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Steroidal Alkaloids from *Veratrum nigrum* Enhance Glucose Uptake in Skeletal Muscle Cells

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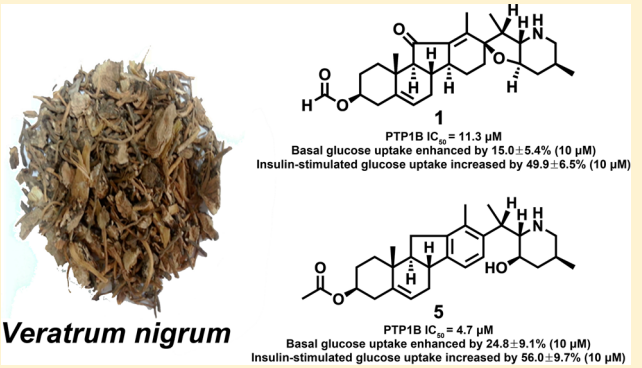
Supporting Information

ABSTRACT: *Veratrum nigrum* is recognized as a medicinal plant used for the treatment of hypertension, stroke, and excessive phlegm. Chemical investigation of the roots and rhizomes led to the isolation of five new steroidal alkaloids, jervine-3-yl formate (**1**), veramarine-3-yl formate (**2**), jerv-5,11-diene-3β,13β-diol (**3**), (1β,3β,5β)-1,3-dihydroxyjervanin-12(13)-en-11-one (**4**), and veratramine-3-yl acetate (**5**). Compounds **1** and **5** exhibited potent inhibitory activity (11.3 and 4.7 μM, respectively) against protein tyrosine phosphatase 1B (PTP1B), which has emerged as a viable target for treatment of type 2 diabetes mellitus. On the basis of their PTP1B inhibitory activity, the compounds were evaluated for their potential to enhance glucose uptake in C2C12 skeletal muscle cells. The insulin-stimulated glucose uptake was enhanced upon treatment with compounds **1** and **5** (10 μM) by 49.9 ± 6.5% and 56.0 ± 9.7%, respectively, in a more potent manner than that with the positive control rosiglitazone (47.3 ± 3.4% at 30 μM). These results suggest that steroidal alkaloids serve as practical antidiabetes mellitus leads capable of enhancing glucose uptake.



The genus *Veratrum* (Liliaceae) comprises 40 species and is broadly distributed in temperate and boreal areas of the northern hemisphere.[1] Several *Veratrum* species are poisonous to humans and animals, and previous investigations have revealed that steroidal alkaloids are responsible for such toxicity.[2] Among many types of steroidal alkaloids, veratrum-type steroidal alkaloids are the most frequently found in the genus *Veratrum*.[3] There are four subtypes of veratrum-type steroidal alkaloids, i.e., the cevanine, veratramine, verazine, and jervine types, which depend upon the linkage between the steroidal and alkaloidal constituents via the E-ring.[3] *Veratrum* spp. are also reported to produce flavonoids, stilbenoids, and phenylpropanoids.[3,4]

Veratrum nigrum L. is a tall, stout plant with cauline, sessile, and broadly elliptic glabrous leaves.[1] Even though previous studies on *V. nigrum* showed the presence of bioactive phytochemicals with hypotensive, emetic, and antifungal activities,[3] such reports are less numerous relative to those on other *Veratrum* spp. The steroidal alkaloids verussurinine and veratroylzygadenine from *V. nigrum* elicited antithrombotic activity[5] and alleviated allergic contact dermatitis,[6] implying that members of this compound class, generally believed to be toxic, also may exert therapeutically beneficial activities.

Globally, the elevated incidence of type 2 diabetes mellitus and obesity has inspired the search for new drug leads to ameliorate these metabolic diseases.[7] Resistance to insulin and leptin is regarded as a common phenotype of type 2 diabetes mellitus and obesity.[8] Among numerous antidiabetic strategies, inhibition of protein tyrosine phosphatase 1B (PTP1B) has been regarded as a suitable approach to treat these metabolic disorders. PTP1B acts as a blocker of insulin signaling through the dephosphorylation of the insulin receptor and its substrate.[8,9] The enzyme also works as a negative regulator of the leptin signaling pathway, involved in food intake and peripheral energy expenditure,[10] and the GLUT 4 transporter involved in glucose uptake.[11] PTP1B knockout murine models exhibit the reduced circulation of insulin, glucose, and triglyceride levels after being injected with insulin, suggesting that inhibition of PTP1B offers the potential for development of antidiabetes mellitus agents.[12,13] Natural products have provided promising leads as anti-PTP1B agents, e.g., alkaloids such as berberine[14] and papaverine[15] and certain isoprenylated flavonoids.[16] A continuing effort to discover antidiabetes

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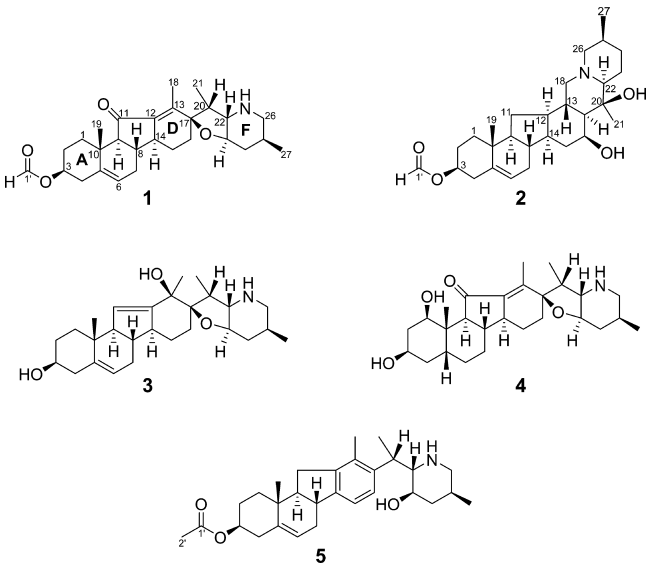
mellitus leads from natural product sources[17] led to the isolation and characterization of five new steroidal alkaloids (**1**–**5**) from *V. nigrum* and the evaluation of their PTP1B inhibitory activity. These compounds were further assessed for their potential to enhance glucose uptake in C2C12 skeletal muscle cells to validate whether they could serve as viable antidiabetes mellitus templates.



RESULTS AND DISCUSSION

A methanol extract of the roots and rhizomes of *V. nigrum* was subjected to a combination of chromatographic techniques to afford five new steroidal alkaloids, namely, jervine-3-yl formate (**1**), veramarine-3-yl formate (**2**), jerv-5,11-diene-3β,13β-diol (**3**), (1β,3β,5β)-1,3-dihydroxyjervanin-12(13)-en-11-one (**4**), and veratramine-3-yl acetate (**5**). The ^{1}H and ^{13}C NMR data of these compounds are summarized in Tables 1 and 2, respectively.

Compound **1** was isolated as a white, amorphous powder. The molecular formula was established as $C_{28}H_{39}NO_4$ based on the ^{13}C NMR data (Table 2) and the HRESIMS quasimolecular ion at m/z 454.2954 (calcd $[M + H]^+$, m/z 454.2957; Figure S7, Supporting Information). The 1D NMR data (Table 1) were similar to those of jervine[18] except for the presence of resonances (δ_H 8.06; δ_C 162.4) and the IR absorption (1708 cm^{-1}) (Figure S8, Supporting Information) for a formate group. This functionality was connected to C-3 (δ_C 74.8) based on the HMBC correlation between H-1′ (δ_H 8.06) and C-3 (δ_C 74.8) (Figure 1). The NOESY correlations from H_3-19 (δ_H 1.06) to H-8 (δ_H 1.53) and from H-9 (δ_H 1.79) to H-14 (δ_H 2.06) (Figure 2) were used to confirm the stereochemical equivalence of the C-nor-D-homosteroid framework[19] in **1** with jervine (Figure 2). The formate group was assigned a β-orientation based on the NOESY correlation of H-3 (δ_H 4.67) with H-1α (δ_H 1.26) (Figure 2). Assignment of the relative configurations of the two heterocyclic E- and F-rings hinged upon the NOESY correlations between H-23 (δ_H 3.57) and H-25 (δ_H 1.87) (Figure 2). The C-25 configuration was validated by its similar ^{13}C NMR chemical shift value to analogous data for its analogues (ca. 30 ppm).[18] In addition, the large vicinal coupling constants of H-22 (δ_H 3.03) with H-20 (δ_H 2.68) and H-23 (δ_H 3.57) ($^3J_{H20,H22} = {}^3J_{H22,H23}$ = 9.6 Hz) and the NOE correlations between H-18 and H-22 and between H-23 (δ_H 3.57) and H-25 indicated that the conformers associated with the C-20–C-22 and C-22–C-23 bonds are *syn* and *anti*, respectively (Figure 2), confirming the configurational assignment of the O- and N-heterocycles. The absolute configurations of the two heterocyclic moieties were assigned on the basis of the consistent absolute configuration of homosteroidal architectures obtained from natural sources[19] and the NOESY correlations from H_2-16 (δ_H 1.63) to H_3-21 (δ_H 1.05) and from H_3-18 (δ_H 2.12) to H-22 (δ_H 3.03) (Figure 2). Consequently, compound **1** was assigned as jervine-3-yl formate.

Compound **2** was isolated as a white, amorphous powder. The ^{13}C NMR data and a quasimolecular ion at m/z 458.3269 (calcd $[M + H]^+$, m/z 458.3270; Figure S14, Supporting Information) in the HRESIMS led to the assignment of a $C_{28}H_{43}NO_4$ molecular formula. The 1D NMR spectra of **2** were found to share significant similarities with those of veramarine,[20] aside from the presence of a formate moiety, as deduced from resonances at δ_H 8.06 and δ_C 162.5 and the IR absorption at 1715 cm^{-1} (Tables 1 and 2; Figure S15, Supporting Information). The HMBC correlation of the formate proton (δ_H 8.06) with C-3 (δ_C 75.1) revealed this functionality to be located at C-3 (Figure 1). The configurational assignment of the C-nor-D-homosteroid core structure in **2** was established via the NOESY correlations between H-8 (δ_H 2.09) and H_3-19 (δ_H 1.11), H-9 (δ_H 1.29) and H-14 (δ_H 1.55), and H-12 (δ_H 1.72) and H-17 (δ_H 1.36), indicating *trans*- and *cis*-fused B/C- and C/D-ring junctions, respectively (Figure 2). The NOESY correlations from H-17 (δ_H 1.36) to H-18α (δ_H 2.64) and H-22 (δ_H 2.98) permitted assignment of the E-ring as shown in Figure 2. The formate group was established with a β-equatorial orientation based on the NOESY correlation from H-1α (δ_H 1.24) to H-3 (δ_H 4.65). The NOESY correlations between H-16 (δ_H 4.46) and H_3-21 (δ_H 1.31) and between H-23β (δ_H 1.93) and H_3-27 (δ_H 1.17) indicated the β-axial orientations of HO-16, HO-20, and Me-27 (Figure 3). This was confirmed by the ^{13}C NMR chemical shift values of the associated C-16 (δ_C 66.8), C-20 (δ_C 73.0), and C-25 (δ_C 27.7) stereogenic centers similar to those of veramarine (δ_C 66.1, 73.2, and 27.6, respectively).[20] The splitting pattern and coupling constants of H-17 (dt, J = 12.2, 2.4 Hz) were reminiscent of its *anti*-relationship with H-13, which established the configuration of C-13 as shown in Figures 1 and 3. The absolute configuration of **2** was established using the same approach as for **1**. Thus, the structure of compound **2** was defined as veramarine-3-yl formate (**2**).

Compounds **1** and **2**, the C-3 formate esters of the known steroidal alkaloids jervine and veramarine, are true natural products since formic acid was not used during the isolation and purification processes. This was validated by an HPLC experiment that indicated both compounds to be present in the crude *V. nigrum* extract (see the Experimental Section; Figure S39, Supporting Information).

Compound **3** was obtained as a white, amorphous powder. The molecular formula of **3** was deduced as $C_{27}H_{41}NO_3$ from its ^{13}C NMR data and a quasimolecular ion at m/z 428.3162 (calcd $[M + H]^+$, m/z 428.3165; Figure S22, Supporting Information) in the HRESIMS. The 1D NMR data were similar to those of jervine, implying that compound **2** is a jervine-type steroidal alkaloid carrying a C-nor-D-homosteroid moiety. The 1D NMR spectra of **3** indicated the presence of two olefinic protons (δ_H 5.41, 5.72) and an additional oxygenated tertiary

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Table 1. ^{1}H NMR Spectroscopic Data for Compounds 1−5 in Methanol-d_4

	1	2	3	4	5
position	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)
1α	1.26 td (11.9, 4.3)	1.24 m	1.20 td (14.0, 3.3)	4.64 br t	1.37 td (13.6, 3.5)
1β	2.21 dt (11.5, 3.3)	1.74 m	1.86 dt (14.0, 3.5)		1.93 dt (13.6, 3.5)
2	1.92 s	1.85 m	1.55 m, 1.84 m	1.87 d (2.8)	1.75 dd (12.1, 3.5), 1.92 m
3	4.67 sep (5.4)	4.65 sep (4.9)	3.41 tt (11.2, 4.6)	4.09 br t (2.5)	4.58 tt (11.6, 4.8)
4	2.33 m, 2.45 dd (11.0, 3.7)	2.34 m	2.14 m	1.63 m	2.38 dq (12.9, 2.5)
		2.44 m	2.32 dd (12.9, 2.8)	1.89 m	2.45 ddd (12.9, 5.0, 1.8)
5				2.02 m	
6	5.9 d (5.1)	5.45 d (4.8)	5.41d (5.0)	1.26 m, 1.96 br s	5.56 dt (5.3, 2.0)
7	1.96 m	2.03 m	1.89 m, 2.14 m	1.49 m (α), 1.86 m (β)	2.03 m, 2.58 m
8	1.53 m	2.09 m	1.56 m	1.50 m	2.96 td (11.6, 5.2)
9	1.79 m	1.29 m	2.04 d (11.3)	2.08 d (12.6)	1.83 m
10					
11		1.70 m	5.72 br s		2.64 m, 2.88 dd (14.9, 7.3)
12		1.72 m			
13		1.65 m			
14	2.06 m	1.55 m	2.21 m	2.06 m	
15	1.98 m	2.35 d (7.7)	1.86 m	1.84 m	7.08 d (7.8)
	1.31 m	1.80 m	1.46 m	1.40 m	
16	1.63 m	4.46 br s	1.65 td (14.1, 4.1) (α)	1.47 m	7.07 d (7.8)
			1.94 m (β)	1.92 m	
17		1.36 dt (12.2, 2.4)			
18	2.12 s	2.64 t (10.9) (α), 3.33 m (β)	1.22 s	2.11 d (2.1)	2.35 s
19	1.06 s	1.11s	0.98 s	1.07 (s)	1.18 s
20	2.68 m		2.83 m	2.63 quin (7.3)	3.8 qd (7.3, 4.5)
21	1.05 d (6.6)	1.31 s	1.04 d (7.7)	1.03 d (6.0)	1.44 d (7.3)
22	3.03 t (9.6)	2.98 m	2.90 t (9.5)	2.95 t (9.5)	3.02 dd (10.0, 4.5)
23	3.57 td (10.6, 3.6)	1.06 m (α), 1.93 m (β)	3.56 td (10.7, 3.7)	3.51 dd (11.1, 6.0)	3.43 td (10.0, 4.4)
24	1.26 m	1.68 m	1.44 d (11.7)	2.18 dd (13.9, 6.9)	1.26 d (13.1)
	2.54 m	1.73 m	2.21 m	1.28 d (13.9)	2.12 d (13.1)
25	1.87 m	2.20 m	1.92 m	1.83 m	1.81 s
26	3.22 dd (12.7, 3.9)	3.22 d (12.8)	3.32 m	3.20 quin (1.7)	3.10 dd (12.2, 2.4)
	2.60 d (13.9)	3.14 d (9.9)	2.63 t (12.4)	2.54 t (12.5)	2.57 d (12.2)
27	1.05 d (6.6)	1.17 d (7.4)	1.06 d (6.7)	1.07 d (8.5)	0.97 d (6.6)
1′	8.06 s	8.06 s			
2′					2.02 s

carbon (δ_C 76.0) and the absence of the carbonyl functionality compared to jervine, implying different locations of the oxygenated and olefinic functionalities in **3** and jervine. The HMBC correlations from H-11 (δ_H 5.72) and H$_3$-18 (δ_H 1.22) to the oxygenated (δ_C 76.0) and olefinic quaternary carbons (δ_C 154.0) (Figure 1) were indicative of the structural differences between **3** and jervine (Figure 1). The same configurational assignments of **3** were made as for **1** except for the additional C-13 stereogenic center. The orientation of the C-13 hydroxy group was assigned as β-equatorial based on the NOESY correlations from H$_3$-18 (δ_H 1.22) to H-14 (δ_H 2.21), H-16α (δ_H 1.65), and H-20 (δ_H 2.83) (Figure 3). Thus, the structure of **3** was assigned as jerv-5,11-diene-3β,13β-diol.

Compound **4** was acquired as a white, amorphous powder. The HRESIMS data showed a quasimolecular ion at m/z 444.3108 (calcd [M + H]$^+$, m/z 444.3114; Figure S29, Supporting Information), which, along with its ^{13}C NMR data, specified its molecular formula as $C_{27}H_{41}NO_4$. The 1D NMR data (Table 1) were in close agreement with those of (1β,3α,5β)-1,3-dihydroxyjervanin-12(13)-en-11-one[21] except for the A-ring ^{13}C NMR resonances. This was indicative of **4** being a stereoisomer of the known compound with different configurations of the two A-ring hydroxylated methine carbons. The configurations of these methine carbons were established by homocoupling constant analysis and from the NOESY spectrum. The broad triplet coupling patterns of H-1 (δ_H 4.64) and H-3 (δ_H 4.09) suggested the β-axial orientations of the C-1 and C-3 hydroxy groups, which was confirmed by the NOESY correlations from H-1 (δ_H 4.64) to H-9 (δ_H 2.08) and H$_3$-19 (δ_H 1.07) and from H-3 (δ_H 4.09) to Hα-7 (δ_H 1.49) (Figure 3). The NOESY correlations from H$_2$-2 (δ_H 1.87) to H-9 (δ_H 2.08) and from H-5 (δ_H 2.02) to H$_3$-19 (δ_H 1.07) and the ^{13}C NMR chemical shift of C-5 (δ_C 32.8) compared to that of (1β,3α,5β)-1,3-dihydroxyjervanin-12(13)-en-11-one (δ_C 36.7) revealed that rings A and B are *cis*-fused in compound **3** (Figure 3). (For *trans*-fused A/B-rings in a C-nor-D-homosteroidal structure, the ^{13}C NMR chemical shift of C-5 is ca. 42 ppm.[22]) The remaining stereochemical features of **4** were established as for **1**. Thus, compound **4** is the C-3 epimer of (1β,3β,5β)-1,3-dihydroxyjervanin-12(13)-en-11-one.

Compound **5** was isolated as a white, amorphous powder. The molecular formula, $C_{29}H_{41}NO_3$, was suggested based on the ^{13}C NMR data and the HRESIMS quasimolecular ion at m/z 452.3167 (calcd [M + H]$^+$, m/z 452.3165; Figure S37, Supporting Information). The 1D NMR data (Tables 1 and 2) and IR absorptions (3448, 2921, 1637, and 1540 cm^{-1}) (Figure

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Table 2. ^{13}C NMR Spectroscopic Data for Compounds 1−5 in Methanol-d_4

	1	2	3	4	5
position	δ_C, type	δ_C, type	δ_C, type	δ_C, type	δ_C, type
1	38.4, CH_2	38.9, CH_2	39.7, CH_2	73.0, CH	38.9, CH_2
2	28.3, CH_2	28.7,[a] CH_2	32.0, CH_2	33.2, CH_2	28.5, CH_2
3	74.8,CH	75.1, CH	72.6, CH	69.0, CH	75.5,CH
4	38.2,CH_2	38.6, CH_2	42.6, CH_2	31.5, CH_2	38.8,CH_2
5	142.1, C	142.0, C	143.2, C	32.8, CH	142.9, C
6	123.6, CH	124.4, CH	123.4, CH	25.0, CH_2	123.9, CH
7	31.5 CH_2	32.1, CH_2	31.0, CH_2	25.9, CH_2	31.3,[a] CH_2
8	39.6, CH	39.7, CH	47.7, CH	43.4, CH	42.5, CH
9	63.4, CH	55.7, CH	61.5, CH	55.6, CH	58.6, CH
10	38.5, C	38.2, C	38.3, C	42.0, C	38.2, C
11	208.1, C	28.4,[a] CH_2	122.4, CH	208.9, C	31.4,[a] CH_2
12	139.1, C	41.5, CH	154.0, C	138.5, C	134.0, C
13	145.6, C	32.2, CH	76.0, C	145.6, C	144.6, C
14	45.5, CH	44.6, CH	48.2, CH	45.5, CH	145.9, C
15	25.3, CH_2	31.9, CH_2	28.2, CH_2	27.9, CH_2	126.4, CH
16	31.5 CH_2	66.8, CH	28.8, CH_2	35.0, CH_2	121.3, CH
17	87.4, C	49.5, CH	92.0, C	87.4, C	139.1, C
18	12.2, CH_3	60.6, CH_2	25.2, CH_3	12.2, CH_3	16.0, CH_3
19	18.5, CH_3	19.2, CH_3	19.7, CH_3	18.1, CH_3	19.5, CH_3
20	40.1, CH	73.0, C	35.7, CH	40.5, CH	36.2, CH
21	10.9, CH_3	22.4, CH_3	10.4, CH_3	11.0, CH_3	21.0, CH_3
22	65.4, CH	71.5, CH	63.8, CH	66.0, CH	67.6, CH
23	75.0, CH	18.4, CH_2	74.3, CH	75.5, CH	69.6, CH
24	37.8 CH_2	28.3, CH_2	37.8, CH_2	38.8, CH_2	43.5, CH_2
25	30.2, CH	27.7, CH	29.4, CH	30.6, CH	29.9, CH
26	53.1,CH_2	60.9, CH_2	52.6, CH_2	53.7, CH_2	53.1,CH_2
27	18.6, CH_3	16.4, CH_3	18.4, CH_3	18.6, CH_3	18.6, CH_3
1′	162.4, C	162.5, C			172.4, C
2′					21.2, CH_3

[a]Values within the column may be interchanged.

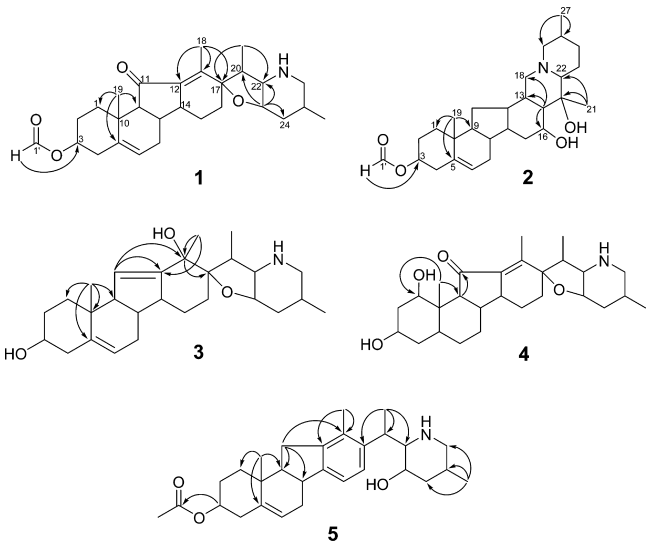

Figure 1. Key HMBC correlations of compounds **1**−**5**. Stereodescriptors were deliberately omitted for a clear presentation of correlations.

S38, Supporting Information) exhibited significant similarities to those of veratramine,[23] carrying the C-nor-D-homosteroid scaffold in which the D-ring is aromatized. The only difference was the presence of an acetyl group, as deduced from NMR spectroscopic resonances (δ_H 2.02; δ_C 21.2, 172.4) and the IR absorbance at 1730 cm^{-1}. The HMBC correlation of H-3 (δ_H 4.58) with the acetyl carbonyl carbon (δ_C 172.4) indicated that the acetoxy group is located at C-3. The configurational assignment of **5** was carried out as for compounds **1**−**4**. The splitting pattern of H-3 (tt, 11.6, 4.8 Hz) and the NOESY correlations from H-3 (δ_H 4.58) to H-1α (δ_H 1.37) (Figure 3) implied a β-equatorial orientation of the C-3 acetoxy group. The configurations of C-20 and C-22 were established by comparison of the coupling constant and the spin pattern of H-20 (qd, 7.3, 4.5 Hz) with those of veratramine. The absolute configuration of **5** was established based on the specific rotation of **5** (−11, *c* 0.1, MeOH) with analogous data for veratramine (qd, 7, 4 Hz; −60.2, *c* 0.3, MeOH).[23] Consequently, compound **5** was identified as veratramine-3-yl acetate. Compound **5** is a true natural product based on an HPLC profile of the crude extract prior to our usage of HOAc in the isolation and purification processes (see the Experimental Section; Figure S39, Supporting Information).

Compounds **1**−**5** were assessed for their inhibitory potential against protein tyrosine phosphatase 1B. The known PTP1B inhibitors RK-682 (IC_{50} = 4.5 μM) and ursolic acid (IC_{50} = 3.2 μM)[16] were used as positive controls in the assay. Compounds **1** and **5** showed inhibitory activities against PTP1B, with IC_{50} values of 11.3 and 4.7 μM, respectively, while compounds **2**−**4** were inactive (IC_{50} > 30 μM). On the basis of the enzyme inhibition assay results, the anti-PTP1B-active alkaloids were tested for their glucose uptake capability to evaluate their potential as viable drug leads for the treatment of diabetes

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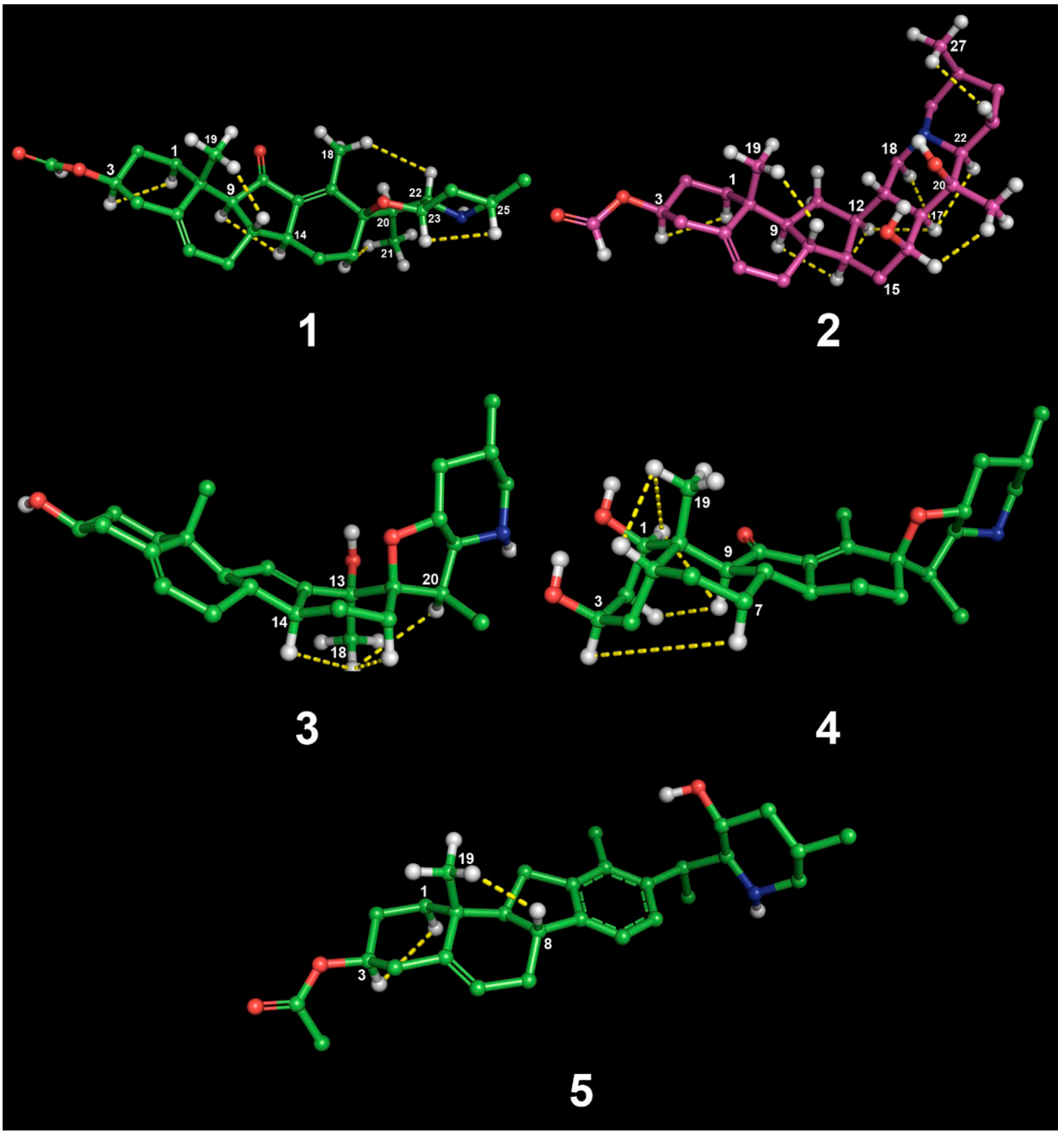

Figure 2. . Key NOESY correlations of compounds **1**−**5**. Stereostructures were minimized at the MMFF force field (see Experimental Section), and some hydrogens were removed for a clearer demonstration of the 3D structures.

mellitus and obesity. The glucose uptake evaluation was performed using C2C12 skeletal muscle cells and 2-deoxy-[^{3}H]-glucose as a probe, with rosiglitazone as the positive control. Initial assessment showed that basal glucose uptake increased upon treatment with compound **1** at 5 μM and reached a maximal level 2 h after treatment. Co-treatment of **1** at 5 μM with insulin enhanced the glucose uptake activity (Figure 3A). These preliminary data led to the evaluation of various concentrations of compounds **1**−**5** to investigate the concentration−response relationship for glucose uptake. As shown in Figure 3B, compounds **1** and **5** at a 10 μM concentration, found to exhibit anti-PTP1B activity, increased basal glucose uptake by 15.0 ± 5.4% and 24.8 ± 9.1%, respectively, and were of comparable potency to rosiglitazone at 30 μM. Insulin-stimulated glucose uptake was also enhanced upon treatment with compounds **1** and **5** at 10 μM by 49.9 ± 6.5% and 56.0 ± 9.7%, respectively, and was compared with rosiglitazone at 30 μM (47.3 ± 3.4%). On the basis of these assay results, compounds **1** and **5** enhanced the glucose uptake process at least in part via impeding the action of PTP1B action and by increasing insulin sensitivity.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured utilizing a JASCO DIP-1000 polarimeter (Tokyo, Japan). IR data were recorded on a Thermo Electron US/Nicolet380 (Madison, WI, USA). NMR experiments were implemented on a Bruker DMX 300 (300 MHz) or Bruker Avance III (600 MHz) spectrometer. HRESIMS data were obtained on a JMS 700 high-

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J. Nat. Prod. XXXX, XXX, XXX−XXX

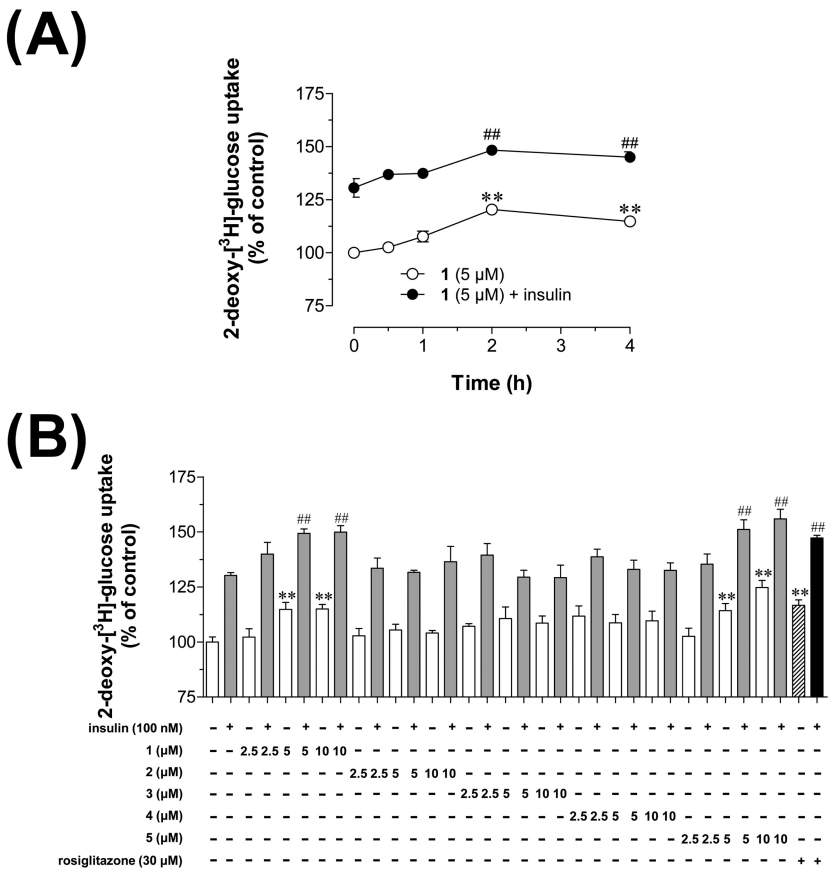

Figure 3. Effects of compounds **1**−**5** on glucose uptake in C2C12 myotubes. (A) Time course of the stimulatory effect of compound **1** on glucose uptake in C2C12 myotubes. Cells were treated with 5 μM **1** alone or 5 μM **1** followed by 100 nM insulin for 30 min. (B) Concentration−response relationship of compounds **1**−**5** on glucose uptake in C2C12 myotubes. Cells were treated with indicated concentrations of **1**−**5** for 2 h. Rosiglitazone (30 μM) was used as a positive control. The results are expressed as mean ± SEM of three independent experiments, each performed in triplicate (**, $p < 0.01$ *vs* basal glucose uptake (no insulin stimulation) and ##, $p < 0.01$ *vs* insulin-stimulated glucose uptake).

resolution mass spectrometer (JEOL, Tokyo, Japan). Thin-layer chromatography (TLC) was performed on glass plates precoated with silica gel 60 F_{254} and RP-18 F_{254} (20 × 20 cm, 200 μm, 60 Å, Merck). Vacuum-liquid chromatography (VLC) was conducted on Merck silica gel (70−230 mesh), and medium-pressure liquid chromatography (MPLC) was carried out utilizing a Biotage Isolera apparatus equipped with a reversed-phase C_{18} SNAP Cartridge KP-C_{18}-HS and normal-phase SNAP Cartridge KP-Sil (340 g, Biotage AB, Uppsala, Sweden). High-performance liquid chromatography (HPLC) was performed on a Gilson system using an OptimaPak SIL-51002546 (250 × 4.6 mm, 5 μm) column, equipped with a Gilson prepELS II detector (Middleton, WI, USA). For the glucose uptake inhibition bioassay, Dulbecco's modified Eagle's cell culture medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Gibco BRL (Grand Island, NY, USA), 2-deoxy-[^{3}H]-glucose was obtained from PerkinElmer Life Sciences (Boston, MA, USA), and 2-deoxy-D-glucose was obtained from Sigma (St. Louis, MO, USA). Rosiglitazone was purchased from Masung & Co., Ltd. (Seoul, Korea). PTP1B (human, recombinant) was purchased from BIOMOL International LP (Plymouth Meeting, PA, USA).

Plant Material. The roots and rhizomes of *Veratrum nigrum* were collected from a medicinal plant market in Daejeon, Korea, in 2013 and identified by one of the authors (M.N.). A voucher specimen (CNU0694) has been deposited at the Pharmacognosy Laboratory of the College of Pharmacy, Chungnam National University, Daejeon, Korea.

Extraction and Isolation. The dried roots and rhizomes (4.5 kg) of *V. nigrum* were soaked in a 14% aqueous NH_4OH solution (11.5 L) and extracted with $CHCl_3$/MeOH (1:1, 42 L) at room temperature for 1 week. The crude extract was concentrated under vacuum to yield a dark brownish slurry (748 g), and the slurry was suspended in H_2O (4 L), acidified with HCl (pH 3), and filtered. The filtrate was basified with NH_4OH (pH 10) and extracted with $CHCl_3$. The alkaloid-enriched $CHCl_3$ fraction (88 g) was subjected to silica gel VLC (800 g) and eluted with the upper phase of an EtOAc/H_2O/HOAc (45:1.5:1.0 → 10:2.7:2.5) mixture to produce seven fractions (Fr. 1−Fr. 7). Fr. 4 (10 g) was purified by MPLC (silica SNAP cartridge KP-Sil, 340 g) eluting with gradient mixtures of EtOAc/MeOH/H_2O (12:0.1:0.1 → 10:1:1) to afford three subfractions, Fr. 4-1−Fr. 4-3. Fr. 4-1 (1 g) was chromatographed over a silica gel column (15 mm × 75 cm), eluting with EtOAc/H_2O/HOAc (40:2.7:2.2) to afford **5** (1.9 mg). Fr. 4-2 (4 g) was purified utilizing silica gel column chromatography (15 mm × 80 cm) with the upper phase of an EtOAc/H_2O/HOAc (30:2:3) mixture as a mobile phase to afford **2** (14 mg). Fr. 6 (11 g) was chromatographed over MPLC (silica SNAP cartridge KP-Sil, 340 g) eluting with EtOAc/*n*-BuOH/HOAc (9:1:0.2 → 2:1:0.2) to afford six subfractions, Fr. 6-1−Fr. 6-6. Fr. 6-6 (4.4 g) was fractionated to generate nine subfractions, Fr. 6-6-1−Fr. 6-6-9, by MPLC (silica SNAP cartridge KP-Sil, 100 g), eluting with a gradient of hexanes/CH_2Cl_2/MeOH (30:1:1 → 10:1:1). Fr. 6-6-3 (1.1 g) was subjected to silica gel column chromatography (15 mm × 75 cm) eluting with the upper phase of a mixture of EtOAc/H_2O/HOAc (50:2.7:2.2) to afford **1** (53 mg). Fr. 6-6-5 (1.1 g) was applied to silica gel column chromatography (15 mm × 75 cm), eluting under isocratic conditions of EtOAc/H_2O/HOAc (50:2.7:2.2), to afford **3** (43.3 mg, R_f 0.4). Fr. 6-6-7 (500 mg) was purified by silica gel column chromatography (15 mm × 65 cm) eluting with the upper phase of an EtOAc/H_2O/HOAc mixture (40:2.7:2.2) to give **4** (9.8 mg, R_f 0.4).

For HPLC profiling for validation of compounds **1**, **2**, and **5** being naturally occurring (Figure S39, Supporting Information), the $CHCl_3$-

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J. Nat. Prod. XXXX, XXX, XXX−XXX

soluble extract of the initial $CHCl_3$/MeOH (1:1) crude extract and each of the compounds were analyzed by normal-phase HPLC (OptimaPak SIL-51002546, 250 × 4.6 mm, 5 μm), eluting with a gradient of hexanes/2-propanol (9:1 → 0:1, 60 min), and detection was carried out using evaporative light scattering detection (Gilson prepELS II detector).

*Jervine-3-yl formate (**1**):* white, amorphous powder; $[\alpha]_D^{24}$ −151 (*c* 0.5, MeOH); IR ($CHCl_3$) ν_{max} 3392, 1708, 1632, 1557 cm^{-1}; ^1H and ^{13}C NMR, see Table 2; HRESIMS *m*/*z* 454.2954 $[M + H]^+$ (calcd for $C_{28}H_{40}NO_4$, 454.2957).

*Veramarine-3-yl formate (**2**):* white, amorphous powder; $[\alpha]_D^{24}$ −41 (*c* 0.5, MeOH); IR ($CHCl_3$) ν_{max} 3321, 1715, 1597 cm^{-1}; ^1H and ^{13}C NMR, see Table 2; HRESIMS *m*/*z* 458.3269 $[M + H]^+$ (calcd for C_{28} $H_{44}NO_4$, 458.3270).

*Jerv-5,11-diene-3β,13β-diol (**3**):* white, amorphous powder; $[\alpha]_D^{24}$ −27 (*c* 0.5, MeOH); IR ($CHCl_3$) ν_{max} 3388, 1710, 1631, 1561 cm^{-1}; ^1H and ^{13}C NMR, see Table 2; HRESIMS *m*/*z* 428.3162 $[M + H]^+$ (calcd for $C_{27}H_{42}NO_3$, 428.3165).

*(1β,3β,5β)-1,3-Dihydroxyjervanin-12(13)-en-11-one (**4**):* white, amorphous powder; $[\alpha]_D^{24}$ −32 (*c* 0.5, MeOH); IR ($CHCl_3$) ν_{max} 3354, 1737, 1707, 1631 cm^{-1}; ^1H and ^{13}C NMR, see Table 2; HRESIMS *m*/*z* 444.3108 $[M + H]^+$ (calcd for $C_{27}H_{42}NO_4$, 444.3114).

*Veratramine-3-yl acetate (**5**):* white, amorphous powder; $[\alpha]_D^{24}$ −11 (*c* 0.1, MeOH); IR ($CHCl_3$) ν_{max} 3457, 3448, 2921, 1637, 1540 cm^{-1}; ^1H and ^{13}C NMR, see Table 2; HRESIMS *m*/*z* 452.3167 $[M + H]^+$ (calcd for $C_{29}H_{42}NO_3$, 452.3165).

Computational Details. All conformational searches were carried out employing the Macromodel (version 9.9, Schrodinger LLC) program with "mixed torsional/low mode sampling" in the MMFF force field. The searches were performed in the gas phase with a 50 kJ/mol energy window limit and a maximum number of 10 000 steps to fully explore all low-energy conformers. All minimization processes were carried out utilizing the Polak-Ribiere conjugate gradient (PRCG) method, 10 000 maximum iterations, and a 0.001 convergence threshold, and global minimized structures were used for the NOESY analysis shown in Figure 2.

Cell Culture and Differentiation. Mouse C2Cl2 skeletal muscle myoblasts (ATCC CRL-1772, Manassas, VA, USA) were grown in DMEM with 10% FBS at 37 °C in a 5% CO_2 atmosphere. One-hundred percent confluent myoblasts were differentiated by incubation of 1% FBS in DMEM. The medium was changed every day until day 4.[24,25] Test compounds were dissolved in DMSO to a final concentration of 0.1%.

PTP1B Inhibitory Activity. The enzyme activity was assessed utilizing *p*-nitrophenyl phosphate (*p*-NPP), as described previously.[26] To each of the 96 wells in a microtiter plate was added 2 mM *p*-NPP and PTP1B (0.05−0.1 μg) in a buffer containing 50 mM citrate (pH 6.0), 0.1 M NaCl, 1 mM EDTA, and 1 mM dithiothreitol with or without a test compound. After incubation at 37 °C for 30 min, the reaction was quenched with addition of 10 M NaOH. The amount of *p*-nitrophenol produced was evaluated by measuring the absorbance at 405 nm.

Glucose Uptake Assay. The glucose uptake assay was performed as described previously, with some modifications.[27] Differentiated mature C2C12 myotubes (ATCC CRL-1772TM, Manassas, VA, USA) cultured in a 24-well microplate were incubated in serum-free DMEM with 0.2% bovine serum albumin for 3 h and again incubated in Krebs-Ringer phosphate-HEPES (KRPH) buffer (10 mM HEPES, pH 7.4, 136 mM NaCl, 4.7 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM phosphate buffer) for 1 h. After treatment with a test compound for the appropriate time intervals, 100 nM insulin was added and incubated for an additional 30 min. Glucose uptake was initiated by the addition of 0.5 μCi/mL 2-deoxy-[^3H]-glucose with 100 μM 2-deoxy-D-glucose to each well. After 10 min, the cells were washed three times with ice-cold phosphate-buffered saline (pH 7.4) and lysed with 0.1% sodium dodecyl sulfate and 0.5 M NaOH. The radioactivity of the lysate was determined employing liquid scintillation counting in a β-counter and normalized to the total protein level.

Statistical Analysis. All values are expressed as means ± standard error of the mean (SEM). Statistical comparisons of the results were tested using Dunnett's multiple comparison test using the GraphPad Prism software (San Diego, CA, USA). Data with $p < 0.05$ are considered statistically significant.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

NMR, HRESIMS, and IR spectra of compounds **1**−**5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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