidation of the basic carbon skeleton of **5** from the above subunits was achieved on the basis of a series of ^1J (HSQC) and ^3J (HMBC) correlations, which also allowed the assignment of the resonances in the ^{13}C NMR spectrum to the pertinent carbons (Table 4). Key correlation peaks were observed between H-3 and C-4, C-5, C-1; Me-19 and C-1, C-5, C-9, C-8; Me-18 and C-17, C-14; H-15 and C-8, C-17, C-13; Me-21 and C-17, C-22; H-28 and C-29, C-25, C-23; and Me-26 and C-24, C-27.

A 2D ROESY[16] experiment showing NOE cross-peaks among protons spatially correlated (particularly those correlating Me-18, Me-19 and H-6$_{ax}$ and H-3 with H-5) led us to establish the relative stereochemistry. These data were confirmed by the chemical shifts of carbons of a cyclopentanoperhydrophenanthrene system which matched well with those of related steroids.[17] NOE effects observed in the ROESY spectrum between H-28 and Me-27 allowed the Z stereochemistry at the $\Delta^{24(28)}$ double bond to be ascertained. Thus, compound **5** was established as 3β,25,-29-trihydroxy-5α-stigmasta-8(9),14(15),24Z(28)-triene.

Compound **6** in the ESIMS revealed a molecular ion at m/z 459 $[M + H]^+$. The molecular formula, deduced also by ^{13}C and ^{13}C DEPT NMR data, was $C_{29}H_{46}O_4$. The MS and NMR data demonstrated that **6** possesses the same steroidal structure from C-1 to C-19 as compound **5**.

The ^{1}H NMR spectrum of **6** showed the presence of two tertiary methyls (δ 1.37 and 1.40 ppm) and two secondary methyl signals (δ 1.08, d, $J = 6.0$ Hz and 1.70, d, $J = 5.5$ Hz) as well a proton at δ 4.32 (1H, t, $J = 7.0$ Hz). The tertiary methyls, linked to a carbinol (C-25), were assigned to C-26 and C-27 for a *tert*-hydroxypropyl group (Table 4). A methyl signal resonating at δ 1.70, which was coupled in the 1D TOCSY experiment to a proton at δ 3.06 (1H, q, $J = 5.5$ Hz), was placed at C-28, forming an epoxide with C-24 (Table 4), and the remaining methyl signal at δ 1.08 was assigned to Me-21 of an open side chain on the basis of 2D NMR experiments. Analysis of the 1D TOCSY and DQF-COSY NMR data starting from the Me-21 signal suggested the partial sequence C-21, C-20, C-22, C-23 and led to the assignment of a C-23 hydroxy substituent at δ 4.32 (1H, t, $J = 7.0$ Hz).[15] To confirm the proposed structure of the side chain of compound **6**, a HMBC experiment was conducted, in which significant cross-peaks were seen between H-21 and C-17, C-22; H-15 and C-8, C-17; H-23 and C-20, C-28, C-25; Me-27 and C-24; and Me-26 and C-24. The relative stereochemistry of the epoxide ring was ascertained by evaluation of NOE effects recorded between the signal at H-28 and the signals of isopropyl protons. The configuration of the C-23 OH was undetermined. Thus the structure of compound **6** was defined as 3β,23,25-trihydroxy-24,28-epoxy-5α-stigmasta-8(9),14(15)-diene.

Pure compounds **1**−**4**, tested using a carrageenan-induced rat paw model,[5] were inactive, while compounds **5** and **6** caused weak inhibition at 3 h of the inflammatory response. As the tested doses were low (1.8; 1.3; 1.08; 1.7; 2.08; 1.67 mg/kg, respectively), a further set of experiments was performed at higher doses (4 times the calculated doses); however, no significant increase in the inhibition of edema was seen, while the reference compound, indo-

methacin (5 mg/kg), was active. On the basis of our results, we can hypothesize that the strong anti-inflammatory activity of the $CHCl_3$−MeOH (9:1) extract and the initial fractions may be due to the presence of a combination of compounds acting synergistically or vehicles enhancing the biological activity. However, we cannot rule out that the activity of the extracts and fractions could be due to the very minor compounds or compounds not isolated.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 241 polarimeter equipped with a sodium lamp (589 nm) and a 1 dm microcell. UV spectra were recorded on a Beckman DU 670 spectrophotometer. A Bruker DRX-600 NMR spectrometer, operating at 599.19 MHz for ^{1}H and 150.86 MHz for ^{13}C, using the UXNMR software package was used for NMR experiments; chemical shifts are expressed in δ (ppm) referring to solvent peaks at δ_H 3.34 and δ_C 49.0 for CD_3OD. DEPT ^{13}C, 1D TOCSY, ^{1}H−^{1}H DQF-COSY, ^{1}H−^{13}C HSQC, and HMBC NMR experiments were carried out using the conventional pulse sequences as described in the literature. ESIMS (positive mode) were obtained from a Finningan LC-Q Deca Thermoquest spectrometer, equipped with Xcalibur software. FABMS were recorded in a glycerol matrix in the negative ion mode on a VG ZAB instrument (Xe atoms of energy 2−6 kV). GC analyses were performed using a Perkin- Elmer Sigma-115 gas chromatograph with a data-handling system and FID. Elemental analysis was performed using a MOD.1106 elemental analyzer. Column chromatography was performed over Sephadex LH-20 (Pharmacia). HPLC separations were conducted on a Waters 590 series pumping system equipped with a Waters R401 refractive index detector and with a Waters μ-Bondapak C_{18} column and U6K injector.

Plant Material. Leaves of *V. colorata* were collected from the V region of Mali, near Bandiagara, in 1998. The plant material was identified by Prof. N'Golo Diarra, Botanist of DMT, Bamako, Mali, where a voucher specimen has been deposited (voucher number DMT196).

Extraction and Isolation. The leaves of *V. colorata* (500 g) were dried at 40 °C, and the dried material was then powdered and submitted to sequential extraction with petroleum ether (3 g), chloroform (10.5 g), chloroform−methanol (9:1) (17.5 g), and methanol (10.5 g) by extensive maceration (3 times × 2 L), corresponding to 0.6, 2.1, 3.5, and 2.1% of the dry plant material, respectively. The $CHCl_3$−MeOH (9:1) residue (8 g) was chromatographed on a Sephadex LH-20 column (100 × 4 cm) with MeOH as the eluent. Fractions (10 mL) were eluted and combined to give five main fractions, I−V (2.2 g; 0.5 g; 0.6 g; 1.1 g; 2.4 g, respectively). Fraction II (500 mg) was purified by HPLC on a Waters μ-Bondapack C_{18} column (30 cm × 7.8 mm, flow rate 3 mL min^{-1}) using MeOH−H_2O (1:1) as the eluent to yield pure compounds **1** (t_R = 26 min, 60 mg), **2** (t_R = 29 min, 44 mg), **3** (t_R = 19 min, 36 mg), and **4** (t_R = 12.5 min, 56 mg). Fraction III was separated by RP-HPLC with MeOH−H_2O as the eluent (7:3) to yield pure compounds **4** (t_R = 10 min, 58 mg), **5** (t_R = 28 min, 74 mg), and **6** (t_R = 30 min, 56 mg).

Compound 1: white amorphous powder (MeOH); $[\alpha]^{25}_D$ +109° (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 236, 242, 250 nm; ^{1}H and ^{13}C NMR data (CD_3OD, 600 MHz), see Tables 2 and 3; FABMS *m/z* 759 [M − H]$^-$, 597 [(M − H) − 162]$^-$, 273 [(M − H) − (162 + 162 + 162)]$^-$; *anal.* C 58.38%, H 7.98%, O 33.64%, calcd for $C_{37}H_{60}O_{16}$, C 58.41%, H 7.95%, O 33.64%.

Compound 2: white amorphous powder (MeOH); $[\alpha]^{25}_D$ +70° (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 236, 242, 250 nm; ^{1}H and ^{13}C NMR data (CD_3OD, 600 MHz), see Tables 2 and 3; FABMS *m/z* 597 [M − H]$^-$, 273 [(M − H) − (162 + 162)]$^-$; *anal.* C 62.14%, H 8.46%, O 29.40%, calcd for $C_{31}H_{50}O_{11}$, C 62.19%, H 8.42%, O 29.39%.

Compound 3: white amorphous powder (MeOH); $[\alpha]^{25}_D$ +57° (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 236, 242, 250 nm; ^{1}H and ^{13}C NMR data (CD_3OD, 600 MHz), see Tables 2 and 3; FABMS *m/z* 809 [M − H]$^-$, 647 [(M − H) − 162]$^-$, 485 [(M − H) − (162 + 162)]$^-$; ESIMS *m/z* 811 [M + H]$^+$, 254; *anal.* C 60.70%, H 7.76%, O 31.54%, calcd for $C_{41}H_{62}O_{16}$, C 60.73%, H 7.71%, O 31.57%.

Compound 4: white amorphous powder (MeOH); $[\alpha]^{25}_D$ +61° (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 236, 242, 250 nm; ^{1}H and ^{13}C NMR data (CD_3OD, 600 MHz), see Tables 2 and 3; FABMS *m/z* 851 [M − H]$^-$, 809 [(M − H) − 42]$^-$, 647 [(M − H) − (162 + 42)]$^-$; *anal.* C 60.49%, H 7.60%, O 31.91%, calcd for $C_{43}H_{64}O_{17}$, C 60.55%, H 7.56%, O 31.89%.

Compound 5: white crystals (MeOH); $[\alpha]^{25}_D$ +48° (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 236, 242, 250 nm; ^{1}H and ^{13}C NMR data (CD_3OD, 600 MHz), see Table 4; ESIMS *m/z* 443 [M + H]$^+$, 254; *anal.* C 78.62%, H 10.50%, O 10.88%, calcd for $C_{29}H_{46}O_3$, C 78.68%, H 10.47%, O 10.84%.

Compound 6: white crystals (MeOH); $[\alpha]^{25}_D$ +25° (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 236, 244, 252 nm; ^{1}H and ^{13}C NMR data (CD_3OD, 600 MHz), see Table 4; ESIMS *m/z* 459 [M + H]$^+$, 271; *anal.* C 75.91%, H 10.15%, O 13.94%, calcd for $C_{29}H_{46}O_4$, C 75.94%, H 10.11%, O 13.95%.

Acid Hydrolysis of Compounds 1−4. A solution of compounds **1**−**4** (2.0 mg each) in 1 N HCl (1 mL) was stirred at 80 °C in a stoppered reaction vial for 4 h. After cooling, the solution was concentrated by blowing with N_2. The residue was dissolved in 1-(trimethylsilyl)imidazole and pyridine (0.2 mL), and the solution was stirred at 60 °C for 5 min. After drying the solution, the residue was separated by water and $CHCl_3$. The $CHCl_3$ layer was analyzed by GC using a 1-Chirasil-Val-column (0.32 mm × 25 m). Temperatures of the injector and detector were 200 °C for both. A temperature gradient system was used for the oven, starting at 100 °C for 1 min and increasing up to 180 °C at a rate of 5 °C/min. Peaks of the hydrolysate were detected by comparison with retention times of authentic samples of D-galactose and D-glucose (Sigma Aldrich, St. Louis, MO) after being treated with 1-(trimethylsilyl)imidazole in pyridine.

In Vivo Anti-inflammatory Activity. Male Wistar rats (Nossan strain, Correzzana, Italy) were housed in an environment with controlled temperature (21−24 °C), lighting (12:12 light:darkness cycle), standard laboratory chow, and drinking water ad libitum, for a period of 7 days before any experimental manipulation. Their body weights ranged from 120 to 140 g. All experiments were conducted according to guidelines established by the Animal Care Committee. A basal recording of paw volume was made using a hydroplestymometer (Ugo Basile, Milano, Italy) to establish in vivo rat paw edema. Groups of least eight animals each received po tested extracts, fractions, and pure compounds of *V. colorata* (200 mg/kg corresponding to 200 mg of extract) or indomethacin (5 mg/kg, Sigma) suspended in 0.5% carboxymethylcellulosa (Sigma) as a reference drug, while the control group received vehicle only. For the evaluation of the effects of extracts of *V. colorata* and indomethacin on edema formation, the rats were treated with extracts, fractions, and pure compounds of *V. colorata* (200 mg/kg or corresponding to 200 mg of extract) and indomethacin (5 mg/kg po) 1 h before the subplantar injection of carrageenan. The above-mentioned test sample (1% w/v in NaCl 0.9%; 0.1 mL/rat) was injected into the subplantar region of the paw. The paw volume was determined immediately before and then each hour for 5 h after the carrageenan injection. Edema was calculated in comparison to the initial value according to the method of Winter et al.[5] Results are reported as means ± SEM of measurements of at least eight different rats. Comparisons among groups were performed using a two-sided Student's test with Bonferroni correction. With all statistical analyses, an associated probability (*p* value) of less than 5% ($p < 0.05$) was considered as significant.

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