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Hypoglycemic Diterpenoids from *Tinospora crispa*

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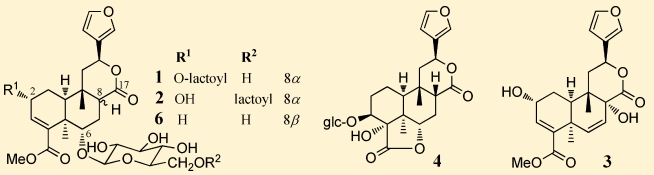
Supporting Information

ABSTRACT: Three new diterpenoids, 2-*O*-lactoylborapetoside B (**1**), 6′-*O*-lactoylborapetoside B (**2**), and tinocrispol A (**3**), and nine known diterpenoids (**4**−**12**) were isolated from an EtOH extract of *Tinospora crispa* vines. Their structures were elucidated by spectroscopic analyses. The C-6 glucosyloxy group in borapetoside C (**6**) was revised to be α-oriented. The in vivo hypoglycemic activities of the major components, borapetosides A−C (**4**−**6**), were examined. Intraperitoneal injection of **4** and **6** (5 mg/kg) showed significant lowering of plasma glucose levels in normal and streptozotocin-induced type 1 diabetic mice. Borapetoside C increased glucose utilization in peripheral tissues and reduced hepatic gluconeogenesis, accounting for the hypoglycemic effect.



Diabetes mellitus (DM) is a metabolic disorder ranked among the top 10 causes of mortality worldwide. Diabetes often leads to neurological and vascular complications that, in severe cases, can result in limb amputation.[1] The World Health Organization estimated that more than 180 million people worldwide suffer from diabetes, a figure that could double by 2030 due to population growth, aging populations, urbanization, unhealthy diets, and soaring incidences of obesity and sedentary lifestyles.[2] Diabetes is associated with abnormal insulin release and sensitivity, which result in increased hepatic glucose production and reduced glucose uptake in muscles.[3] The use of herbal remedies to treat DM has been practiced since ancient times.[4] Currently more than 1000 plant species are being used as folk medicines for DM throughout the world.[5] In Malaysia, extracts from *Tinospora crispa* Miers (syn. *T. tuberculata* Beumee) (Menispermaceae) are often used to treat malaria[6−8] and filariasis.[9] It has also been shown to reduce plasma glucose levels in moderately diabetic rats,[10] ascribable to its stimulus efficacy on β cell insulin release.[11] Further characterization of the insulinotropic activity of this extract, using HIT-T15 cells[11] as an insulin-secreting cell model, has demonstrated its influence on Ca^{2+} handling in β cells.[12] Also, it has been found that such insulin release at the basal condition may be mediated by closure of the ATP-sensitive K^+ channels to depolarize the membrane of β cells and in turn open the voltage-sensitive Ca^{2+} channels. These results suggest a potential for this natural substance to be developed as an oral antihyperglycemic agent. Diterpenoids, such as borapetosides A−G and borapetols A−C,[13−16] have been isolated from this plant. However, the active components of this plant remained poorly understood. Hence, this study was initiated to isolate the active compounds for further pharmacological studies.

The BuOH-soluble fraction of an EtOH extract of *T. crispa* vines was chromatographed repeatedly using centrifugal partition chromatography (CPC), Sephadex LH-20, and a reversed-phase Lobar column to give three new (**1**−**3**) and nine known (**4**−**12**) *cis*-clerodane-type furanoditerpenoids. The known diterpenoids were identified as borapetosides A−F (**4**−**6**, **12**, **8**, **9**), columbin (**10**), and borapetols A (**11**) and B (**7**).[13−17] Although borapetoside C (**6**) had been reported,[15] its structure and some ^1H and ^{13}C NMR assignments were revised in this study. The following describes the structural characterization of compounds **1**−**3** and **6** and the hypoglycemic activity of borapetosides A−C (**4**−**6**) on normal, streptozotocin-induced type 1 diabetic (T1DM) and diet-induced type 2 diabetic (T2DM) mice.

RESULTS AND DISCUSSION

Compound **1** had the molecular formula $C_{30}H_{40}O_{14}$, as suggested by HRFABMS, a $C_3H_4O_2$ unit more than borapetoside B (**5**). The ^1H NMR spectrum of **1** was similar to that of **5**,[14] except for additional signals indicating a $-CH(OH)CH_3$ residue (δ 1.36, 3H, d; δ 4.23, 1H, q) and the H-2 signal being downfield shifted (δ 5.64 vs 4.52, **5**) (Table 1, S1). The ^{13}C NMR spectrum of **1** was also similar to that of **5**,[14] except for the additional signals of the lactoyl group (δ 20.5, CH_3; δ 67.9, CH; δ 176.0, qC). These data established **1** to be 2-*O*-lactoylborapetoside B. The HMBC spectrum of **1** showed the correlation of H-2 and H-2″/C-1″ (Figure S1, Supporting Information), confirming the lactoyl group to be ester linked to C-2 of the borapetoside B moiety.

The NOESY spectrum of **1** showed correlation between H-10 and Me-19, but none between H-8 and Me-20 (Figure 1A), supporting rings A/B and B/C to be *cis*- and *trans*-fused,

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Table 1. ^{1}H NMR Data for Compounds 1−3 and 6

H atom	1[a]	1[b]	2[a]	3[a]	6[a]	6[c]
1α	2.04 m	2.15 m	2.01 m	1.98 ddd (14.4, 10.2, 6.0)	1.80 dd (15.3, 8.5)	1.65 m[d]
1β	2.31 m	2.27 m	2.22 m	2.20 br dd (14.4, 6.0)	2.07 m	2.01 m
2	5.64 dt (8.4, 4.1)	5.83 dt (8.4, 4.1)	4.51 dt (9.4, 3.9)	4.46 ddt (10.2, 7.4, 2.2)	2.33 m	2.29 m
					2.38 dt (8.7, 4.1)	
3	6.36 d (4.1)	6.51 d (4.1)	6.43 d (3.9)	6.63 dd (2.2, 0.8)	6.79 t (3.7)	6.74 t (3.7)
6	4.55 br d (2.4)	5.16 br d (2.2)	4.58 br d (3.1)	6.40 d (10.3)	4.65 br d (4.5)	4.60 br d (4.5)
7β	1.45 m	1.92 m	1.44 m	6.22 d (10.3)	1.73 dd (13.8, 6.5)	1.65 m[d]
7α	2.29 dt (14.6, 2.5)	2.92 dt (14.4, 3.8)	2.19 dt (14.2, 3.0)		2.65 ddd (13.8, 4.5, 1.8)	2.60 br dd (13.5, 4.0)
8	3.41 dd (11.8, 2.5)	3.71 dd (11.9, 2.6)	3.36 br t (9.1)		2.28 dd (6.5, 1.8)	2.15 br d (5.8)
10	2.37 br d (6.5)	2.33 m	2.28 br d (6.1)	2.53 dd (6.0, 2.0)	2.44 br d (6.5)	2.31 br d (6.8)
11α	2.08 m	1.69 dd (14.2, 5.8)	2.09 dd (14.2, 5.9)	2.26 dd (13.2, 8.4)	2.22 dd (14.8, 1.8)	2.09 br d (13.1)
11β	2.00 dd (13.8, 11.2)	1.89 m	1.97 dd (14.0, 11.3)	1.93 dd (13.2, 8.4)	1.60 dd (14.8, 12.2)	1.45 dd (14.4, 12.6)
12	5.52 dd (11.2, 6.1)	5.38 dd (11.2, 5.8)	5.49 dd (11.3, 5.9)	5.97 t (8.4)	5.78 dd (12.2, 1.8)	5.65 br d (12.8)
14	6.51 d-like (1.1)	6.64 br s	6.52 d-like (1.1)	6.49 d (1.6)	6.53 d-like (1.7)	6.43 d-like (1.2)
15	7.50 t-like (1.5)	7.67 d-like (1.3)	7.51 t-like (1.7)	7.50 t (1.6)	7.48 t-like (1.7)	7.38 t-like (1.4)
16	7.59 br s	7.78 br s	7.60 br s	7.59 br s	7.59 br s	7.52 br s
19	1.55 s	2.01 s	1.54 s	1.53 s	1.42 s	1.35 s
20	0.99 s	1.08 s	0.95 s	0.88 s	1.13 s	1.04 s
OMe	3.76 s	3.61 s	3.75 s	3.72 s	3.72 s	3.70 s
1′	4.42 d (7.8)	5.12 d (7.5)	4.41 d (7.8)		4.41 d (7.6)	4.38 d (7.5)
2′	3.23 br t (8.3)	4.14 dd (14.0, 6.5)	3.21 dd (9.1, 7.8)		3.16 dd (9.3, 7.6)	3.23 br t (8.1)
3′	3.36 t (7.9)	4.21 t (9.0)	3.36 t (9.1)		3.39 t (9.3)	3.35 t (9.3)
4′	3.36 m	4.30 d (9.0)	3.24 t (9.1)		3.32 t (9.3)	3.57 t (9.3)
5′	3.27 m	3.85 m	3.47 m		3.20 m	3.12 m
6′	3.79 dd (11.9, 2.4)	4.34 dd (11.7, 4.4)	4.50 dd (11.8, 2.1)		3.80 dd (11.9, 2.5)	3.72 m
	3.69 dd (11.9, 4.7)	4.34 dd (11.7, 3.4)	4.17 dd (11.8, 6.5)		3.69 dd (11.9, 3.8)	3.72 m
2″	4.23 q (7.0)	4.67 q (6.8)	4.29 q (7.0)			
3″	1.36 d (7.0)	1.64 d (6.8)	1.38 d (7.0)			

[a]In CD_3OD, 600 MHz. [b]In Pyr-d_5, 400 MHz. [c]In $CDCl_3$, 600 MHz. [d]Signals are interchangeable in each column.

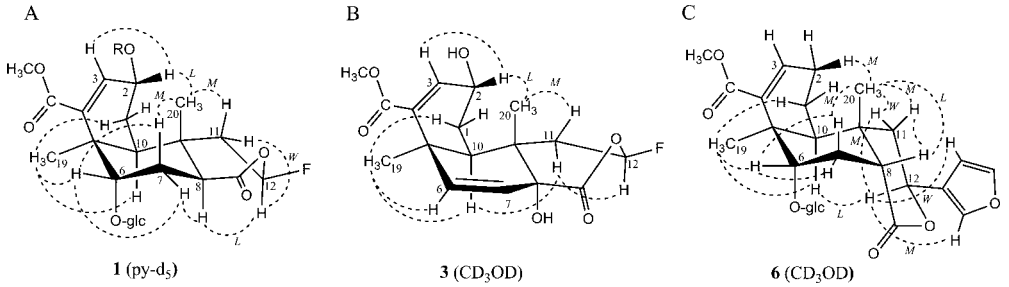
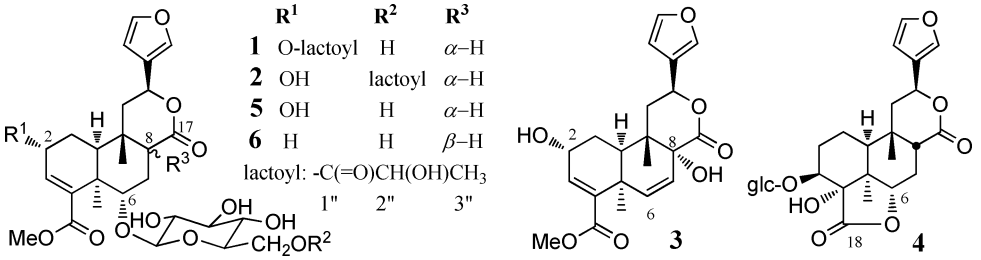


Figure 1. NOESY correlations of **1** (A), **3** (B), and **6** (C).



respectively, based on a Dreiding stereomodel study. Analysis of the coupling patterns and constants of H-6 (br d, J = 2.4 Hz), H-8 (dd, J = 11.8 and 2.5 Hz), H-11β (dd, J = 13.8 and 11.2 Hz), and H-12 (dd, J = 11.2, 6.1 Hz) suggested H-6 to be equatorially oriented and H-8, H-11β, and H-12 to be axially oriented. These analyses established the conformation of the A−B−C ring to be half-boat−chair−twisted boat, as shown in Figure 1A.

Compound **2** had the same molecular formula as **1**, $C_{30}H_{40}O_{14}$, as deduced from HRESIMS. The ^{1}H NMR spectrum of **2** was similar to that of **1**, except for the resonances of H-2 and H-6′ being upfield ($\delta_{H\text{-}2}$ 4.51 vs 5.64, **1**) and downfield shifted ($\delta_{H\text{-}6s}$ 4.17 and 4.50 vs 3.69 and 3.79, **1**), respectively (Table 1). Accordingly, the lactoyl group at C-2 in **1** was designated at the glc C-6 (C-6′) in **2**. The HMBC spectrum of **2** showed the correlations of glc H_2-6 and the

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Table 2. ^{13}C NMR Data (δ) for Compounds 1−3 and 6

pos.	1[a]	1[b]	2[a]	3[a]	6[a]	6[c]
1	26.1, CH_2	25.5, CH_2	29.2, CH_2	28.8, CH_2	17.5, CH_2	16.6, CH_2
2	68.7, CH	67.4, CH	64.6, CH	65.0, CH	24.9, CH_2	23.9, CH_2
3	135.2, CH	134.8, CH	141.0, CH	141.2, CH	142.8, CH	141.2, CH
4	143.6, qC	142.5, qC	140.1, qC	138.0, qC	137.2, qC	135.9, qC
5	42.8, qC	42.2, qC	42.7, qC	38.8, qC	41.2, qC	39.8, qC
6	79.8, CH	78.8, CH	80.1, CH	137.3, CH	78.8, CH	76.7, CH
7	27.7, CH_2	27.8, CH_2	27.5, CH_2	125.0, CH	29.5, CH_2	28.6, CH_2
8	42.0, CH	41.4, CH	42.1, CH	73.3, qC	47.0, CH	45.8, CH
9	38.2, qC	37.4, qC	38.3, qC	40.0, qC	36.4, qC	35.2, qC
10	50.9, CH	49.9, CH	51.4, CH	45.7, CH	37.8, CH	36.5, CH
11	46.0, CH_2	45.1, CH_2	46.1, CH_2	39.9, CH_2	43.3, CH_2	42.6, CH_2
12	71.8, CH	70.1, CH	71.9, CH	72.7, CH	71.4, CH	68.3, CH
13	126.0, qC	125.5, qC	125.9, qC	127.9, qC	127.0, qC	125.1, qC
14	109.9, CH	109.7, CH	109.9, CH	109.7, CH	109.7, CH	108.6, CH
15	145.1, CH	144.3, CH	145.0, CH	145.2, CH	144.8, CH	143.5, CH
16	141.5, CH	140.6, CH	141.6, CH	141.6, CH	141.2, CH	140.2, CH
17	178.2, qC	174.9, qC	178.3, qC	174.0, qC	177.4, qC	175.4, qC
18	168.9, qC	167.6, qC	169.2, qC	168.6, qC	168.9, qC	167.3, qC
19	29.4, CH_3	29.2, CH_3	29.6, CH_3	30.5, CH_3	27.8, CH_3	27.3, CH_3[d]
20	23.9, CH_3	23.6, CH_3	23.8, CH_3	24.2, CH_3	27.8, CH_3	27.6, CH_3[d]
OMe	52.6, CH_3	52.0, CH_3	52.5, CH_3	52.2, CH_3	52.1, CH_3	51.6, CH_3
1′	106.3, CH	106.8, CH	106.0, CH		105.8, CH	103.5, CH
2′	75.6, CH	75.9, CH	75.5, CH		75.4, CH	73.7, CH
3′	78.1, CH	78.7, CH	77.8, CH		78.0, CH	76.5, CH
4′	71.3, CH	71.6, CH	71.7, CH		70.6, CH	69.9, CH
5′	77.7, CH	78.2, CH	75.2, CH		77.1, CH	75.0, CH
6′	62.4, CH_2	62.5, CH_2	64.9, CH_2		62.3, CH_2	60.7, CH_2
1″	176.0, qC	175.6, qC	176.2, qC			
2″	67.9, CH	67.5, CH	67.8, CH			
3″	20.5, CH_3	21.0, CH_3	20.8, CH_3			

[a]In CD_3OD, 150 MHz. [b]In Pyr-d_5, 100 MHz. [c]In $CDCl_3$, 150 MHz. [d]Signals are interchangeable in each column.

lactoyl H-2 to the lactoyl C-1, thus confirming **2** to be 6′-*O*-lactoylborapetoside B.

Attempts to elucidate the absolute configuration of the lactoyl residue in **1** and **2** were made. Since the specific optical rotation of L-(+)-lactic acid is small, +2.6 (*c* 8.0),[18] and only minor amounts of **1** and **2** were isolated, a CD method was developed. (+)-Lactic acid benzyl ester was used as the model compound, whose CD spectrum showed strong negative and positive Cotton effects at 206 and 202 nm (MeCN), respectively. The CD spectrum of the benzylation product of the lactic acid obtained from hydrolysis of **1** under either acidic (1 N HCl) or alkaline conditions (10% KOH),[19] followed by *O*-benzylation, however, indicated that the benzyl ester was racemic (Figure S42). The glucose residue obtained from acidic hydrolysis of borapetoside B (**5**)[20] was established as D-form by its positive specific rotation. The glucosyl moieties in the other glucosides in the same plant were assumed to have the same form.

Compound **3** had the molecular formula $C_{21}H_{24}O_7$. The ^1H and ^{13}C NMR spectra of **3** (Tables 1 and 2) were similar to those of borapetol B (**7**) (Table S1) for the proton and carbon signals in rings A and D, indicating the same ring structures. The ^1H NMR spectrum of **3** showed signals for two *cis*-coupled olefinic protons (δ 6.22 and 6.40, J = 10.7 Hz), and the ^{13}C NMR spectrum showed an additional oxygenated quaternary carbon (δ 73.3) but lacked the signals for the C-6 carbinoyl, C-7 methylene, and C-8 methine when compared to the corresponding spectrum of **7**. These data and the degree of unsaturation, 10 in total, as deduced from the molecular formula, established **3** to be a Δ^6,8-hydroxy derivative of **7**. The HMBC spectrum of **3** showed correlation of Me-20 (δ 0.88) and the olefinic H-6 (δ 6.40) to the oxygenated C-8 (δ 73.3) and of Me-19 (δ 1.53) to C-4 (δ 138.0), C-5 (δ 38.8), C-10 (δ 45.7), and C-6 (δ 137.3) (Figure S1), supporting this suggestion. The NOESY spectrum of **3** showed correlations of Me-20/H-2 and H_β-11 and of H-12/H_α-11, but not H_β-11. A Dreiding model study, based on these observations and the coupling constant of H-12 (t, J = 8.4 Hz), established the 8-OH to be α-oriented and the conformation of the A−B−C rings to be twisted boat−twisted chair−boat (Figure 1B). Consequently, the structure of compound **3** was established as 6-dehydroxy-6,7-didehydro-8-hydroxyborapetol B, which was named tinocrispol A after its plant origin.

While comparing the ^{13}C NMR data of compound **6** (Table 2, Figure S36) and borapetoside C in $CDCl_3$ (Table 2S),[15] their chemical shifts were superimposable. However, the multiplicity of some carbon signals and assignments should be revised, including δ 36.6 (CH, not CH_3), 28.6 (CH_2, not CH_3), 27.7 (CH_3, not CH_2), and 27.3 (CH_3, not CH). The ^1H and ^{13}C NMR assignments of **6** (Tables 1 and 2) (CD_3OD) were unambiguously made on the basis of the 2D NMR analysis. The result indicated that the chemical shifts of C-1 and C-2 and those of C-7, C-10, Me-19, and Me-20 should be revised from the previous report (Table 2S).[15] The NOESY spectrum of **6** (Figures S30−32) revealed correlations of H-12 (δ 5.78)/H-10 and H_α-11, but not H_β-11, and Me-20/H-8, H_β-7, H_β-2, and H_2-

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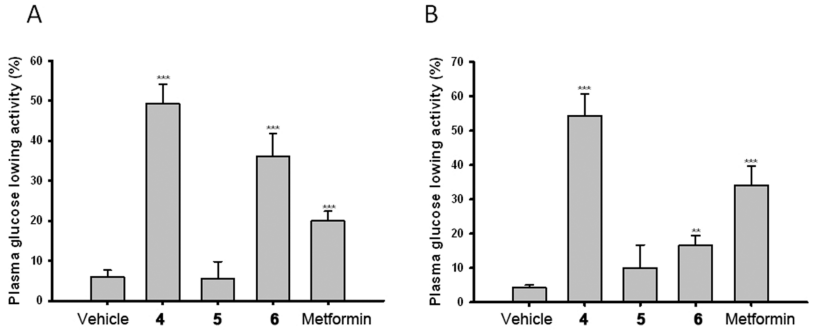

Figure 2. Comparison of plasma glucose lowering activity of **4**–**6** in normal mice (A) and T1DM mice (B). Values are expressed as mean ± SEM from seven animals in each group. $^{**}p < 0.01$, and $^{***}p < 0.001$.

11 (Figure 1C). On the basis of these data, a Dreiding model study established rings A–C of **6** to be in a twisted boat–chair–chair conformation, as shown in Figure 1C. Accordingly, H-8 in ring B was equatorially oriented, supported by the coupling constants (J = 6.5, 1.8 Hz), revising the reported axial orientation.[15] The NOESY spectrum also revealed the correlation of H-6 (δ 4.65) to Me-19 and H_2-7, but not H-10 (Figure 1C), indicating H-6 in **6** to be β- and equatorially oriented, consistent with its coupling pattern and constant (br d, J = 4.5 Hz). Thus, the 6-*O*-glucosyl group in **6** should be α-oriented rather than the reported β-orientation.[15] The ^{1}H NMR assignment of **6** in $CDCl_3$ (Table 1) was also made by analysis of a COSY spectrum (Figure S37, $CDCl_3$) and comparison to the data assigned in CD_3OD. Thus the H-8 signal was designated at δ 2.15 (br d, J = 5.8 Hz) and the reported H-8 signal (δ 2.66, J = 13.0, 5.0 Hz)[15] was assigned to that of H_α-7.

Since *T. crispa* contained mainly clerodane-type furanoditerpenoids from the previous[13−16] and our current studies, these compounds were considered to be the active components. However, our unpublished data indicated that the major compounds, borapetosides A–C (**4**–**6**), could not close the ATP-sensitive K^+ channels and failed to increase insulin secretion in MIN6 cells. To clarify whether these three compounds were active, an in vivo assay was undertaken. Plasma glucose levels of the treated mice were determined before and 60 min after intraperitoneal (ip) administration. Like the positive control, compounds **4** and **6** showed a hypoglycemic effect in normal and type 1 DM mice (Figure 2). Figure 2A and B indicated the hypoglycemic activity of **4** at a dose of 5.0 mg/kg to be nearly 2 times higher than the positive control, metformin (200 mg/kg), in both animal models. Figure 3 indicates that such activity for **6** was dose-dependent in normal, T1DM, and T2DM mice. In contrast, glibenclamide (5.0 mg/kg) lowered the plasma glucose concentration from 170 ± 2.8 mg/dL to 108 ± 8.2 mg/dL in T2DM mice and from 102 ± 3.7 mg/dL to 84 ± 4.3 mg/dL in normal mice.

Borapetoside C (**6**) was found to stimulate insulin release in normal and T2DM mice, but not in T1DM mice (Table 3). The plasma insulin level was significantly increased to almost plateau at 3.0 mg/kg in normal and T2DM mice, such effect being comparable to that of glibenclamide (5 mg/kg) (Table 3). However, in the T1DM mice group neither **6** nor metformin increased the plasma insulin level.

In the ip glucose tolerance test, plasma glucose levels of the **6**-treated mice were significantly lower than those of mice treated with the vehicle 30 min after ip glucose injection in both normal (Figure 4A, 293 ± 11 mg/dL vs 346 ± 15 mg/dL)

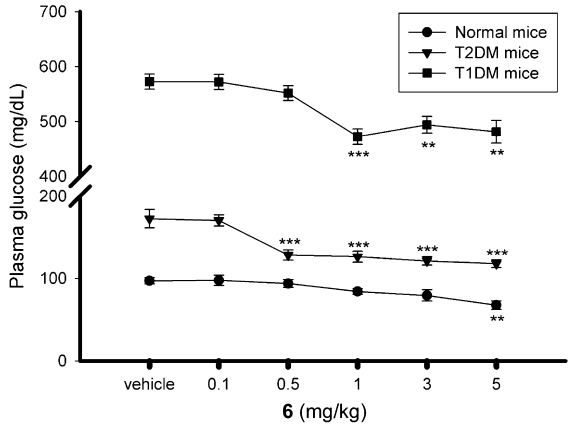

Figure 3. Hypoglycemic effects of **6** on normal and diabetic mice 60 min after ip injection. The data from each experiment, independently repeated seven times, were averaged and presented as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$; $^{***}p < 0.005$.

and T2DM mice (Figure 4B, 343 ± 11 mg/dL vs 406 ± 9 mg/dL). Plasma glucose levels of the **6**-treated mice at 60, 120, and 150 min after treatment were also significantly lower than those of the control mice. These results indicated that **6** enhanced in vivo glucose utilization significantly in both normal and T2DM mice.

The effect of **6** on liver glucose synthesis in T1DM mice was examined by measuring the level of phosphoenolpyruvate carboxykinase (PEPCK). Treatment with **6** (5.0 mg/kg, twice per day, for 7 days) caused the PEPCK level in the liver to decrease to an amount close to that of the insulin-treated group (0.75 vs 0.64) (Figure 5).

In this study, 12 *cis*-clerodane-type furanoid terpenoids were isolated from *T. crispa* vines. Three of them (**1**–**3**) are new natural products, and the configuration at C-6 of borapetoside C (**6**) is revised. Borapetosides A (**4**) and C (**6**) at 5.0 mg/kg were found to possess hypoglycemic activity via ip injection, while borapetoside B (**4**) was inactive. The effect of **6** was demonstrated to cover insulin-dependent and -independent pathways. Both the increase of glucose utilization in peripheral tissue and the reduction of hepatic gluconeogenesis contribute to the lowering of plasma glucose. Although detailed molecular mechanisms of **6** must be determined, the results of its antidiabetic activity and acute toxicity evaluation suggest that this compound could become a useful agent for the treatment of diabetic disorders. The active compounds **4** and **6** possess 8*R*-chirality, but inactive **5** has 8*S*-chirality. Their 3D structures are shown in Figure 1, where the conformation of **5** is similar to

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Table 3. Plasma Insulin Levels (pmol/L)[a] in Normal and Diabetic Mice before and 60 min after ip Treatment with Compound 6

	normal mice		T1DM mice		T2DM mice	
group	pretreatment	post-treatment	pretreatment	post-treatment	pretreatment	post-treatment
vehicle-treated	56.3 ± 3.9	61.4 ± 4.9	14.6 ± 0.2	14.6 ± 0.5	114.4 ± 6.9	124.5 ± 9.3
6, mg/kg						
0.1	54.0 ± 3.4	71.5 ± 5.3	15.2 ± 0.8	16.3 ± 0.9	111.8 ± 5.2	111.3 ± 6.9
0.5	53.1 ± 3.3	70.7 ± 9.9	16.2 ± 0.7	15.7 ± 0.6	120.8 ± 4.3	170.8 ± 3.1
1.0	60.3 ± 3.7	63.0 ± 5.1	15.7 ± 1.0	15.9 ± 0.3	111.6 ± 2.7	173.0 ± 10.7**
3.0	52.0 ± 3.7	142.6 ± 29.2***	13.8 ± 1.6	14.8 ± 0.8	112.1 ± 3.2	253.9 ± 24.9***
5.0	57.6 ± 2.4	149.6 ± 27.6***	14.7 ± 0.4	14.3 ± 0.6	112.1 ± 6.3	257.6 ± 13.9***
positive control[b]	52.5 ± 4.6	145.8 ± 14.6***	13.5 ± 1.1	15.1 ± 1.5	123.5 ± 10.2	208.8 ± 17.3***

[a]Values are expressed as mean ± SEM from six animals in each group. **p < 0.01, ***p < 0.005. [b]Positive controls: for normal and T2DM models, glibenclamide (5.0 mg/kg); for T1DM models, metformin (200 mg/kg).

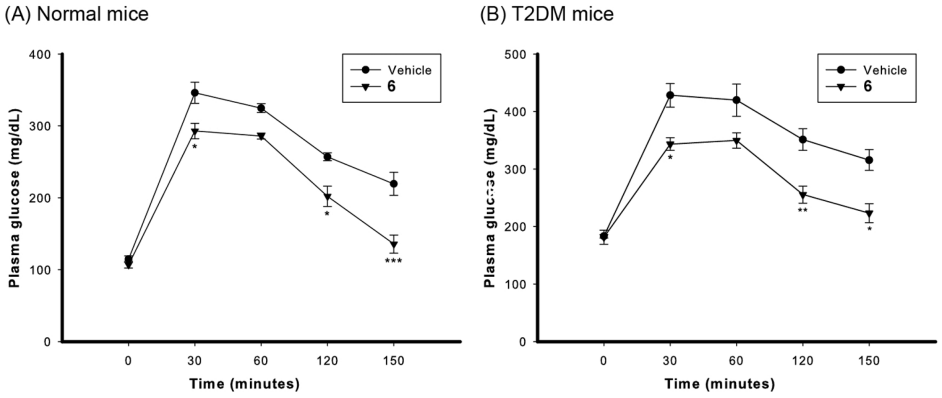

Figure 4. Effects of **6** on plasma glucose concentration in normal (A) and T2DM (B) mice that received an IPGTT. Data are expressed as mean ± SEM in each group. *p < 0.05, **p < 0.01, ***p < 0.005.

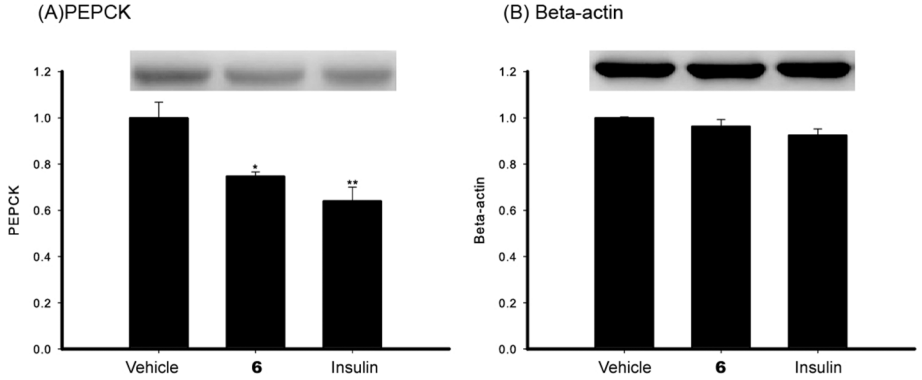

Figure 5. Expression levels of PEPCK and actin protein in the liver of T1DM mice. The amounts of PEPCK protein (A) and the beta-actin protein (B) are presented as means ± SEM of n = 3; *p < 0.05, **p < 0.01.

that of **1**. Whether such differences and/or other substitutions at C-2−6 will affect hypoglycemic activity will require additional studies.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations: JASCO DIP-370 polarimeter. CD: JASCO J-720 spectropolarimeter. IR: JASCO IR Report-100 infrared spectrometer. UV: Hitachi 150-20 double beam spectrophotometer. 1D and 2D NMR spectra: Bruker AV-400 and AVIII-600 with a cryo-probe (CD_3OD, δ_H 3.30 and δ_C 49.0 ppm; C_5D_5N, δ_H 8.71 and δ_C 149.9 ppm; $CDCl_3$, δ_H 7.24 and δ_C 77.0 ppm). HRFABMS: JEOL JMS-700 mass spectrometer. HRESIMS: Bruker Daltonik micrOTOF orthogonal ESI-TOF mass spectrometer. CPC: Sanki LLN type, equipped with six cartridges. Lobar column chromatography: size B, 310 × 25 mm, LiChroprep RP-18, 40−63 μm (Merck). Sephadex LH-20: Pharmacia Co. Silica gel: 230−400 mesh (Merck). Sepabeads SP700: Mitsubishi Chemical. Analytical HPLC column: RP-18 Phenomenex Prodigy ODS3 column (250 mm × 4.6 mm i.d., 5 μm). Streptozotocin, glibenclamide, metformin, and sodium pentobarbital: Sigma Chemical Co., St. Louis, MO, USA. Insulin Actrapid HM: Novo Nordisk, Denmark. Glucose kit reagent: Biosystems S.A., Barcelona, Spain. Glucose analyzer: Synergy HT, BioTek, USA. Mammalian insulin ELISA kit: Mercodia AB, Uppsala, Sweden. PEPCK antibody: a gift from Professor D. K. Granner. Prism 5.0 demo software: GraphPad Software Inc., La Jolla, CA, USA.

Plant Material. The vines of *Tinospora crispa* Miers (Menispermaceae) were collected in March 2004 in Qi-Du, New Taipei City, Taiwan, and authenticated by the author (S.S.L.). The voucher

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specimen (NTUSP20040301) is deposited in School of Pharmacy, College of Medicine, National Taiwan University.

Extraction and Isolation. Fresh vines of *T. crispa* (50.80 kg) were ground by blender, and the resultant mass was stirred with water (30 L) and 95% EtOH (40 × 2) in sequence. The suspensions were centrifuged, and the supernatants were combined and condensed to give 1314 g of extract. Part of the extract (182.0 g) was partitioned between water (1.3 L) and *n*-BuOH (1.3 L × 3) to yield BuOH (22.7 g) and water (150.1 g) soluble fractions. The BuOH-soluble fraction (7.3 g × 3) was fractionated via a Sanki CPC, using $CHCl_3$–MeOH–H_2O (10:10:5) as a delivery system with a flow rate of 3 mL/min and rotation speed of 800 rpm, to give eight fractions, of which fractions 1–5 and 6–8 were obtained using the aqueous and organic layers as the mobile phase, respectively. An aliquot (1.83 g) of fraction 2 (5.90 g) was separated on the RP-18 Lobar column, eluted with 30% $MeOH_{aq}$ to MeOH, and yielded eight subfractions. Subfraction 8 (24.1 mg) yielded the precipitate **1** (5.9 mg). Subfraction 4 (56.4 mg) yielded **2** (5.0 mg) via a Sephadex LH-20 column (40 g, 50% MeOH in H_2O). Subfractions 2 and 6 were **5** (222.1 mg) and **4** (58.9 mg), respectively. Fraction 3 (1.30 g) yielded another crop of **5** (413.8 mg) and **12** (47.6 mg) using RP-18 Lobar column chromatography. Fraction 7 (1.10 g) was chromatographed over a Sephadex LH-20 column (70 g, MeOH–H_2O, 10:3) repeatedly to yield **6** (399.2 mg), **9** (13.6 mg), and **8** (50.3 mg), respectively. Fraction 8 (1.30 g) was subjected to repeated Sephadex LH-20 column chromatography, which yielded **3** (2.1 mg) and a subfraction (149.2 mg) that yielded **7** (13.5 mg), **10** (4.5 mg), and **11** (10.0 mg) upon separation over the RP-18 Lobar column (30–85% MeOH–H_2O).

*2-O-Lactoylborapetoside B (**1**):* white, amorphous powder; $[\alpha]_D^{25}$ +20.0 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 273 (4.14) nm; CD (*c* 3.21 × 10^{-6} M, MeOH) $[\theta]_{251}$ −33 249, $[\theta]_{219}$ +198 567; IR (KBr) ν_{max} 3443, 2952, 1717, 1649, 1453, 1214, 1026, 876 cm^{-1}; ^1H and ^{13}C NMR, see Tables 1 and 2; HMBC and NOESY, see Figures S1 and 1A; (+)ESIMS *m*/*z* (rel int %) 647.4 (100, $[M + Na]^+$); (−)HRFABMS *m*/*z* 623.2332 $[M - H]^-$, calcd for $C_{30}H_{39}O_{14}$ − H, 623.2345.

*6′-O-Lactoylborapetoside B (**2**):* white, amorphous powder; $[\alpha]_D^{25}$ +2.0 (*c* 0.4, MeOH); UV (CH_3CN) λ_{max} (log ε) 208 (4.01) nm; CD (*c* 3.21 × 10^{-6} M, MeOH) $[\theta]_{251}$ −12 714, $[\theta]_{218}$ + 39 504; IR (KBr) ν_{max} 3417, 2950, 1713, 1645, 1503, 1443, 1382, 1249, 1214, 1074, 1031, 875 cm^{-1}; ^1H and ^{13}C NMR, see Tables 1 and 2; (+)ESIMS *m*/*z* (rel int %) 646.9 (100, $[M + Na]^+$); (+)HRESIMS *m*/*z* 647.2285 $[M + Na]^+$, calcd for $C_{30}H_{40}O_{14}$ + Na, 647.2310.

*Tinocrispol A (**3**):* yellowish, amorphous powder; $[\alpha]_D^{25}$ +67.0 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 279 (3.43) nm; CD (*c* 2.58 × 10^{-5} M, MeOH) $[\theta]_{250}$ −14 818, $[\theta]_{219}$ +91 045; ^1H and ^{13}C NMR, see Tables 1 and 2; COSY and NOESY, see Figures S1 and 1B; (+)ESIMS *m*/*z* 410.9 ($[M + Na]^+$); (−)HRFABMS *m*/*z* 387.1459 $[M - H]^-$, calcd for $C_{21}H_{24}O_7$ − H, 387.1449.

Preparation of L-(+)-Lactic Acid Benzyl Ester. The mixture of L-(+)-lactic acid (8.0 mg), benzyl chloride (30 μL), and K_2CO_3 (26.7 mg) in DMF (0.6 mL) was reacted at 95 °C for 18 h. The reaction mixture was evaporated under reduced pressure to give a residue, which was separated over a silica gel column ($CHCl_3$), followed by an analytical RP-18 column eluted with a linear gradient of 50% to 100% CH_3CN–H_2O in 15 min, to give (*S*)-benzyl-2-hydroxypropanoate (1.4 mg): CD (*c* 3.3 × 10^{-4} M, MeCN) $[\theta]_{206}$ −210 974, $[\theta]_{202}$ + 422 050 (Figure S41); ^1H and ^{13}C NMR and HMBC, see Figures S38–S40.

Benzylation of the Hydrolysis Product of 1 under Acid and Alkaline Conditions. The solution of **1**-containing fraction (12 mg) in 1 N HCl(aq)–MeOH (1:1, 1 mL) was heated at 55 °C for 2 h. The mixture was partitioned between H_2O (10 mL) and $CHCl_3$ (10 mL × 3). The aqueous layer was evaporated to dryness, and the residue in DMF (1 mL) was reacted with benzyl chloride (30 μL) and K_2CO_3 (26.7 mg) at 95 °C for 18 h. The product was separated in the same manner as described above for L-(+)-lactic acid benzyl ester to yield benzyl-2-hydroxypropanoate. The same product was obtained by treating the **1**-containing fraction (6 mg) with 10% KOH–MeOH[19] (1:1, 1 mL) at rt for 2 h, followed by benzylation and separation. The CD spectra of these two products showed no apparent curve (Figure S42).

Acid Hydrolysis of 5. Compound **5** (15.8 mg) was heated at 105 °C in 2 mL of 2 M TFA for 4 h.[20] The reaction mixture was extracted by EtOAc (2 mL × 3). The residue of the aqueous layer (13.3 mg), obtained after evaporation under reduced pressure, was separated using Sephadex LH-20 (20 g, 70% MeOH–H_2O) and SP700 columns (3 g, H_2O) to yield D-glucose (<0.8 mg), $[\alpha]_D^{22}$ > +12.5 (*c* < 0.08, H_2O), with ^1H NMR data (D_2O, 600 MHz) identical to the authentic sample.

Experimental Animals. Four-week-old male ICR mice were purchased from BioLasco Taiwan Co., Ltd., and kept in the National Taiwan University College of Medicine Experimental Animal Center at 22 ± 1 °C and 60 ± 5% relative humidity with a 12 h light–dark cycle. Animals were given ad libitum access to food (Laboratory Rodent Diet 5001) and water throughout the study. The investigation followed the University guidelines for the use of animals in experimental studies and conformed to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH publication no. 85-23, revised 1996). The animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of National Taiwan University (IACUC No. 20070004).

Induction of Diabetes in Mice. Type 1 diabetic mice (T1DM) were induced in 4-week-old ICR mice by ip injection with a freshly prepared solution of streptozotocin (STZ).[21] All studies were carried out 4 weeks after STZ injection. Fasting mice with plasma glucose level greater than 350 mg/dL were considered diabetic and used in the study. Diet-induced diabetic (T2DM) mice were induced by feeding with fat-rich chow and 20% fructose-sweetened water for 4 weeks.[22] Control mice received a regular diet.

Intraperitoneal Treatment of Mice with Borapetoside C (6). On the basis of the body weight and plasma glucose level, the mice were assigned randomly into three groups, seven animals each. They were ip injected with vehicle (2% DMSO/normal saline) (group I), **6** (5 mg/kg) (group II), and glibenclamide (5 mg/kg) (group III), or metformin (200 mg/kg), or insulin (0.5 IU/kg).[23] In the acute study, mice under anesthesia received a single ip injection. In the subacute study, mice received ip injection of **6** or other positive control twice daily for seven days.

Blood Sample Collection and Plasma Assay. The blood samples were collected from the orbital vascular plexus of mice under anesthesia by ip injection of sodium pentobarbital (80.0 mg/kg). Blood samples were centrifuged at 13 000 rpm for 5 min, and the supernatant was collected and placed on ice or at −20 °C for later assays.[24] Plasma glucose concentrations were determined using the glucose kit reagent and a glucose analyzer. Plasma insulin concentrations were determined using a mammalian insulin ELISA kit.[25]

Intraperitoneal Glucose Tolerance Test (IPGTT). The ip glucose tolerance test was performed on animals after fasting overnight. The glucose concentrations were quantified prior to ip injection of glucose (2 g/kg) and at 0, 30, 60, 90, and 120 min postinjection. Mice were maintained under anesthesia by pentobarbital throughout the procedure.[26]

Determination of Protein Level by Western Blot Analysis. T1DM mice were treated with **6** (5.0 mg/kg, twice per day), insulin, or a vehicle for 7 days. Liver tissue homogenates were prepared, and the protein contents were determined.[27] Sixty micrograms of homogenates were separated in 8% SDS-PAGE gels using the Bio-Rad Mini-Protein II system (60 and 100 V during the stacking and separation gels, respectively), and the Western blot analysis was performed using PEPCK antibody (diluted in 1:1000).[28] Blots were developed using the ECL-Western blotting system. The obtained chemiluminescent densities at 70 kDa representing PEPCK and at 43 kDa representing β-actin were quantified using ImageQuant.[27]

Statistical Analysis. The results of plasma glucose lowering activity were calculated as percentage decrease of the initial value according to the formula $(G_i - G_t)/G_i \times 100\%$, where G_i and G_t stand for the initial and post-treatment glucose concentrations, respectively.

Results are presented as mean ± SEM for the number (n) of animals in the group as indicated in the tables and figures. Statistical differences between the means of the various groups were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's multiple test with Prism 5.0 demo software. Data were considered statistically significant at $*p < 0.05$.[28]

■ ASSOCIATED CONTENT

Supporting Information

Structures of **7**−**12**, ^{1}H and ^{13}C NMR data of **5** and **7**, 1D and 2D NMR spectra of **1**−**3** and **6**, ^{1}H and ^{13}C NMR spectra of **5**, and CD of lactic acid benzyl ester. This information is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

Co-authors Sio-Hong Lam and Chi-Tun Ruan contributed equally to this study.

Notes

The authors declare no competing financial interest.

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