

Vernomigeodiins A–D, highly oxygenated stigmastane-type steroids isolated from *Vernoniastrum migeodii* with anti-herpes simplex virus activity

Morteza Yazdani ^{a, ID}, Anita Barta ^{a, b}, Anasztázia Hetényi ^c, Muhammad Bello Saidu ^{a, ID}, Umar Shehu Gallah ^d, Róbert Berkecz ^e, Antal Csámpai ^f, Katalin Burián ^g, Dóra Paróczai ^{g, h}, Sara H.H. Ahmed ^{i, ID}, Judit Hohmann ^{a, b, *}

^a HUN-REN–USZ Biologically Active Natural Products Research Group, University of Szeged, Eötvös Str. 6, 6720, Szeged, Hungary

^b Department of Pharmacognosy, University of Szeged, Eötvös Str. 6, 6720, Szeged, Hungary

^c Department of Medical Chemistry, University of Szeged, Dóm Square 8, 6720, Szeged, Hungary

^d Bioresource Department, National Research Institute for Chemical Technology (NARICT), Zaria, Nigeria

^e Institute of Pharmaceutical Analysis, University of Szeged, Somogyi Str. 4, 6720, Szeged, Hungary

^f Department of Organic Chemistry, Eötvös Loránd University, Pázmány Péter sétány 1/a, 1117, Budapest, Hungary

^g Department of Medical Microbiology, Albert Szent-Györgyi Health Center and Albert Szent-Györgyi Medical School, University of Szeged, Dóm Square 10, 6720, Szeged, Hungary

^h Department of Pulmonology, Faculty of Medicine, University of Szeged, Alkotmány Str. 36, 6772, Deszk, Hungary

ⁱ Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Khartoum, AlQasr Avenue, 11111, Khartoum, Sudan

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ABSTRACT

Previously undescribed steroids vernomigeodiins A–D (1–4), were isolated from the African medicinal plant *Vernoniastrum migeodii* along with known sterols 5–10 and the tripeptide aurantiamide acetate (11). The isolated steroids featured a stigmastane skeleton with a unique conjugated $\Delta^{7,9(11)}$ -diene segment and a highly oxidized side chain, occasionally forming a bi- or tricyclic ring system. Sterols 1–3, 5–9 are glucosylated, whereas 4 and 10 are aglycons. The structures of the compounds were elucidated on the basis of 1D and 2D NMR spectroscopy and HRESIMS analyses. The isolated sterols were evaluated for their antiviral activities against *Herpes simplex* virus type 2. First, their cytotoxicity was assessed in Vero cells using the standard MTT assay, and direct quantitative PCR was then used to evaluate the antiviral activity. Vernomigeodiins A (1) and B (4) and vernonioside B₁ (6) were not cytotoxic up to a concentration of 10 μ M but reduced the HSV-2 viral DNA levels with IC₅₀ values between 0.25 and 0.56 μ M. In addition, vernomyoside B (9) showed a direct virucidal effect (IC₅₀ = 1.23 $\pm$ 0.15 μ M). The interactions between the antiviral compounds and specific viral targets were investigated using molecular docking.

1. Introduction

Herpes simplex virus type 2 (HSV2), a member of the Herpesviridae family, establishes lifelong latency in the sacral and trigeminal ganglia following primary infection. Periodic reactivation leads to recurrent genital ulcerations and contributes to the virus's widespread prevalence in both immunocompetent and immunocompromised individuals. According to the World Health Organization, approximately 431 million people between the ages 15 and 49 years are infected with HSV2. In addition to mucocutaneous manifestations, active infection can result in severe complications such as meningitis, encephalitis, and increased

susceptibility to HIV (Horváth et al., 2024).

Computer-aided drug design constitutes an important tool that allows scientists to investigate possible interactions of lead compounds against certain targets and to anticipate the reasons beyond observed biological activities that might be exhibited by these compounds (Zhang et al., 2022). The HSV-2 protease enzyme and the HSV-2 gD protein constitute two important targets that have prime role in viral replication and entry to host cells, respectively (Stamos et al., 2022).

Plant-derived triterpenes have shown promising antiviral activity against herpesviruses. Notably, lanostane-type triterpenes isolated from *Euphorbia deightonii* have demonstrated effective inhibition of HSV2

* Corresponding author. HUN-REN–USZ Biologically Active Natural Products Research Group, University of Szeged, Eötvös Str. 6, 6720, Szeged, Hungary.
E-mail address: hohmann.judit@szte.hu (J. Hohmann).

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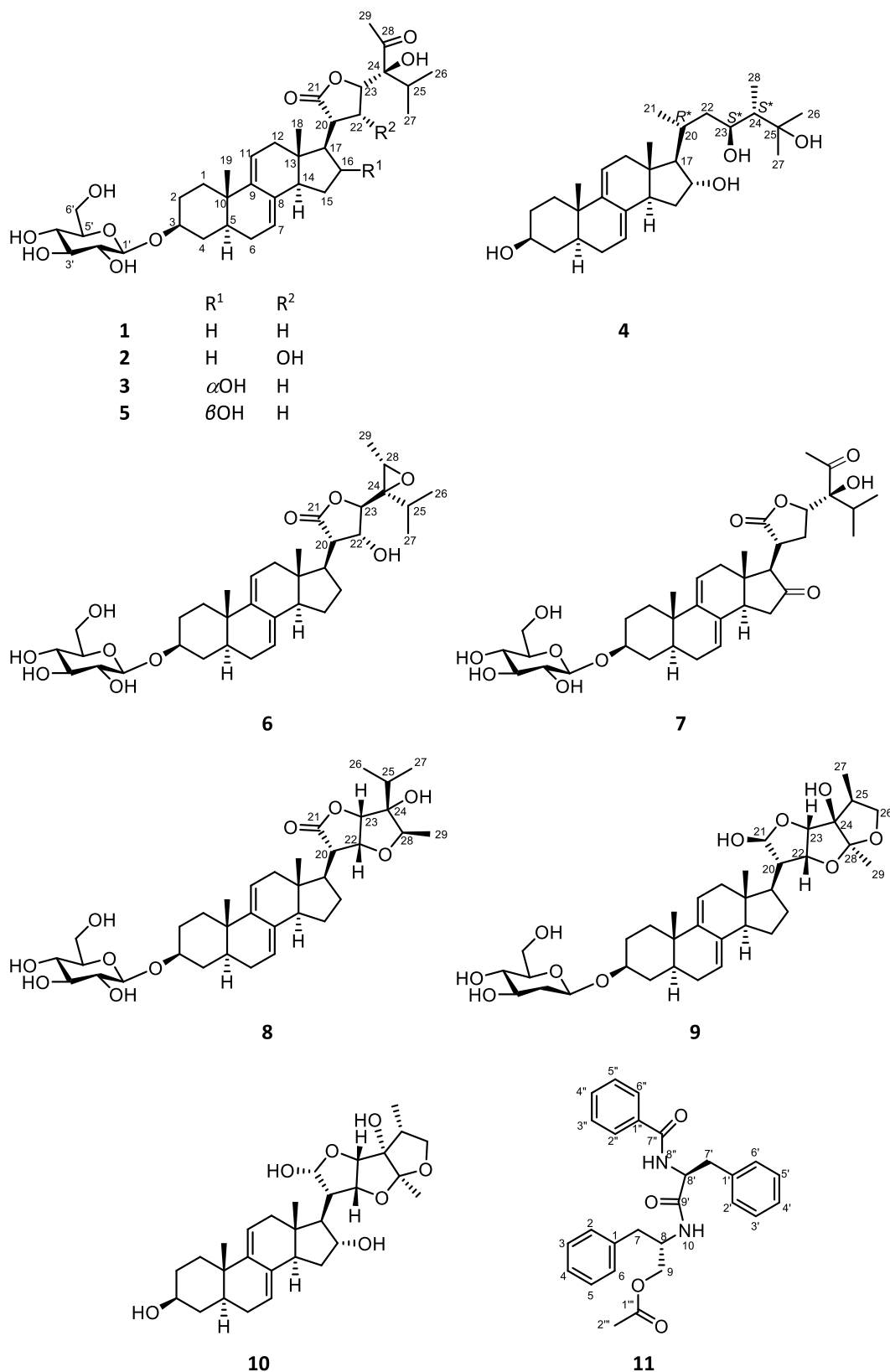


Fig. 1. Structures of compounds 1–11.

replication (Saidu et al., 2022). Similarly, lupenone, a triterpene isolated from *Euphorbia segetalis*, exhibited potent plaque-reduction activity against both HSV-1 and HSV-2 (Madureira et al., 2003). Screening of *Eucalyptus globulus* revealed that tereticornate, its triterpenoid

constituent, demonstrated considerable antiviral activity against HSV-1, boasting a selectivity index (SI) slightly higher than that of acyclovir (Brezáni et al., 2018). Cucurbitacin B has emerged as one of the most potent compounds, which exhibited excellent antiviral efficacy (IC₅₀ =

Table 1¹H NMR data of compound 1–4 (CD₃OD, δ ppm, J = Hz).

No.	1 ^a	2 ^b	3 ^b	4 ^b
δ_H, mult. (J in Hz)				
1 α	1.36 m	1.37 m	1.33 m	1.34 m
1 β	2.01 m	2.00 m	1.86 m	1.98 dt (11.5, 2.9)
2 α	1.98 m	1.95 m	1.99 m	1.85 m
2 β	1.61 m	1.29 m	1.60 m	1.48 m
3	3.70 m	3.70 m	3.71 m	3.52 m
4 α	1.89 m	1.88 m	1.95 m	1.71 m
4 β	1.34 m	1.34 m	1.32 m	1.29 m
5	1.41 m	1.41 m	1.41 m	1.43 m
6	1.93 m (2H)	1.93 m (2H)	1.93 m (2H)	1.92 m (2H)
7	5.42 brs	5.42 brs	5.40 s	5.40 s
11	5.54 d (6.9)	5.53 d (6.8)	5.54 d (6.5)	5.50 d (7.0)
12	2.04 d (16.9)	2.04 m	2.00 d (17.7)	2.21
	2.41 dd (16.9, 6.9)	2.52 dd (17.9, 6.8)	2.44 dd (17.7, 6.5)	d (17.6) 2.30 dd (17.6, 7.0)
14	2.23 m	2.24 m	2.54 br t (6.9)	2.47 m
15 α	1.86 m	1.85 m	1.74 ddd	1.72 m
15 β	1.49 m	1.50 m	(13.2, 7.4, 5.7) 1.95 m	1.85 m
16 α	1.77 m	1.92 m	4.24 t (7.7)	4.16 dd (9.6, 6.0)
16 β	1.59 m	1.78 m		
17	2.04 m	2.05 m	1.92 t (7.7)	1.35 m
18	0.53 s	0.63 s	0.53 s	0.53 s
19	0.92 s	0.93 s	0.92 s	0.91 s
20	2.86 ddd (11.2, 9.2, 6.1)	2.58 dd (8.8, 6.2)	2.92 ddd (12.0, 9.0, 6.5)	1.75 m
22 α	2.22 m	4.27 t (6.2)	2.42 m	1.29 m
22 β	2.07 m		2.10 m	1.86 m
23	4.76 dd (10.1, 5.6)	4.57 d (5.3)	4.81 dd (9.7, 6.7)	4.17t (6.5)
24				3.14 q (6.5)
25	2.14 sept (6.9)	2.14 sept (6.7)	2.15 m	
26	1.00 d (6.9)	1.00 d (6.7)	1.01 d (7.0)	1.37 s
27	0.94 d (6.9)	1.00 d (6.7)	0.96 d (7.0)	1.36 s
28	–	–	–	1.56 d (6.5)
29	2.23 s	2.24 s	2.24 s	
1'	4.40 d (8.1)	4.40 d (8.1)	4.40 d (8.0)	
2'	3.14 t (8.1)	3.15 t (8.1)	3.15 t (8.0)	
3'	3.35 dd (9.5, 8.1)	3.35 dd (10.6, 8.1)	3.35 dd (10.6, 8.0)	
4'	3.26 m	3.26 m	3.26 m	
5'	3.27 m	3.27 m	3.27 m	
6'	3.65 dd (11.6, 5.3)	3.66 dd (11.8, 5.3)	3.66 dd (11.8, 5.3)	
	3.85 dd (11.6, 1.1)	3.85 dd (11.8, 1.5)	3.86 br d (11.8)	

^a 500 MHz.^b 600 MHz.

0.94 μ M and SI = 127.7) as well as notable antibacterial effects (Hassan et al., 2017). Furthermore, ursolic acid isolated from *Mallotus peltatus*, appeared to act during the early stage of HSV replication (0–5 h post-infection), achieving an SI of 20, supporting its potential as a valuable antiviral agent (Bag et al., 2012).

The genus *Vernoniastrum* H. Rob. was first published in 1999 (Robinson, 1999) and includes 13 accepted species native to Tropical and South Africa and Madagascar. *Vernoniastrum migeodii* (S. Moore) Isawumi (syn. *Vernonia migeodii* S. Moore, *Vernonia courtetii* O. Hoffm. et Muschl.) (Asteraceae) is a perennial herb widely distributed in Benin, Cameroon, Central African Republic, Chad, Ghana, and Nigeria. *V. migeodii* was traditionally used to treat malaria, diarrhea, and skin infections (Orabueze et al., 2016). However, despite its cultural and medicinal relevance, the phytochemical profile of this species remains largely unexplored. In a previous study, only the composition of the essential oils distilled from the leaf and stem bark was investigated, finding sesquiterpenoids germacrene D, β -caryophyllene, and caryophyllene oxide as the main compounds in the leaf oil and the same quantity of monoterpenes α -phellandrene, *p*-cymene, and α -pinene

Table 2¹³C NMR data of compounds 1–4, 6, 8, and 10 (CD₃OD, δ ppm).

No.	1 ^a	2 ^b	3 ^b	4 ^b	6 ^a	8 ^a	10 ^a
δ_C, type							
1	36.1, CH ₂	36.0, CH ₂	35.0, CH ₂	35.9, CH ₂	35.0, CH ₂	36.0, CH ₂	35.9, CH ₂
2	30.6, CH ₂	30.6, CH ₂	30.6, CH ₂	32.2, CH ₂	30.6, CH ₂	30.6, CH ₂	32.2, CH ₂
3	78.9, CH	79.0, CH	78.9, CH	71.5, CH	79.0, CH	79.0, CH	71.4, CH
4	35.0, CH ₂	35.1, CH ₂	36.0, CH ₂	38.4, CH ₂	36.0, CH ₂	35.0, CH ₂	38.5, CH ₂
5	40.5, CH	40.5, CH	40.6, CH	40.7, CH	40.5, CH	40.5, CH	40.6, CH
6	31.0, CH ₂	31.0, CH ₂	31.0, CH ₂	31.0, CH ₂	31.1, CH ₂	31.0, CH ₂	31.0, CH ₂
7	121.7, CH	121.5, CH	121.8, CH	121.8, CH	121.3, CH	121.3, CH	122.0, CH
8	137.3, C	137.4, C	136.7, C	136.9, C	137.6, C	137.6, C	136.7, C
9	145.5, C	145.3, C	145.3, C	145.3, C	144.8, C	145.0, C	145.2, C
10	37.2, C	37.1, C	37.2, C	37.0, C	37.0, C	37.0, C	37.0, C
11	119.5, CH	119.9, CH	119.4, CH	119.4, CH	120.5, CH	120.4, CH	119.1, CH
12	41.5, CH ₂	41.7, CH ₂	41.9, CH ₂	43.8, CH ₂	42.8, CH ₂	42.4, CH ₂	41.5, CH ₂
13	43.5, C	43.5, C	44.1, C	44.4, C	43.3, C	43.3, C	44.6, C
14	52.2, CH	52.5, CH	49.7, CH	50.0, CH	52.8, CH	52.7, CH	49.2, CH
15	24.1, CH ₂	24.0, CH ₂	36.0, CH ₂	36.7, CH ₂	24.2, CH ₂	24.2, CH ₂	35.2, CH ₂
16	25.4, CH ₂	27.1, CH ₂	75.3, CH	76.8, CH	28.5, CH ₂	29.5, CH ₂	77.5, CH
17	51.5, CH	50.9, CH	60.7, CH	66.6, CH	46.3, CH	47.7, CH	59.6, CH
18	13.1, CH ₃	12.4, CH ₃	14.0, CH ₃	12.9, CH ₃	12.6, CH ₃	12.4, CH ₃	13.9, CH ₃
19	19.9, CH ₃	19.9, CH ₃	19.9, CH ₃	19.9, CH ₃	19.9, CH ₃	19.9, CH ₃	19.9, CH ₃
20	41.5, CH	51.0, CH	40.6, CH	32.9, CH	51.4, CH	47.6, CH	54.0, CH
21	180.5, C	178.4, C	180.4, C	19.8, CH ₃	177.8, C	178.6, C	105.2, CH
22	28.1, CH ₂	72.9, CH	28.5, CH ₂	39.0, CH ₂	73.8, CH	80.1, CH	85.2, CH
23	82.4, CH	88.4, CH	82.3, CH	70.5, CH	80.5, CH	83.8, CH	86.3, CH
24	85.2, C	85.1, C	85.2, C	57.4, CH	65.2, C	83.0, C	88.2, C
25	35.5, CH	35.6, CH	35.7, CH	71.1, C	31.0, CH	31.6, CH	41.7, CH
26	17.4, CH ₃	17.4, CH ₃	17.6, CH ₃	28.3, CH ₃	18.4, CH ₃	17.3, CH ₃	73.7, CH
27	17.6, CH ₃	17.9, CH ₃	17.5, CH ₃	26.9, CH ₃	18.6, CH ₃	17.8, CH ₃	11.2, CH ₃
28	215.0, C	214.4, C	214.8, C	14.4, CH ₃	57.6, CH	81.9, CH	118.3, C
29	29.6, CH ₃	29.2, CH ₃	29.5, CH ₃		13.1, CH ₃	14.0, CH ₃	21.8, CH ₃
1'	102.4, CH	102.4, CH	102.4, CH		102.4, CH	102.4, CH	
2'	75.2, CH	75.2, CH	75.2, CH		75.2, CH	75.2, CH	
3'	78.1, CH	78.2, CH	78.2, CH		78.1, CH	78.1, CH	
4'	71.7, CH	71.8, CH	71.8, CH		71.7, CH	71.7, CH	
5'	77.9, CH	77.9, CH	77.9, CH		77.9, CH	77.9, CH	
6'	62.8, CH ₂	62.9, CH ₂	62.9, CH ₂		62.8, CH ₂	62.8, CH ₂	

^a 125 MHz.^b 150 MHz.

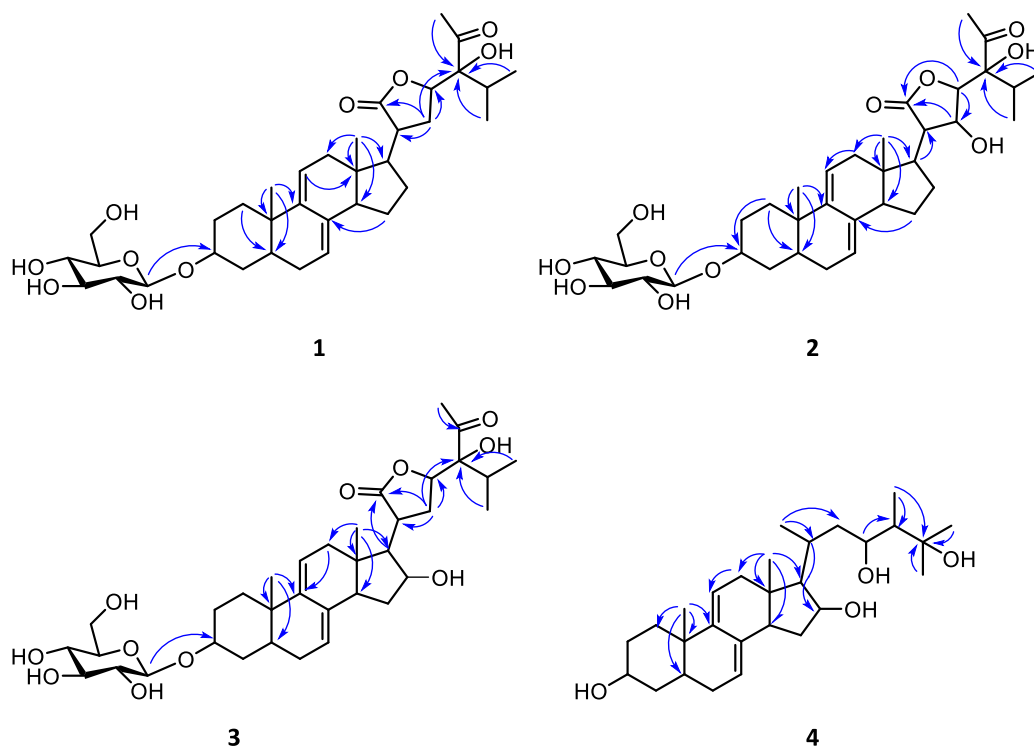


Fig. 2. Key HMBC correlations of vernomigeodiin A-D (1–4).

along with sesquiterpenes germacrene D, (*E*)- β -farnesene, caryophyllene oxide, and β -caryophyllene in the stem oil (Ogunbinu et al., 2009).

This study presents the first investigation of the non-volatile compounds of the aerial parts of *V. migeodii*, with a primary focus on the triterpene-containing chloroform phase of the methanolic extract, and the evaluation of the cytotoxic and anti-HSV2 activities of the isolated compounds.

2. Results and discussion

2.1. Compound identification

The chloroform fraction of the MeOH extract prepared from the aerial part of *V. migeodii* was subjected to multistep chromatographic purification, affording compounds 1–11, including previously undescribed vernomigeodiins A–D (1–4) (Fig. 1). The structures of the isolated compounds were determined using spectroscopic analyses,

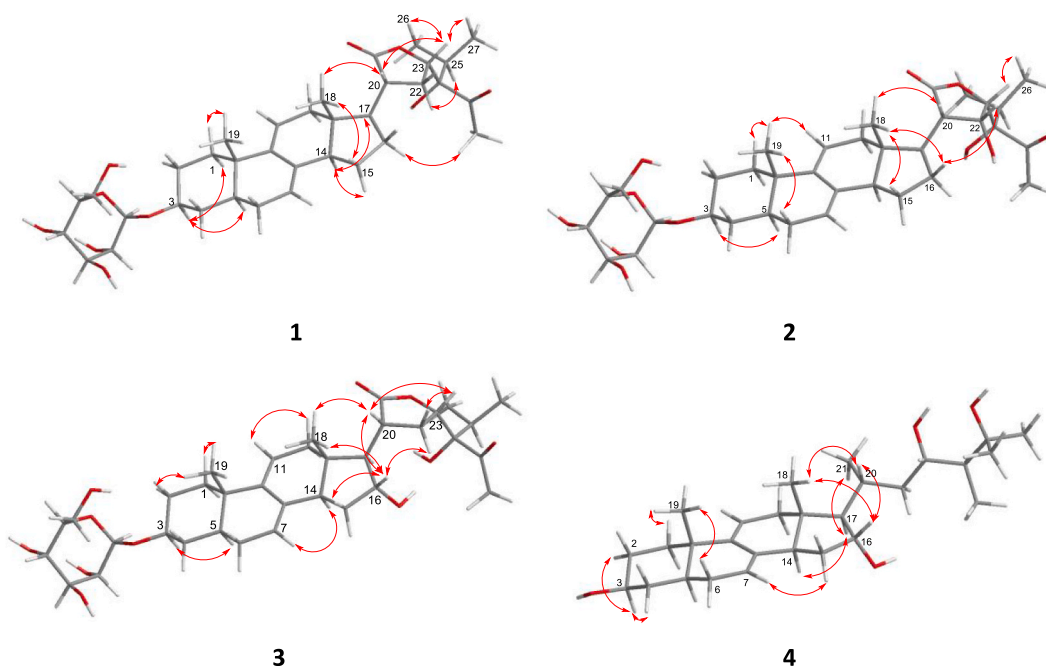


Fig. 3. Key NOESY correlations of vernomigeodiin A-D (1–4).

including HRESIMS, 1D [^1H and ^{13}C J -modulated spin-echo (JMOD)] and two-dimensional (2D) NMR experiments, i.e., correlation (^1H - ^1H COSY), nuclear Overhauser effect (NOESY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC) spectra.

Vernomigeodiin A (**1**) was isolated as a colorless amorphous solid. The molecular formula of compound **1** was determined to be $\text{C}_{35}\text{H}_{52}\text{O}_{10}$ on the basis of the peak at m/z 633.3638 $[\text{M} + \text{H}]^+$ (calcd for $\text{C}_{35}\text{H}_{53}\text{O}_{10}^+$ 633.3638) in positive-ion HRESIMS, corresponding to 10 degrees of unsaturation. The ^1H NMR, ^{13}C NMR JMOD, and HSQC spectra of compound **1** revealed the presence of seven quaternary carbons, nine methines, eight methylenes, five methyls, and a glucose moiety in the molecule (Tables 1 and 2).

The NMR spectra of **1** contained signals for one isopropyl group [δ_{H} 2.14 sept (1H, $J = 6\text{--}9$ Hz), 1.00 d (3H, $J = 6.9$ Hz), 0.94 d (3H, $J = 6.9$ Hz; δ_{C} 35.5, 17.4, 17.6], one acetate group [δ_{H} 2.23 s (3H); δ_{C} 29.6, 215.0], two angular methyls [δ_{H} 0.53 s (3H), 0.92 s (3H); δ_{C} 13.1, 19.9], and two trisubstituted olefins [δ_{H} 5.42 brs (1H), 5.54 d (1H, $J = 6.9$ Hz); δ_{C} 119.5, 121.7, 137.3, 145.5]. Typical carbon (δ_{C} 102.4, 75.2, 78.1, 71.7, 77.9, and 62.8) and proton signals (δ_{H} 4.40 d, 3.14 t, 3.35 dd, 3.26 m, 3.27 m, 3.65 dd, 3.85 dd) indicated the presence of a glucose moiety (Anh et al., 2021; Quasie et al., 2016). These data showed that compound **1** might be a stigmastane-type steroid glycoside. The HMBC and ^1H - ^1H COSY spectra provided information on the position of the substituents. Thus, a HMBC correlation of the anomeric proton H-1' [δ_{H} 4.40 d (1H, $J = 8.1$ Hz)] with C-3 (δ_{C} 78.9) indicated that the glucose moiety was connected to C-3 (Fig. 2).

The position of the olefin groups was determined from the sequence of correlated protons in the ^1H - ^1H COSY spectrum, which showed signals at δ_{H} 5.42 brs, 1.93 m, 1.41 m and δ_{H} 5.54 d, 2.04 d, and 2.41 dd, indicating the partial structures of $=\text{CH}-\text{CH}_2-\text{CH}-(=\text{C}-7-\text{C}-6-\text{C}-5-)$ and $=\text{CH}-\text{CH}_2-(=\text{C}-11-\text{C}-12-)$, and HMBC correlations of H₂-12 and H₃-19 with C-9 (δ_{C} 145.5) and of H-15a with C-8 (δ_{C} 137.3). With regard to the 10 degrees of unsaturation, the structural fragment connecting to C-17 must be a monocyclic moiety. Specifically, the HMBC correlations of H-22 (δ_{H} 2.04) with C-21 (δ_{C} 180.5) and of H-22 (δ_{H} 2.22 m) with C-20 (δ_{C} 41.5) and C-23 (δ_{C} 82.4) revealed the presence of a disubstituted γ -butyrolactone ring (C-20–C-23). Meanwhile, the acetate and isopropyl groups are connected to the quaternary carbon at δ_{C} 85.2 (C-24), as confirmed by the HMBC cross-peaks of the isopropyl methyls (H₃-26, H₃-27) and acetate methyl (H₃-29) with C-24. The chemical shift of C-24 indicates that this quaternary carbon must be connected to a hydroxy group and to C-23, according to the three-bond correlation of H-22 (δ_{H} 2.22 m) with C-24. Taken together, these spectral data allowed us to elucidate the planar structure of compound **1**.

The relative configuration of compound **1** was determined by means of a NOESY experiment. The NOESY correlations of H-3/H-5, H-3/H-1 α , H-1 β /H₃-19, H-14/H-15 α , H-15 β /H₃-18, H-14/H-17, and H₃-18/H-20 proved the A/B and C/D *trans* ring junction with α -oriented H-3, H-5, and H-14 and β -oriented side chains at C-17 and the 18- and 19-methyl groups (Fig. 2). The Overhauser effects between H-23/H₃-27, H-23/H₃-26, H-20/H-23, H₃-29/H-17, and H-22 α /H-25 evidenced the stereochemistry of the lactone ring (Fig. 3). The β -orientation of the anomeric H-1' proton was assigned based on the coupling constant $J_{\text{H-1'}/\text{H-2}} = 8.1$ Hz (Anh et al., 2021; Quasie et al., 2016).

Vernomigeodiin B (**2**) was isolated as a colorless amorphous solid with the molecular formula $\text{C}_{35}\text{H}_{52}\text{O}_{11}$, as revealed by its positive-ion HRESIMS peak at m/z 671.3416 $[\text{M} + \text{Na}]^+$ (calcd $\text{C}_{35}\text{H}_{52}\text{O}_{11}\text{Na}^+$ 671.3407). The ^1H NMR and ^{13}C NMR JMOD spectra were similar to those of **1**, except for the lactone fragment of the molecule. In compound **2**, instead of the 22-methylene, a methine group appeared at δ_{H} 4.27 t (1H, $J = 6.2$ Hz) and δ_{C} 72.9, indicating the presence of a hydroxyl group in position C-22 (Tables 1 and 2). This was confirmed by the sequence of correlated protons in the ^1H - ^1H COSY spectrum (δ_{H} 2.05 m, 2.58 dd, 4.27 t, 4.57 d), which allowed establishing the $-\text{CH}-\text{CH}-\text{CH}-\text{CH}-$ structure (C-17–C-20–C-22–C-23). The α -position of the 22-OH group

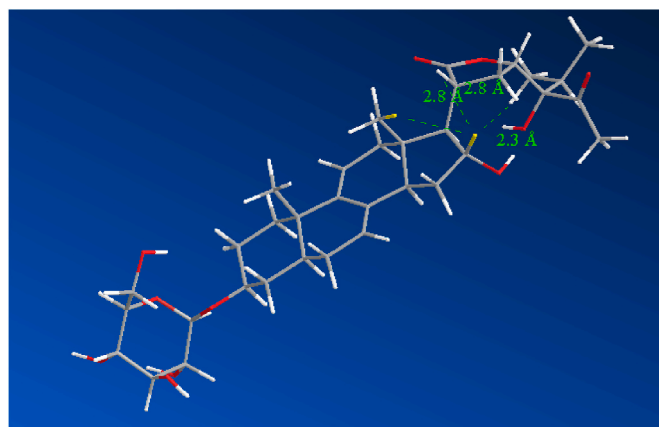
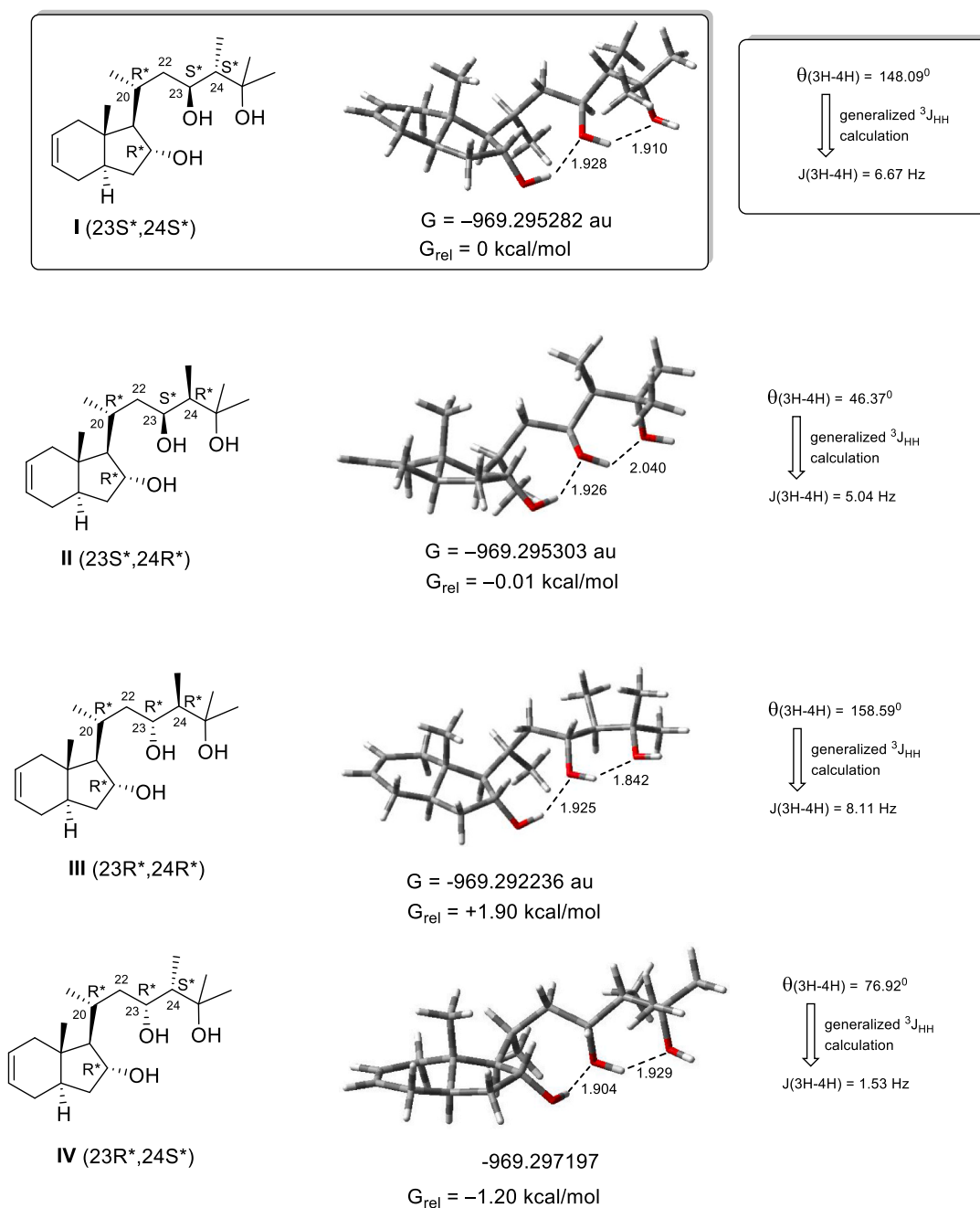


Fig. 4. Energy-minimized 3D structure of vernomigeodiin C (**3**) and atom distances between H-16, and H₃-18, H-20, and H-22 α .

was determined by the NOESY correlation between H-22/H-16 β , and H-16 β /H₃-18. The key HMBC and NOESY correlations for compound **2** are presented in Figs. 2 and 3, respectively. NMR data of the C-24–C-29 and glucose parts of the molecule agreed well with those of vernomigeodiin A (**1**).

Vernomigeodiin C (**3**) was isolated as a colorless amorphous solid. The molecular formula of **3** was determined to be $\text{C}_{35}\text{H}_{52}\text{O}_{11}$ according to the positive-ion HRESIMS peak at m/z 649.3567 $[\text{M} + \text{H}]^+$ (calcd for $\text{C}_{35}\text{H}_{53}\text{O}_{11}^+$ 649.3582). The ^1H and ^{13}C NMR JMOD spectra revealed characteristic resonances for a $\Delta^{7,9(11)}$ -stigmastane glucoside similar to compound **1** (Tables 1 and 2). A comparison between the ^1H and ^{13}C NMR chemical shifts of **1** and **3** showed that the compounds differ in the C-16 position. In this position, compound **3** contains a hydroxyl group, as indicated by the chemical shifts of H-16 [δ_{H} 4.24 t (1H, $J = 7.7$ Hz)] and C-16 (δ_{C} 75.3). The HMBC correlations of H-16 with C-14 and C-20 and of H-15 and H-17 with C-16 further verified the existence of a hydroxyl group at C-16 (Fig. 2). The NOESY correlations between H-16/H₃-18, H-16/H-22 α , H-20/H-23 confirmed the α -position of the hydroxyl group at C-16 and β position of H-23 (Fig. 3). The α -position of the 16-OH group was further corroborated by the atom distances 2.8, 2.8, and 2.3 Å between H-16/H₃-18, H-16/H-20, and H-16/H-22 α , respectively, which were obtained using the energy-minimized 3D model generated by the Chem3D 20.1.1. program (Fig. 4).

Vernomigeodiin D (**4**) was obtained as a colorless amorphous solid. The molecular formula $\text{C}_{28}\text{H}_{46}\text{O}_4$ was determined on the basis of the HRESIMS peak at m/z 447.3440 $[\text{M} + \text{H}]^+$ (calcd for $\text{C}_{28}\text{H}_{47}\text{O}_4^+$ 447.3469) and 469.3294 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{28}\text{H}_{46}\text{O}_4\text{Na}^+$ 469.3289). This molecular composition and the ^1H NMR and ^{13}C NMR JMOD spectra, in particular the upfield-shifted H-3 and C-3 NMR signals relative to those of compound **3** [$\delta_{\text{H-3}}$ 3.52 m (**4**), 3.71 m (**3**); $\delta_{\text{C-3}}$ 71.5 (**4**), 78.9 (**3**)] (Tables 1 and 2), indicated that compound **4** is not a glycoside. The close similarity of the other NMR signals confirmed that **4** contains the same 3,16-dihydroxy-7,9(11)-diene-stigmastane structure. Regarding the side chain at C-17, the HMBC correlations between H₃-21/C-22, H₃-21/C-20, H-23/C-24, H₃-26/C-25, H₃-27/C-25, H₃-28/C-25, and H₃-28/C-24 indicated the presence of a 23,25-dihydroxylated C₉ moiety (Fig. 2). Moreover, the absence of C-29 allowed establishing the whole planar structure as a nor-stigmastane. The stereochemistry of **4** was elucidated from the NOESY cross-peaks between H-16/H₃-18, H-16/H-20, H₃-18/H-20, H-17/H-14, and H-14/H₃-21 proving α -oriented 16-OH, H-14, H-17, and 21-methyl group and β position of 18-methyl and H-20 (Fig. 3). The relative configuration of C-23 and C-24 inside the side chain of vernomigeodiin D (**4**) cannot unambiguously be determined based on the NMR data alone. To disclose the conformation- and configuration-dependent stereostructure of this side chain we undertook a comparative modelling study on four simplified



The O...H distances indicated by dashed lines are given in Angström.

Fig. 5. Comparative modelling study of four simplified diastereomeric structures I–IV of vernomigeodiin D (4) for determination of the stereochemistry of side chain.

diastereomeric structures comprising hydroxylated indene fragment of the steroid skeleton (I–IV; Fig. 5). It was reasonable to assume that introduction of two sequential intramolecular H-bonds involving the three hydroxyl groups present in the simplified models poses substantial restrictions on the conformational diversity facilitating the generation of optimal spatial arrangement of the side chain with well-defined torsional angles. In the H-bond-stabilized optimal chain-conformation with highly restricted flexibility the dihedral angle $\theta(\text{H}_{23}\text{--H}_{24})$ is expected to be reliably correlated with the vicinal $^3J_{\text{H}_{23}\text{--H}_{24}}$ coupling constant. Since this parameter is of diagnostic importance in the determination of the relative configuration of the adjacent C-23 and C-24 stereogenic centres we attempted to identify such a diastereomer in which the dihedral angle $\theta(\text{H}_{23}\text{--H}_{24})$ is in good correlation with the

experimental $^3J_{\text{H}_{23}\text{--H}_{24}}$ coupling constant obtained by ^1H -NMR spectroscopy. Thus, taking all the considerations discussed above into account, we constructed the optimized structures of I–IV stabilized by two sequential H-bonds with highly similar O...H distances. These diastereomers are of comparable thermodynamic stability with similar conformational characteristics and steric congestion in the critical terminal region of the side chain. The diagnostic theoretical coupling constants $^3J_{\text{H}_{23}\text{--H}_{24}}$ were obtained by generalized $^3J_{\text{HH}}$ calculation (Haasnoot et al., 1980) using the dihedral angles found in the optimized structures diastereomers I–IV. The value calculated for I (6.67 Hz) practically matches perfectly with the experimental one (6.5 Hz) providing compelling evidence for the relative configuration (20R*, 23S*, 24S*) of the stereogenic centres in the side chain of compound 4.

Table 3¹H NMR data of compounds **6**, **8** and **10** (500 MHz, CD₃OD, δ ppm, J = Hz).

No.	6	8	10
δ_{H} , mult. (J in Hz)			
1	1.34 m 1.87 m	1.36 m 2.01 m	1.98 dt (11.5, 2.9) 1.33 m
2	1.61 dt (12.5, 3.2) 1.98 m	1.30 m 1.62 m	α : 1.85 m β : 1.46 m
3	3.71 m	3.71 ddd (11.2, 10.8, 4.7)	3.50 m
4	1.34 m 2.01 m	1.35 m 1.88 m	β : 1.28 m α : 1.70 brd (12.1)
5	1.40 m	1.41 m	1.42 m
6	1.93 m (2H)	1.93 m	1.91 m (2H)
7	5.42 brs	5.42 s	5.42 br s
11	5.56 d (6.9)	5.55 d (6.5)	5.51 d (6.6)
12	2.13 d (17.7) 2.80 dd (17.7, 6.9)	2.05 d (18.0) 2.84 dd (18.0, 6.5)	β : 2.39 dd (17.6, 6.6) α : 2.11 d (17.6)
14	2.26 m	2.23 m	2.53 m
15	1.49 m 1.83 m	1.48 m 1.82 m	β : 1.91 m α : 1.78 dd (12.0, 8.8)
16	1.53 m, 2.17 m	1.56 m, 2.22, m	4.22 ddd (14.2, 8.8, 6.9)
17	1.98 m	2.01, m	1.83 m
18	0.59 s	0.65 s	0.60 s
19	0.93 s	0.93 s	0.91 s
20	2.84 dd (10.6, 4.1)	2.78 dd (11.2, 6.7)	2.31 m
21	—	—	5.30 d (5.9)
22	4.44 dd (4.1, 2.8)	4.66 dd (6.7, 5.2)	4.87 dd (4.4, 2.7)
23	4.73 d (2.8)	4.76 d (5.2)	4.40 d (2.7)
25	1.84 m	1.96 m	2.23 m
26	1.18 d (7.2)	1.02 d (7.2)	4.00 dd (8.5, 7.4) 3.47 t (8.5)
27	1.14 d (7.2)	1.05 d (7.2)	1.04 d (7.0)
28	3.35 m	3.87 m	—
29	1.35 d (5.7)	1.20 d (6.5)	1.36 s
1'	4.40 d (7.9)	4.40 d (8.0)	—
2'	3.15 dd (9.0, 7.8)	3.15 t (8.0)	—
3'	3.35 m	3.35 m	—
4'	3.26 m	3.26 m	—
5'	3.27 m	3.27 m	—
6'	3.66 dd (12.0, 5.4) 3.85 dd (12.0, 2.2)	3.66 dd (11.8, 5.1) 3.86 d (11.8)	—

Known compounds **5**–**11** were also isolated from the chloroform extract of *V. migeodii* and identified using a detailed NMR analysis. Vernomyoside D (**5**) (Quasie et al., 2016; Wang et al., 2018), vernonyoside B₁ (**6**) (Jisaka et al., 1992), vernomyoside A (**7**) (Quasie et al., 2016), vernomyoside E (**8**) (Zhao et al., 2021; Anh et al., 2021), vernomyoside B (**9**) (Quasie et al., 2016), (3 β ,5 α ,16 α ,21R,22R,23S,25R,28S)-21,23:22,28:25,28-triepoxy-3,16,21,24-tetrahydroxystigmasta-7,9(11)-diene (**10**) (Nelson, 2003), and the tripeptide aurantiamide acetate (**11**) were obtained for the first time from this species. Our NMR investigations allowed assigning the ¹H and ¹³C NMR signals for compounds **6**, **8**, **10**, and **11** in solvents other than those previously published (Tables 2–4). Steroids **5**–**10** were previously isolated only from the leaves of *V. amygdalina*. Aurantiamide acetate (**11**) is a selective and orally active cathepsin inhibitor (Isshiki et al., 2001) with anti-inflammatory activity (Fang et al., 2022). Meanwhile, weak anti-inflammatory activity was found for vernomyoside D (**5**), vernomyoside A (**8**), and vernomyoside B (**10**) by measuring the inhibition of NO production in LPS-induced RAW264.7 macrophages (Quasie et al., 2016). Compound **10** was reported to be effective in the treatment of hyperglycemia (Nelson, 2003). In a previous study, vernomyoside E (**9**) considerably inhibited the activity of both α -amylase and α -glycosidase (Anh et al., 2021).

2.2. Cytotoxic and antiviral activity

To evaluate the cytotoxic potential of these compounds, Vero cells were incubated with a series of two-fold dilutions of each compound (Fig. 6A–C). A 75 % cell viability threshold relative to untreated control cells was applied to determine nontoxic concentrations. Among the

Table 4NMR data of aurantiamide acetate (**11**) [500 MHz (¹H), 125 MHz (¹³C), CDCl₃, δ ppm].

No.	¹ H NMR δ_{H} , mult. (J in Hz)	¹³ C NMR δ_{C} , type
1	—	136.8 ^a , C
2	7.07 d (7.3)	129.3, CH
3	7.18 t (7.3)	128.7, CH
4	7.14 t (7.3)	126.9, CH
5	7.18 t (7.3)	128.7, CH
6	7.07 d (7.3)	129.3, CH
7	2.73 dd (13.7, 6.6) 2.77 dd (13.7, 6.6)	37.6, CH ₂
8	4.35 m	49.6, CH
9	3.82 dd (11.3, 4.2) 3.93 dd (11.3, 4.9)	64.7, CH ₂
1'	—	136.6 ^a , C
2'	7.26 d (7.4)	129.4, CH
3'	7.29 t (7.4)	128.9, CH
4'	7.24 t (7.4)	127.3, CH
5'	7.29 t (7.4)	128.9, CH
6'	7.26 d (7.4)	129.4, CH
7'	3.05 dd (13.5, 8.3) 3.22 dd (13.5, 5.7)	38.2, CH ₂
8'	4.75 ddd (8.3, 7.7, 5.7)	55.1, CH
9'	—	170.4, C
1''	—	133.8, C
2''	7.71 d (7.5)	127.2, CH
3''	7.44 t (7.5)	128.8, CH
4''	7.53 t (7.5)	132.1, CH
5''	7.44 t (7.5)	128.8, CH
6''	7.71 d (7.5)	127.2, CH
7''	—	167.2, C
1'''	—	170.9, C
2'''	2.03 s	20.9, CH ₃
8''-NH	7.61 d (7.7)	—
10-NH	5.90 d (8.6)	—

^a Interchangeable signals.

tested compounds, compounds **7** and **8** showed the lowest cytotoxicity at a concentration of 250 μ M with a cell viability of 85.9 % and 76.6 %, respectively. In contrast, compound **1** was the most cytotoxic, exhibiting a nontoxic threshold of 3.9 μ M. Overall, the nontoxic concentration ranges for the compounds spanned between 10 and 0.078 μ M. These concentrations were subsequently used for further antiviral assays.

To assess the intracellular antiviral activity, Vero cells were infected with HSV-2 at a multiplicity of infection (MOI) of 0.01 and exposed to two-fold serial compound dilutions ranging from 10 to 0.078 μ M for 24 h. A subsequent MTT assay confirmed that none of the tested concentrations caused statistically significant cytotoxicity.

Within the 0.3125–0.625 μ M range, vernomigeodiin D (**4**) reduced HSV-2 viral DNA copy numbers by an average of 9.82-fold with a calculated IC₅₀ value of 0.56 $\pm$ 0.08 μ M. The IC₉₀ was between 0.625 and 1.25 μ M (1.12 $\pm$ 0.1 μ M), resulting in a 21.5-fold decrease in the viral DNA levels (p < 0.01). Compound **4** demonstrated the strongest statistically significant antiviral activity among the tested compounds at concentrations of 0.625–10 μ M; in particular, a concentration of 10 μ M led to a dramatic 2130-fold viral DNA reduction (p < 0.001). Meanwhile, vernomigeodiin D (**4**) did not induce cytotoxicity at these concentrations, as revealed by the MTT assay (Fig. 7A–C) (Table 5).

Similarly, vernonyoside B₁ (**6**) showed a potent antiviral activity at a concentration of 10 μ M with a 499.15-fold decrease in the viral copy number (p < 0.01). The calculated IC₅₀ of 0.30 $\pm$ 0.06 μ M was associated with a 3.67-fold decrease in the viral DNA levels. The IC₉₀ value ranged from 0.625 to 1.25 μ M (1.17 $\pm$ 0.1 μ M), resulting in a 14.79-fold reduction in the viral DNA levels. Vernonyoside B₁ (**6**) showed no signs of cytotoxicity at these concentrations according to the MTT assay (Fig. 7D–F).

Although to a lesser extent, vernomigeodiin A (**1**) also showed antiviral activity at 10 μ M, leading to a 5.49-fold reduction in the viral

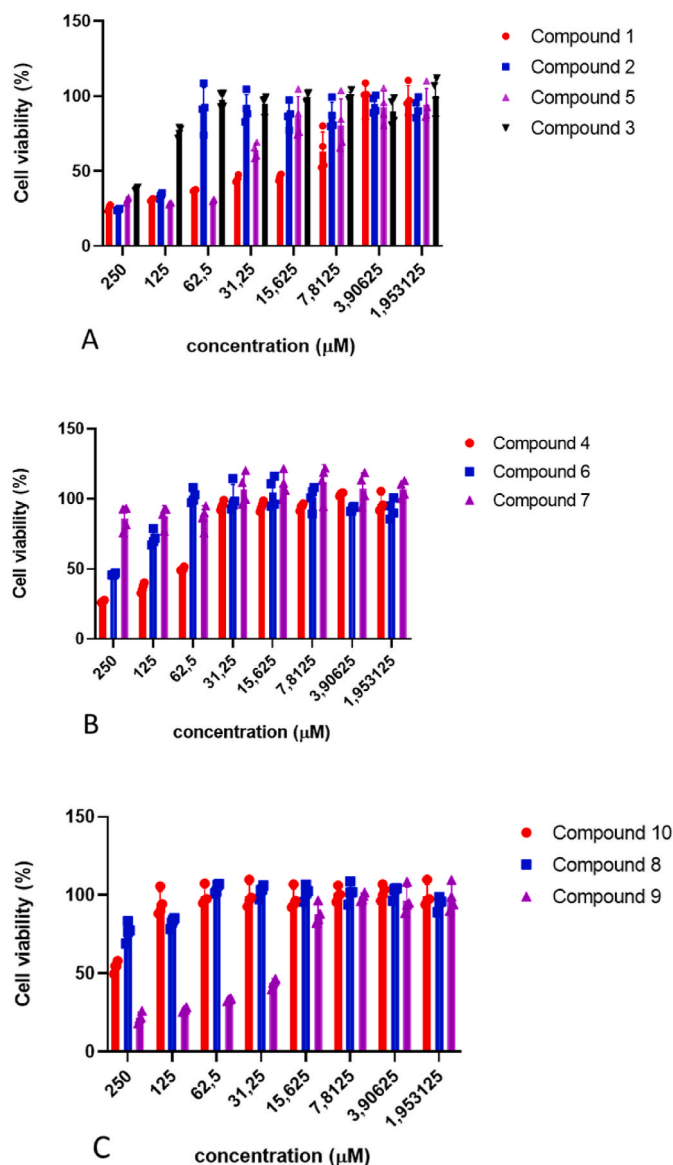


Fig. 6. Cell viability assessment of the tested compounds (1–10) in Vero cells using the MTT assay. Vero cells were seeded at a density of 4×10^4 cells/well in 96-well plates. After 24 h, cells were treated with serial two-fold dilutions of the tested compounds and were incubated at 37 °C with 5 % CO₂ for 24 h. Cell viability was evaluated in comparison to untreated controls. Compounds were grouped and displayed based on similarities in their chemical structure to facilitate comparison of the molecules. Data are expressed as percentages and presented as mean $\pm$ SD.

DNA levels ($p < 0.05$). The IC₅₀ value was 0.25 ± 0.05 μM, with a slight 1.79-fold decrease in the viral DNA levels, and the IC₉₀ value of 0.3125 – 0.625 μM (0.60 ± 0.07 μM) was associated with a 3.69-fold reduction in the viral DNA levels. Vernomigeodiin A (1) did not alter the cell viability at these concentrations (Fig. 7G–I).

To evaluate whether the compounds exhibited direct virucidal activity, HSV-2 virions were preincubated with the compounds for 30 min at concentrations ranging from 10 to 0.078 μM.

Compounds 1, 4, and 6 had no measurable effect on extracellular virions, whereas compound 9 showed virucidal activity with an IC₅₀ value of 1.23 ± 0.15 μM, causing a 2.59-fold decrease in the viral DNA levels, and an IC₉₀ value of 2.47 ± 0.35 μM associated with a 4.48-fold viral DNA reduction (data not shown). The remaining compounds (2, 3, 5, and 7–10) did not show significant antiviral activity against HSV-2 under the experimental conditions and were therefore considered

inactive.

With regard to the structure–activity relationships, the active compounds were established to belong to the $\Delta^{7,9(11)}$ -stigmastane diene group featuring a γ -lactone ring (1 and 6) or an aliphatic C₉ side chain at C-17 (4). Compounds with two or three condensed heterocyclic rings at C-17 exhibited no antiviral activity; only compound 9 showed a direct virucidal effect in *H. simplex* type-2 virus. The 16- or 22-hydroxy or 16-keto groups of the γ -lactone ring-containing steroids (2, 3, 5, 6, and 7) with an α -oriented C-24–C-29 fragment are not conducive to antiviral activity (2, 3, 5, and 7), whereas the epoxy group and/or the 16-hydroxy group of the C-24–C-29 moiety in β position (6) is favourable for the antiviral effect.

2.3. Molecular docking study

In an effort to better understand the mechanism of the antiviral activity of the tested compounds; molecular docking study was performed against key viral targets, specifically HSV-2 protease enzyme and HSV-2 gD protein to unveil possible binding interactions and mechanistic pathways (Čulenová et al., 2020). The HSV-2 protease represents an important enzyme for the viral replication. Targeting this protein would interrupt the replication cycle and allows for antiviral effects (Melk and El-Sayed, 2024). While the surface glycoprotein; HSV-2 gD plays a prime role for the viral attachment and entry into host cells via binding to Nectin-1; a cellular adhesion protein (Wu et al., 2022). Our *in silico* study of compounds 1, 4, and 6 against both targets showed promising results that come in accordance with the *in vitro* ones (Table 5).

Vernomigeodiin A (1) which scored the lowest antiviral IC₅₀ value showed the best binding affinity of -11.17 kcal/mol against HSV-2 protease enzyme followed by vernonioside B₁ (6) and vernomigeodiin D (4), (-10.59 and -9.83 , respectively), compared to -4.91 kcal/mol for acyclovir used as a standard. These compounds showed hydrogen bonding with the amino acids VAL A:70, LEU A:120, LEU A:223 and, GLN A:246 (Fig. 7). Alkyl, Pi-alkyl and carbon-hydrogen bonds were also detected (Fig. 8). Though, no interactions with the SER–HIS–HIS triad that was described in literature to be important for the enzyme's catalytic activity (Čulenová et al., 2020; Hassan et al., 2019), nevertheless, these compounds achieved high binding affinities indicating strong interactions with the amino acids of the active site which have been previously reported in literature (Kant et al., 2019). Compound 4 specifically dock to a site that is relatively far from where the other two compounds interact, which might suggest its interaction with another binding pocket within the enzyme, or may be an allosteric site.

A similar binding energy profile was obtained for the docking of these compounds against HSV-2 gD protein, with both compounds 1 and 6 achieving better results (-12.32 and -11.42 kcal/mol; respectively) compared to the standard; epigallocatechin gallate (-10.22 kcal/mol). Compound 4 scored a binding energy of -8.82 kcal/mol, which indicates its binding to the active site of the protein, however, with lower affinity compared to the others (Fig. 9). Investigations into the binding interactions showed hydrogen bonding interactions with VAL A:61, ARG A:64, LEU A:70, HIS A:72, SER A:75, TYR B:115, ARG B:130, THR B:131, GLU B:146, and ASN B:148. As for the interactions with the HSV-2 protease, other interactions were also detected, which plays a great role in stabilizing the ligand interactions with the binding pocket.

Despite, the highest viral DNA reduction caused by compound 4 (Table 5), it demonstrated the least binding affinities for both targets. This might suggest a different mechanism of action, and it would be beneficial to investigate the binding modes of this compound against HSV-2 polymerase enzyme upon the availability of its structure in the databases. To conclude, through these docking results we can anticipate that these compounds might exert their action by affecting both viral entry and replication.

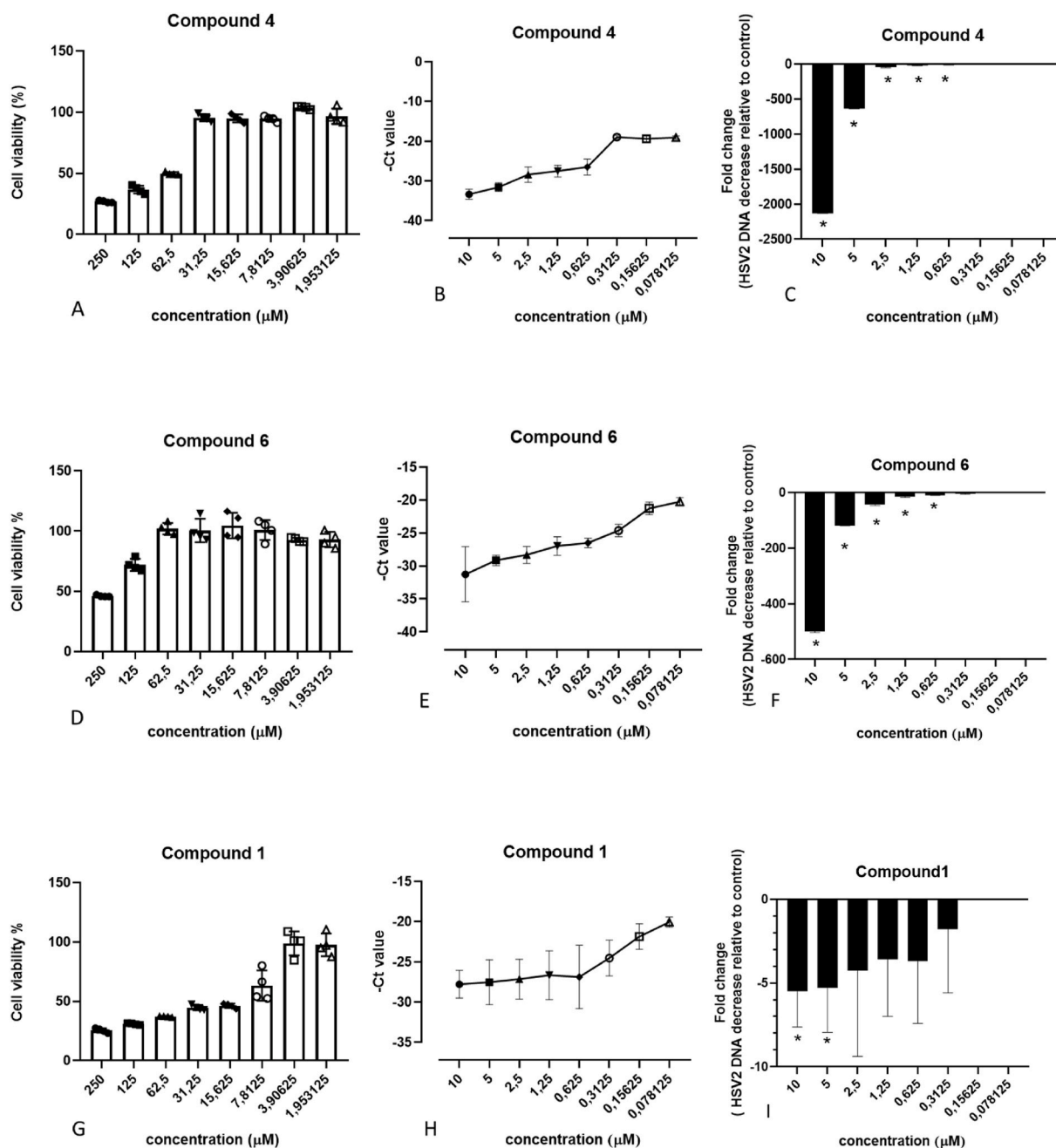


Fig. 7. Analysis of cell viability and inhibition of HSV-2 replication by antiviral compounds 1, 4, and 6 using direct qPCR. Cell viability was assessed by MTT assay before antiviral testing (A, D, G). Vero cells were infected with HSV-2 at an MOI of 0.01 and treated with serial two-fold dilutions of each compound. The effect of compound treatment on HSV-2 replication, assessed by direct qPCR 24 h post-infection (B, E, H). Viral DNA levels were quantified using the Δ Ct method and fold reduction was calculated relative to the untreated infected control (C, F, I). Fold changes in viral DNA quantity are shown as mean $\pm$ SD. Several compounds demonstrated dose-dependent antiviral activity, with compound 4 exhibiting the most significant reduction in viral DNA at higher concentrations. Statistical significance was determined using two-way ANOVA with Sidak's post hoc test $*p < 0.05$.

3. Conclusions

In summary, 10 steroids, including previously undescribed vernomigeodiins A–D (1–4) were isolated from the aerial parts of *V. migeodii* along with the tripeptide aurantiamide acetate (11). The isolated compounds are rarely occurring stigmastane steroids with a unique conjugated $\Delta^{7,9(11)}$ -diene segment and a highly oxidized side chain, occasionally forming a bi- or tricyclic ring system. The isolated compounds were investigated for their antiviral activities against *H. simplex* virus type 2, and found that vernomigeodiins A (1) and B (2) and vernonioside B₁ (6) reduced the HSV-2 viral DNA levels with IC₅₀ values

between 0.25 and 0.56 μ M. In addition, vernomioside B showed a direct virucidal effect (IC₅₀ = 1.23 $\pm$ 0.15 μ M). The interactions between the antiviral compounds and specific viral targets (HSV-2 protease enzyme and gD protein) were studied using molecular docking. The docking results indicate a potential mechanism of action for these compounds involving both viral entry and replication. Our study demonstrates that vernomigeodiins and related stigmastanes can be regarded as antiviral lead scaffolds. These findings not only enrich the structural diversity of plant steroids but also highlight the potential of *V. migeodii* as a source of antiviral agents.

Table 5
Antiviral activity of compounds 1, 4, and 6.

Compd	IC ₅₀ (μM)	IC ₉₀ (μM)	Viral DNA reduction at			
			10 μM	5 μM	2.5 μM	1.25 μM
1	0.25 ± 0.05	0.60 ± 0.07	5.49-fold (p < 0.05)	5.2-fold (p < 0.05)	4.3-fold (n.s., p = 0.14)	3.6-fold (n.s., p = 0.30)
4	0.56 ± 0.08	1.12 ± 0.1	2130-fold (p < 0.001)	637.7-fold (p < 0.001)	44.9-fold (p < 0.001)	21.5-fold (p < 0.01)
6	0.30 ± 0.06	1.17 ± 0.1	499.15-fold (p < 0.01)	499.1-fold (p < 0.01)	118.6-fold (p < 0.01)	42.4-fold (p < 0.01)

4. Experimental

4.1. General experimental procedures

Optical rotations were determined using a Jasco P-2000 polarimeter (Jasco International Co. Ltd., Hachioji, Tokyo, Japan). NMR spectra were recorded on a Bruker Ascend 500 spectrometer with a 5 mm BBO Prodigy Probe at 500 MHz (¹H) and 125 MHz (¹³C) or on a Bruker AVANCE III 600 MHz spectrometer equipped with a 5 mm CP-TCI triple-resonance cryoprobe (Bruker Biospin, Karlsruhe, Baden Württemberg, Germany) at 600 MHz (¹H) and 150 MHz (¹³C). The signals of the deuterated solvent were taken as references. 2D experiments were performed using standard Bruker software. In the ¹H–¹H COSY, NOESY, HSQC, and HMBC spectroscopy experiments, gradient-enhanced versions were applied. The flow injection analysis was performed using a Thermo Scientific Orbitrap Exploris 240 hybrid quadrupole-Orbitrap (Thermo Fischer Scientific, Waltham, MA, USA) mass spectrometer coupled to a Waters Acquity I-Class UPLC™ (Waters, Manchester, UK). Open column chromatography (OCC) was performed using a polyamide stationary phase (MP Biomedicals, particle size 50–160 μm). Vacuum liquid chromatography (VLC) was conducted on reversed-phase (RC) stationary phase (LiChroprep RP-18, 40–63 μm, Merck) and normal-phase stationary phase (silica gel, 15 μm, Merck) for an efficient separation of extracts and fractions. Flash chromatography (FC) was performed on a CombiFlash NEXTGN 300+ instrument (Teledyne Isco, Lincoln, NE, USA) equipped with integrated UV, UV-Vis, and evaporative light scattering detectors using a RediSep C₁₈ Bulk 950 flash column. High-performance liquid chromatography (HPLC) separations were conducted using a Shimadzu LC-20 AD UFLC system equipped with a photodiode array detector and Shimadzu LC-10 AS and LC-2010 CHT systems equipped with UV-vis detector (Shimadzu Co., Ltd., Kyoto, Japan). For the RP-HPLC, a LiChrospher RP-18 column (250 × 4 mm, 5 μm) was used. The TLC plates were detected under UV light at 254 nm and sprayed with concentrated sulfuric acid, followed by 5 min heating. All solvents used for OCC and TLC were of at least analytical grade (VWR Ltd., Hungary).

4.2. Plant material

The aerial parts of *Vernoniastrum migeodii* (S.Moore) Isawumi (Asteraceae) were collected from the outskirts of Zaria, Kaduna State, Nigeria (11.134179, 7.669629) in June 2020. The plant was identified by Umar Shehu Gallah from the National Research Institute for Chemical Technology (NARICT), Zaria, Nigeria. A voucher specimen was deposited at NARICT (Voucher No.: Narict/Biores/323) and in the Herbarium of the Department of Pharmacognosy, University of Szeged, Hungary (Voucher No. 899).

4.3. Extraction and isolation

The dried aerial parts (950 g) of *V. migeodii* were ground using a blender and then percolated with MeOH (20 L) at room temperature. The MeOH extract was concentrated *in vacuo*, yielding 175.49 g of the extract. This extract was dissolved in 50 % aqueous MeOH and subjected to solvent–solvent partitioning using *n*-hexane (6 × 500 mL), chloroform (6 × 500 mL), and then ethyl acetate (6 × 500 mL). The chloroform phase (38.24 g) was chromatographed by OCC on polyamide (125 g) using MeOH–H₂O (1:4, 2:3, 3:2, 4:1, and 5:0) mixtures as the eluent to afford 20 %, 40 %, 60 %, 80 %, and 100 % MeOH fractions, respectively.

The 40 % MeOH fraction (1.6 g) was subjected to reversed-phase flash chromatography (RP-FC) on silica gel (100 g) using a gradient system of H₂O–MeOH (linear from 10 % to 100 % MeOH, t = 66 min). This process led to compound 11 (2.4 mg) and 21 subfractions (A1–21). A14 (147 mg) was subjected to vacuum-liquid chromatography (VLC) on silica gel using a gradient system of cyclohexane–EtOAc–EtOH (85:15:0, 75:25:0, 65:35:0, 50:20:2, 50:20:4, 50:20:8, 50:20:15, 50:20:20, 50:20:25, 45:25:30, 40:30:40, 40:30:60, and 40:30:80), providing 13 fractions (A14/1–13). Fraction A14/12 (25.5 mg) was purified by sequential two-step RP-HPLC, first using an isocratic solvent system of H₂O–MeCN (55:30) and then H₂O–MeCN (56:29) in the second step, leading to the isolation of compound 10 (0.6 mg). Fraction A14/13 (29.5 mg) was purified by RP-HPLC using an isocratic solvent system of H₂O–MeCN (55:30), yielding compound 3 (2.1 mg).

The 60 % MeOH fraction (8.6 g) was separated by RP-VLC using a gradient system of H₂O–MeOH (from 95:5 to 0:100). Fractions with similar compositions were combined on the basis of TLC monitoring (B1–12). RP-FC was employed to further separate the B1 (1.8 g) fractions on silica gel (100 g) by applying a gradient system of H₂O–MeOH (linear from 0 % to 100 % acetone, t = 65 min) to yield fractions B1/1–18. The purification of fraction B1/7 (36 mg) was conducted by RP-HPLC using an isocratic H₂O–MeOH (25:75) solvent system to purify compound 10 (1.7 mg). Compound 4 (0.7 mg) was obtained by further purifying B1/8 (56 mg) via RP-HPLC separation (mobile phase: H₂O–MeOH 28:72). RP-HPLC was employed for the final purification of B1/9 (132 mg) using a H₂O–MeOH (25:75) solvent system, which led to the isolation of compounds 6 (11.6 mg), 1 (2.2 mg), and 8 (2.75 mg). Fraction B1/11 (131.2 mg) was chromatographed by RP-HPLC with a H₂O–MeCN (31:19) solvent system to yield compounds 9 (2.2 mg) and 2 (1.7 mg) along with subfractions B1/11/1–7. Finally, compound 5 (8.6 mg) was isolated from subfraction B1/11/3 (32 mg) via RP-HPLC (mobile phase: H₂O–MeCN, 32:18).

4.4. Spectroscopic data of compounds

Vernomigeodiin A (1): Colorless amorphous solid; [α]_D²⁶ +55.7 (c 0.2, MeOH); for ¹H and ¹³C NMR data, see Tables 1 and 2; positive-ion HRESIMS *m/z* 633.3638 [M + H]⁺ (calcd for C₃₅H₅₃O₁₀⁺ 633.3638).

Vernomigeodiin B (2): Colorless amorphous solid; [α]_D²⁵ +9.5 (c 0.1, MeOH); for ¹H and ¹³C NMR data, see Tables 1 and 2; positive-ion HRESIMS *m/z* 671.3416 [M + Na]⁺ (calcd C₃₅H₅₂O₁₁Na⁺ 671.3407), 631.3473 [M – H₂O + H]⁺ (calcd C₃₅H₅₁O₁₀⁺ 631.3482). negative-ion HRESIMS *m/z* 647.3431 [M – H][–] (calcd for C₃₅H₅₁O₁₁[–] 647.3431).

Vernomigeodiin C (3): Colorless amorphous solid; [α]_D²⁵ +10.5 (c 0.2, MeOH); for ¹H and ¹³C NMR data, see Tables 1 and 2; positive-ion HRESIMS *m/z* 649.3567 [M + H]⁺ (calcd for C₃₅H₅₃O₁₁⁺ 649.3582), HR-APCIMS *m/z* 469.2940 [M – C₆H₁₂O₆ + H]⁺ (calcd for C₂₉H₄₁O₅⁺ 469.3582).

Vernomigeodiin D (4): Colorless amorphous solid; [α]_D²⁵ +4.6 (c 0.05, MeOH); for ¹H and ¹³C NMR data, see Tables 1 and 2; positive-ion HRESIMS *m/z* 447.3440 [M + H]⁺ (calcd for C₂₈H₄₇O₄⁺ 447.3469), 469.3294 [M + Na]⁺ (calcd for C₂₈H₄₆O₄Na⁺ 469.3289).

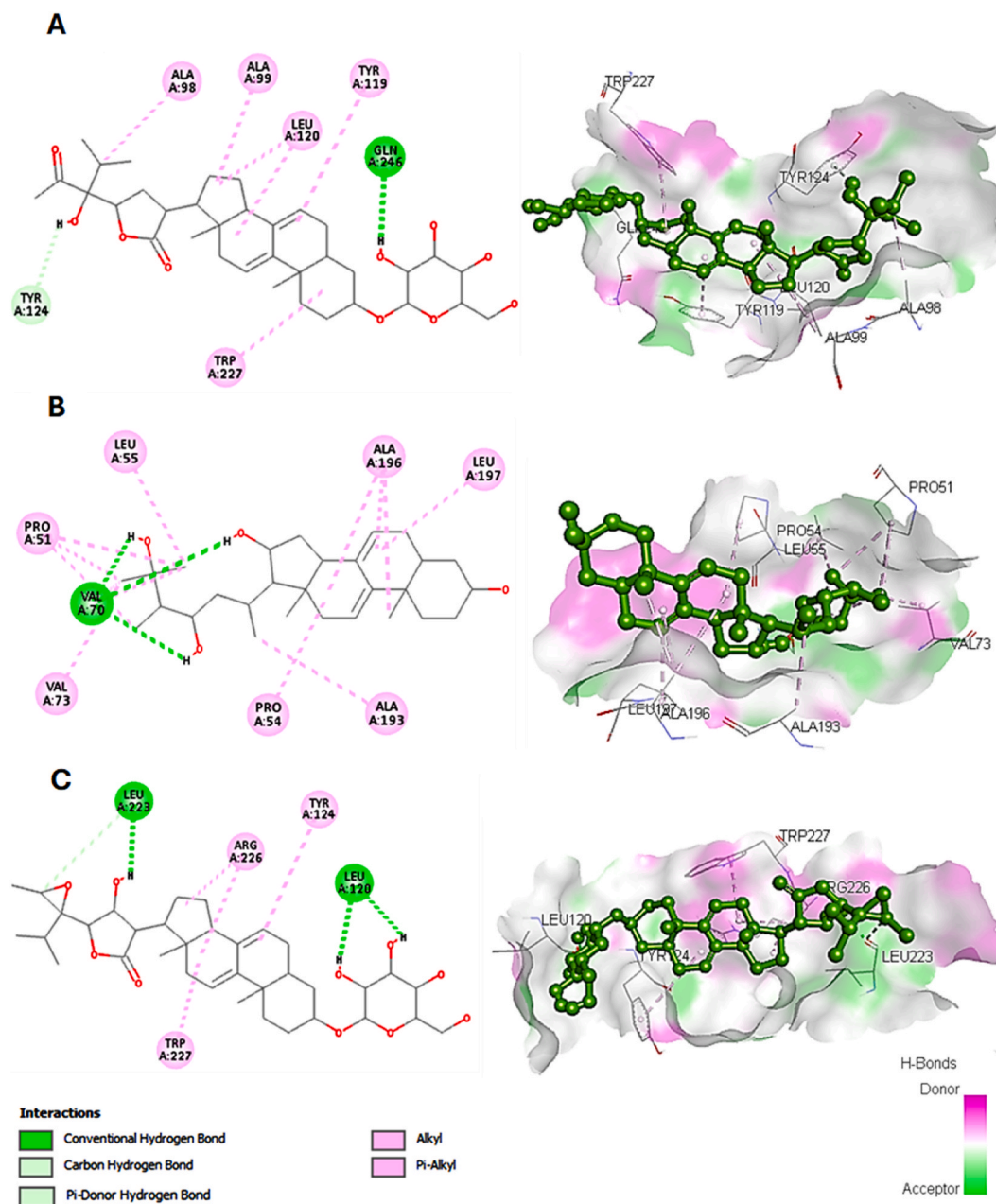


Fig. 8. 2D (left) and 3D (right) representations of compound 1 (A), 4 (B), and 6 (C) best poses in complex with HSV-2 protease enzyme active sites (PDB ID: 1AT3) visualised with Discovery studio visualizer (version 21.1.0.20298). Legend at the lower-left part is for all 2D-representations, A, B and C.

4.5. Comparative modelling study of the stereochemistry of side chain of compound 4

The coupling constants were obtained by generalized $^3J_{HH}$ calculation (Haasnoot et al., 1980). The program available online (Generalized $^3J_{HH}$ calculation acc. Haasnoot et al.). The optimization of the structures I-IV and subsequent frequency calculation resulting in gibbs free energy data were carried out with M06-2X global functional [Zhao and Truhlar (2008) using 6-31 G(d,p) basis set [Hehre et al. (1986)]. All calculations were carried out using the Gaussian 09 software (Gaussian Incorporation, Pittsburgh, USA) package (Frisch et al., 2016).

4.6. Cytotoxicity assay

The cytotoxicity of the tested compounds was assessed in Vero cells using the standard MTT assay. Vero cells [American Type Culture

Collection (ATCC), Manassas, VA, USA] were seeded into 96-well plates at a density of 4×10^4 cells per well in 100 μ L of minimum essential medium (MEM) supplemented with 10 % heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 $\times$ nonessential amino acids, 100 mM sodium pyruvate, and 9 mM nystatin. The cells were incubated at 37 $^{\circ}$ C in a humidified atmosphere containing 5 % CO_2 for 24 h. After incubation, the culture medium was removed and replaced with serial two-fold dilutions of each compound in MEM. The highest tested concentration was 250 μ M, and the dilution series ranged from 250 to 0.98 μ M. Control wells included untreated cells as a positive control and wells containing only the medium served as a negative control. All conditions were tested in quadruplicate.

After 24 h of exposure at 37 $^{\circ}$ C, 20 μ L of MTT reagent (5 mg/mL stock solution, Sigma) was added to each well, and the plates were incubated for an additional 4 h. Subsequently, 100 μ L of SDS solution (10 % SDS in 0.01 M HCl, Sigma) was added and the plates were incubated overnight

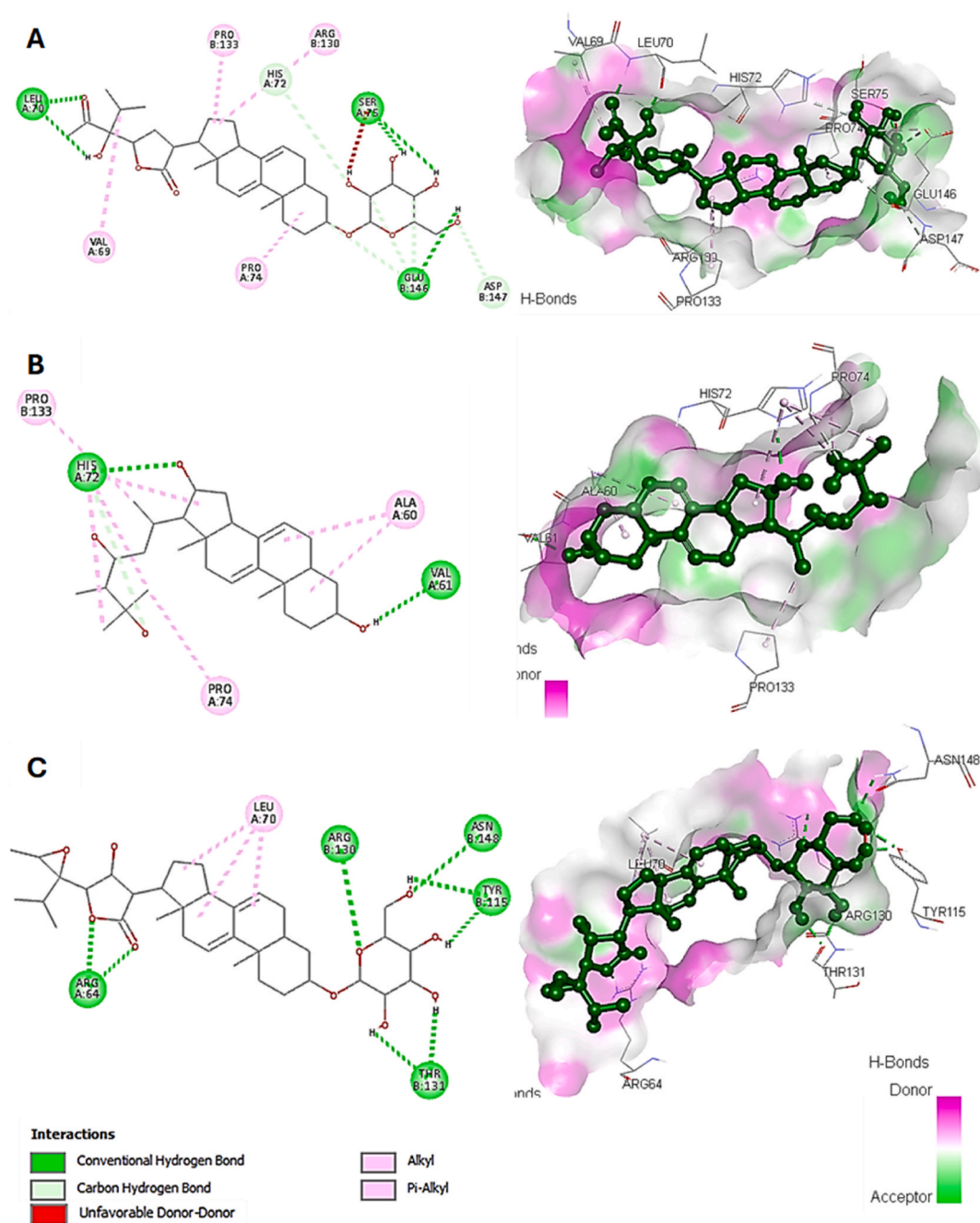


Fig. 9. 2D (left) and 3D (right) representations of compound 1 (A), 4 (B), and 6 (C) best poses in complex with HSV-2 *gD* protein active site (PDB ID: 4MYV) visualised with Discovery studio visualizer (version 21.1.0.20298). Legend at the lower-left part is for all 2D-representations, A, B and C.

under the same conditions. The cell viability was determined by measuring the absorbance at 570 nm with a reference wavelength of 650 nm using an EZ READ 400 microplate reader (Biochrom, Cambridge, UK). The viability was expressed as a percentage relative to untreated control cells.

4.7. HSV-2 cultivation and quantification

The HSV-2 strain, kindly provided by Dr. Ilona Mucsi (University of Szeged, Hungary), was propagated in Vero cells (ATCC) as described earlier (Mucsi et al., 2001), and the viral infectivity was assessed on the same cell line using the plaque assay technique.

4.8. Direct quantitative PCR for antiviral activity assessment

The antiviral potential of the tested compounds was assessed in Vero cell monolayers using noncytotoxic concentrations. Cells were seeded in 96-well plates at a density of 4×10^4 cells per well and infected with HSV-2 at a MOI of 0.01. In the first approach, the compounds were added to the cells simultaneously with HSV-2. In the second setup, to evaluate the direct antiviral effects, HSV-2 was preincubated with each compound for 1 h at 37 °C before the virus-compound mixture was applied to the Vero cells. Virus-infected but untreated cells served as infection controls in both cases.

In both setups, after treatment, the cells were incubated for 1 h to allow viral adsorption to the Vero cells. After incubation, the cells were washed with phosphate-buffered saline to remove unbound virions. The

culture medium was then replaced with fresh medium containing serial two-fold dilutions of each compound, starting from the highest nontoxic concentration. After 24 h of incubation at 37 °C in a 5 % CO₂ atmosphere, the cells were washed and lysed in Milli-Q water (Millipore, Billerica, MA, USA). After performing two freeze–thaw cycles to ensure complete cell lysis, the lysates were collected. Quantification of HSV-2 genomic DNA was performed using qRT-PCR with HSV-2-specific gD2 primers (forward: 5'-TCA GCG AGG ATA ACC TGG GA-3'; reverse: 5'-GGG AGA GCG TAC TTG CAG GA-3'), as previously described (Virók et al., 2017). Amplification was performed using a CFX96 Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA). The cycle threshold (Ct) values were determined for each sample. Relative HSV-2 DNA levels were calculated using the formula $\Delta Ct = Ct(\text{treated}) - Ct(\text{infected control})$. The percentage of viral inhibition was derived from the ΔCt values and used to compare the antiviral efficacy of the tested extracts. The IC₅₀ and IC₉₀ values of the antiviral compounds were defined as the drug concentration that reduced the viral titer by 50 % and 90 %, respectively, in the treated cultures compared with the untreated control. Statistical analysis was performed using two-way Analysis of Variance (ANOVA) followed by Sidak's multiple comparisons test, conducted with GraphPad Prism (version 8.0.1; GraphPad Software, San Diego, CA, USA). A *p* value lower than 0.05 was considered statistically significant.

4.9. Molecular docking study

The docking process was conducted according to a previously reported procedure (Ahmed et al., 2023). In brief, the structures of the compounds under study were drawn and saved in Protein Data Bank (PDB) formats using the ChemDraw 12.0.2 software (ACD/LABS, Advanced Chemistry Development, Inc.). The protein structure [HSV-2 protease (PDB ID: 1AT3)] and HSV-2 gD protein (PDB ID: 4MYV) were procured from the PDB database. The PDB files for the enzyme and compounds were converted to the PDBQT format using the graphical user interface of AutoDock4 (The Scripps Research Institute) after conducting the necessary preprocessing steps (Morris et al., 2009). A grid box which includes the essential amino acids mentioned in literature was applied (Melk and El-Sayed, 2024; Hassan et al., 2019). The 2D and 3D representations for the interactions were investigated using Biovia (Discovery Studio visualizer version 21.1.0.20298; Dassault Systèmes, Vélizy-Villacoublay, France) in PDB formats that were obtained using the OpenBabel Graphical User Interface software version 2.4.1 (O'Boyle et al., 2011).

CRediT authorship contribution statement

Morteza Yazdani: Writing – original draft, Visualization, Validation, Software, Investigation, Formal analysis, Data curation. **Anita Barta:** Visualization, Validation, Software, Data curation. **Anasztázia Hetényi:** Investigation, Data curation. **Muhammad Bello Saidu:** Methodology, Data curation. **Umar Shehu Gallah:** Investigation, Data curation. **Róbert Berkecz:** Investigation, Funding acquisition, Data curation. **Antal Csámpai:** Writing – original draft, Investigation, Data curation. **Katalin Burián:** Writing – review & editing, Writing – original draft, Resources, Funding acquisition, Conceptualization. **Dóra Paróczai:** Writing – original draft, Investigation, Data curation. **Sara H.H. Ahmed:** Writing – original draft, Software, Investigation, Data curation. **Judit Hohmann:** Writing – review & editing, Writing – original draft, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.phytochem.2025.114659>.

Data availability

The NMR data for 1–4 have been deposited in the Natural Products Magnetic Resonance Database (NP-MRD; www.np-mrd.org) and can be found at NP0351080 (1), NP0351081 (2), NP0351082 (3), NP0351083 (4).

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