Original Article

A novel protein tyrosine phosphatase 1B inhibitor from *Tinospora sinensis*

Abstract

Bioassay-directed fractionation led to the identification of a new compound, 4-hydroxy-heptadec-6-enoic acid ethyl ester (1) together with three known compounds (2-4) from *Tinospora sinensis*. The structure of 1 was determined by analysis of spectroscopic data. The isolated compounds were evaluated for their protein tyrosine phosphatase 1B (PTP1B) inhibitory activity. Compounds 1 and 2 displayed significant inhibitory activity with IC_{50} values 61.1 and 74.2 μM, respectively

Key words:
Lignane, menispermaceae, protein tyrosine phosphatase 1B, tinospora sinensis

Introduction

Diabetes mellitus is a chronic, incurable disease, which altered the metabolism of lipid, carbohydrates, and proteins in humans and increased the risk of complications from artery diseases, myocardial infarction, hypertension, and dyslipidemia and is clinically characterized by peripheral hyperglycemia. Diabetes can be classified clinically as insulin-dependent diabetes mellitus (IDDM, or type-1 diabetes) and non-insulin-dependent diabetes mellitus (NIDDM, or type-2 diabetes). Type-2 diabetes is common and found in >90% patient characterized by either normal/abnormal insulin secretion or function. Insulin resistance is central to type-2 diabetes and is known to involve decreased tyrosine phosphorylation of insulin receptors (IRs) despite normal insulin levels.[1] Protein tyrosine phosphatases (PTPases) constitute a diverse family of enzymes and are responsible for the selective dephosphorylation of tyrosine residues.[2] Several PTPs, including protein tyrosine phospahtase 1B (PTP1B), LAR, PTPa, and PTPe, are capable of dephosphorylation the IR, thereby attenuating tyrosine kinase activity. Furthermore, PTP1B has been implicated in the insulin resistance associated with diabetes and obesity[3] by the finding of correlations between insulin resistance and the level of PTP1B in muscle and adipose tissue.[4] This is further supported by a variety of cellular and biochemical studies where PTP1B has been shown to be playing a role in the dephosphorylation of the IR. Therefore, the use of specific PTP1B inhibitors may enhance insulin action and represents a novel strategy for the treatment of type-2 diabetes.[5] Small molecule PTP1B inhibitors may find an important clinical role as novel insulin sensitizers in the treatment of type-2 diabetes.[6]

Tinospora sinensis (syn: *Tinospora malabarica*) is a plant that grows almost throughout India and other South East Asian countries and belongs to the family Menispermaceae. The stem of this plant has great therapeutic value traditionally in treating debility, dyspepsia, fever, inflammation, syphilis, ulcer, bronchitis, jaundice, urinary disease, skin disease and liver disease,[7] and known for its adaptogenic and immunomodulatory properties.[8] The aqueous and alcoholic extracts of this species are reported to have many biological potential, such as anti-inflammatory,[9] anti-diabetic,[10]

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hepotoprotective,[11] and immunomodulatory, and adaptogenic.[12] Previous phytochemical investigations have discovered that this species contains steroids, flavonoides, alkaloids, and most important furano diterpenoids class of compounds including 10α-hydroxy columbin,[13] menispermacide,[14] tinosporicide,[15] malaborolide,[16] malaborolide B1,[17] magnoflorine, quercetin-3-O-glycoside, kaempferol and its glycosides,[18] palmatine,[14] kokusaginine,[19] N-formyl-anonaine,[20] cyclo-euphordenol,[14] tinosinen-I,[21] di-O-methyl syringaresinol,[20] tinosporinone, dibenzoylethane, and allyloxyflavone,[22-23] The tinosporine,[24] and two new lignan glycosides, namely, tinosposide A and tinosposide B have also been reported from the stem of the plant.[25]

In the course of our efforts to discover PTPase 1B inhibitor from natural sources, *T. sinensis* was selected on the basis of its promising antidiabetic activity.[10] In our initial screening ethanol extract and its chloroform and *n*-butanol fractions exhibited considerable *in vitro* PTP1B inhibitory activities of 22%, 32%, and 29%, respectively at 100 µg/mL. Further bioactivity-guided isolation from the chloroform fraction resulted in the isolation of four compounds 1-4 [Figure 1]. Compounds 1 and 2 displayed significant inhibitory activity with IC_{50} values 61.1 and 74.2 µM, respectively. Herein we described the isolation and structure elucidation of 1, and *in vitro* PTP1B inhibitory activity of all the isolated compounds.

Results and Discussion

Compound 1 was obtained as yellow oil. Its molecular formula was determined to be $C_{19}H_{36}O_3$, on the basis of HRESI-MS data (*m/z* 313. 2756 $[M+H]^+$). The IR spectrum revealed absorption bands at 993 cm^{-1} for C = CH bending vibration, 1667 cm^{-1} for C = C stretching and 1735 cm^{-1} for ester group. These data compiled with ^1H NMR spectrum suggested that compound could be an unsaturated carboxylic acid ester with a long hydrocarbon chain.

The ^1H and ^{13}C NMR spectrum [Table 1] with the aid of ^1H-^1H COSY furnished signal at δ_H 4.01 (*q*, *J* = 6.9Hz; δ_C 60.1), assigned to oxygenated methylene coupled with methyl group at δ_H 1.24 (*t*, *J* = 6.8Hz; δ_C 14.9), a two proton triplet at δ_H 2.29 (2H, *t*, *J* = 7.2Hz; δ_C 32.1) assigned to a methylene adjacent to a carbonyl (δ_C 172.9), which further coupled with a methylene at δ_H 1.88 and 2.32 (1H each, *m*; δ_C 32.4). Signals at δ_H 1.91 (*brd*, *J* = 5.3Hz), 2.30 (*m*) and 1.95 (*m*) were assigned to two methylene protons adjacent to a double bond at δ_H 5.35 (2H, *bt*, *J* = 8.6Hz; δ_C 130.5, 129.3). The signal δ_H 0.85 (3H, *t*, *J* = 6.3Hz; δ_C 14.7) was assigned to terminal methyl groups and δ_H 4.22 (1H, *m*; δ_C 70.1) was assigned to one oxygenated methine which showed coupling with the two methylenes adjacent to a double bond and β to carbonyl in COSY spectrum. Other methylenes of chain resonated in the range of δ_C 29.4-22.1. This NMR data confirmed the conclusion drawn from IR and mass spectrum; it also indicated an unsaturated long chain carboxylic acid. The geometry of a double bond in 1 was not clear from ^1H spectrum as both double bond protons resonated at 5.35 (*bt*, *J* = 8.6Hz), however *E* geometry was assigned on the basis of large coupling constant 8.6 Hz of broad triplet together with appearance of two methylene carbons adjacent to a double bond at δ_C 27.9, 33.9.[26] The HMBC spectrum [Table 1, Figure 2] gave the important correlation H-2′/C-1′; H-2/C-1, 4; H-3/C-1, 4, 5 and H-4/C-2, 6 to confirm the structure. Finally, compound 1 was elucidated as 4-hydroxy-heptadec-6-enoic acid ethyl ester.

The known compounds were characterized as 4-methyl-heptadec-6-enoic acid ethyl ester 2 [Table 2],[27] β-sitosterol 3[28] and lirioresino-β-dimethyl ether 4 [Table 2][29] by direct comparison of their NMR data

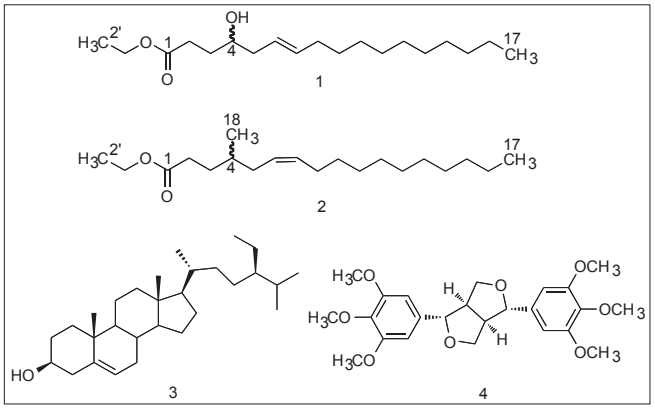

Figure 1: Chemical structure of compounds 1-4

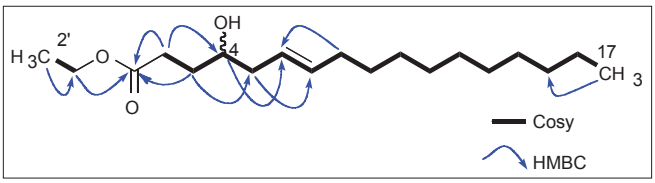

Figure 2: ^1H-^1H COSY and key HMBC correlation

Table 1: ^1H and ^{13}C NMR spectral data of 1[a]

Position	δ_H (*J* in Hz)	δ_C	HMBC	^1H-^1H COSY
1	–	172.9	–	–
2	2.29 (*t*, 7.2)	32.1	1, 4	3
3	1.88 (*m*), 2.32 (*m*)	32.4	1, 4, 5	2, 4
4	4.22 (*m*)	70.1	2, 6	3, 5,
5	1.91 (*brd*, 5.3), 2.30 (*m*)	33.9	3, 7	4; 6
6	5.35 (*bt*, 8.6)	129.3	4, 5	5; 8
7	5.35 (*bt*, 8.6)	130.5		
8	1.95 (*m*), 2.30 (*m*)	32.9	5	7, 9
9-16	1.27-1.18 (*m*)	29.4-22.1	–	–
17	0.85 (*t*, 6.3)	14.7	–	16
1′	4.01 (*q*, 6.9)	60.1	1, 2′	2′
2′	1.24 (*t*, 6.8)	14.9	1′	1′

[a]Recorded in $CDCl_3$ at 300 MHz (TMS as internal standard)

with those reported in literature. The compound 3, was also confirmed by its fragmentation pattern [Figure 3] in FAB-MS spectrum.[28]

In vitro PTP1B inhibitory activity

Compounds 1-4 were evaluated against PTP1B enzyme using sodium vanadate as a standard inhibitor and their results are summarized in Table 3. Vanadate is a non-selective inhibitor of PTPases, and many of the studies have shown that treatment with vanadate can normalize blood glucose level in diabetics.[30] Compounds 1 and 2 demonstrated PTP1B inhibitory activity at 100 µM concentration to around 86% and 69%, respectively. The effect of the test compounds on PTPase was studied by preincubating the test compounds with enzyme in the reaction system for 10 min and the residual protein tyrosine phosphatase activity was determined according to the method of Goldstein *et al*.[31] The 1.0 *m*L *p*-nitrophenylphosphate (pNPP) as the substrate, assay mixture contained 10 *m*M pNPP in 50 *m*M HEPES buffer (pH 7.0), with 1 *m*M EDTA and DTT, respectively. The reaction was stopped by addition of 500 µL of 0.1 M NaOH and the optical density was determined at 410 nm. Control tubes omitting the enzyme were always run in parallel to nullify the nonenzymic reaction and for calculating the concentration of p-nitrophenolate ions produced in the reaction mixture. A molar extinction coefficient of 1.78×10^4 was used to determine the concentration of p-nitrophenolate produced in the system. It is evident from the activity profile [Table 3] that 3 and 4 are a weak PTP1B inhibitor, whereas 1 and 2 were found to show good inhibition. The structure–activity relationship study of compounds 1 and 2 showed that the introduction of OH group at C-4 in compound 1 enhanced the activity profile when compared with compound 2 and the reference compound sodium vanadate.

Conclusions

In conclusion, activity-guided purification of ethanol extract of *T. sinensis* resulted in four compounds 1-4, among them one compound 1 was new. Compounds 1 and 2 have demonstrated significant *in vitro* PTP1B inhibitory activity, whereas 3 and 4 have not shown certain levels of inhibition.

Experimental

General experimental procedures

All NMR spectra were recorded on a Bruker DRX 300 MHz spectrometer. All chemical shifts (d) were referenced internally to the residual solvent peak ($CDCl_3$: ^1H 7.26 ppm; ^{13}C 77.0 ppm). The abbreviations of ^1H-NMR signals pattern are as follows: s, singlet; d, doublet; dd, double doublet; brs, broad singlet; brd, broad doublet; t, triplet; brt, broad triplet; m, multiplates; and q, quartate. Short- and long-range ^1H-^{13}C correlations were determined with gradient-enhanced inverse-detected HSQC and HMBC experiments respectively. Optical rotation was measured on a Jasco P-2000 polarimeter

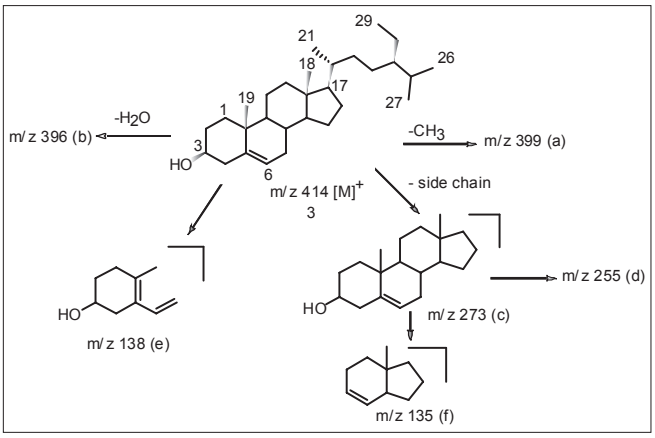

Figure 3: Mass fragmentation of 3

Table 2: ^1H and ^{13}C NMR spectral data of 2[a] and 4[b]

Position	2 (mult, J in Hz)		4 (mult, J in Hz)	
	δ_H	δ_C	δ_H	δ_C
1	–	174.0	3.15 (*m*)	54.1
2	2.24 (*t*, 7.5)	31.8	4.70 (*d*, 3.8)	85.4
3	1.58 (*brt*, 6.9)	30.4	–	–
4	1.72 (*m*)	29.9		
4a			3.92 (*dd*, 3.0, 9.0)	71.6
4b			4.29 (*dd*, 6.6, 8.8)	–
5	1.98 (*brd*, 5.4)	27.5	3.15 (*m*)	54.1
6	5.30 (*t*, 3.6)	130.0[c]	4.76 (*d*, 3.8)	85.4
7	5.30 (*t*, 3.6)	128.6[c]	–	–
8	1.98 (*brd*, 5.4)	34.7		
8a			3.92 (*dd*, 3.0, 9.0)	71.6
8b			4.29 (*dd*, 6.6, 8.8)	
9-16	1.27-1.18 (*m*)	29.7-22.9		
17	0.85 (*t*, 6.3)	14.3		
18	0.85 (*t*, 6.3)	18.0		
1′	4.08 (*q*, 7.2)	60.4		
2′	1.21 (*brs*)	14.5		
1′, 1″			–	137.0
2′, 2″			6.73 (*s*)	103.5
3′, 3″			–	153.2
4′, 4″			–	137.6
5′, 5″			–	153.2
6′, 6″			6.73 (*s*)	103.5
OCH_3-3′, 5′, 3″, 5″			3.86 (*s*)	56.2
OCH_3-4′, 4″			3.72 (*s*)	60.3

[a]Recorded in $CDCl_3$ at 300 MHz (TMS as internal standard). [b]Recorded in DMSO-*d6* at 300 MHz (TMS as internal standard). [c]Value can be interchanged within the column

Table 3: PTP1B inhibitory activity of the compounds 1-4

Test sample	Inhibition %	IC50 (µM)
1	86.0±0.56	61.1
2	69.0±5.61	74.2
3	18.5±6.1	230
4	49.1±5.31	150
Sodium vanadate (Na_2VO_4)	56.4	–

Results are means+SE of three independent experiments

(c g/100 mL) at 589 nm. UV spectra were obtained on a Perkin Elmer λ-15 UV spectrophotometer. IR spectra were recorded on a Perkin-Elmer RX-1 spectrophotometer. The high-resolution ESI mass spectra were performed on an APEX II FTICR mass spectrometer. Column chromatography was performed using silica gel (60—120 and 230—400 mesh); TLC: pre-coated silica gel plates 60 F_{254} or RP-18 F_{254} plates with 0.5 or 1 mm film thickness (Merck). Spots were visualized by UV light or by spraying with H_2SO_4-MeOH, anisaldehyde-H_2SO_4 reagents.

Plant materials

The whole stem of *T. sinensis* was collected from Dehradun, Uttarakhand, India in January 2010. The plant was collected and identified by Dr. Prashant K. Gupta, Horticulture specialist, Krishi Vigyan Kendra, Gwalior, India.

Extraction and isolation

The powdered stems of *T. sinensis* (5.5 kg) were extracted with EtOH, yielded extract 210.0 g. The EtOH extract was dissolved in water and fractionated successively with $CHCl_3$ and *n*-BuOH which resulted in $CHCl_3$ (95.1 g), *n*-BuOH (70.3 g) and aqueous (43.2 g) crude residue respectively. A portion of chloroform fraction (60.0 g) was chromatographed over silica gel (60-120 mesh, 300.0 g) and eluted with gradient of hexane: $CHCl_3$ (95:05) to $CHCl_3$: MeOH (85:15) sequentially. Seventy-eight fractions of (250 ml each) were sampled and monitored by TLC, with those showing similar TLC profile grouped into nine fractions (F-1 to F-6). Purification of F-3 (12.3 g) over silica gel (60—120 mesh, 300.0 g) with gradient of hexane: benzene (90:10) to hexane: benzene (50:50) afforded four pooled fraction (F-7 to F-10) on the basis of TLC profiles from total 40 fractions of 100 mL each. The purification of F-8 (1.2 g) using same gradient over silica gel (60-120- mesh, 100) afforded compound 1(10 mg). Further purification of F-7 (1.8 g) using similar condition over silica gel (60-120 mesh, 45.0 g) yielded compound 2 (90 mg) as yellow-colored oil. Fraction F-4 (8.0 g) was rechromatographed over silica gel (60-120 mesh, 160 g), eluted with gradient of hexane: $CHCl_3$ (100-50%) afforded four fractions (F-11 to F-14) from total 68 fractions of 100 mL each on the basis of TLC profiles. Subsequently fraction F-13 (1.1 g) was purified over silica gel (60—120 mesh, 35.0 g) eluted with hexane: acetone as isocratic eluent. Totally 32 sub-fractions of 50 mL were collected, sub-fractions 8 to 15 containing 3 were dried, afforded 3 (78 mg) as white solid. Compound 4 was crystallized from F-5 at room temperature; it was then recrystallized in cold methanol afforded 4 (135 mg).

Compound 1

Yellow oil (10 mg); IR ν_{max} (Neat) cm^{-1}: 3343, 1735, 1667, 1595 and 993; ESI-MS (pos.): *m/z* 313 $[M+H]^+$; Positive HR-ESIMS: *m/z* $[M+H]^+$ 313.2756 (calculated 313. 2743 for $C_{19}H_{36}O_3$). ^1H NMR ($CDCl_3$, 300 MHz) and ^{13}C NMR ($CDCl_3$, 75 MHz), see Table 1.

Compound 2

Yellow oil (90 mg); IR ν_{max} (Neat) cm^{-1}: 1732, 1660, 1595 and 996; ESI-MS (pos.): *m/z* 311 $[M+H]^+$; Positive HR-ESIMS: *m/z* $[M]^+$ 310.2891 (calculated 310.2872 for $C_{20}H_{38}O_2$). ^1H NMR ($CDCl_3$, 300 MHz) and ^{13}C NMR ($CDCl_3$, 75 MHz), see Table 2

Compound 3

Colorless crystals (78 mg); M.P. 136-137°C; $[\alpha]^{25}_D$: - 36.00° ($CHCl_3$, *c*2.0); IR ν_{max} (KBr) cm^{-1}: 3417, 3022, 1644, 1519, 1427, 1215, 1053; ESI-MS: *m/z* 415 $[M+H]^+$; ^1H NMR ($CDCl_3$, 300MHz): δ_H 5.36 (1H, *m*, H-6), 3.26 (1H, *m*, H-3), 2.03 (2H, *m*, H-2), 1.00 (3H, *s*, H-19), 0.92 (3H, *d*, *J* = 6.0 Hz, H-21), 0.86 (3H, *m*, H-29), 0.84 (6H, *d*, 5.2 Hz, H-26, H-27), 0.88 (3H, *s*, H-18); ^{13}C NMR ($CDCl_3$, 75 MHz): δ_C 36.7 (C-1), 32.0 (C-2), 71.9 (C-3), 42.3 (C-4), 142.7 (C-5), 122.3 (C-6), 29.6 (C-7), 31.7 (C-8), 49.8 (C-9), 36.7 (C-10), 21.0 (C-11), 40.0 (C-12), 43.1 (C-13), 56.8 (C-14), 24.1(C-15), 29.1 (C-16), 57.1 (C-17), 12.5 (C-18), 13.9 (C-19), 36.4 (C-20), 20.8 (C-21), 35.1 (C- 22), 29.6 (C-23), 51.8 (C-24), 27.0 (C-25) 19.4 (C-26), 18.8 (C-27), 24.0 (C-28) 13.0 (C-29).

Compound 4

White crystals (135 mg); m.p. 105-106°C; UV (MeOH) λ_{max} nm: 248, 224; IR ν_{max} (KBr) cm^{-1}: 1595, 1505, 1463, 1240, 895; ESI-MS: *m/z* 446 $[M]^+$; ^1H NMR (DMSO-d_6, 300MHz) and ^{13}C NMR (DMSO-d_6, 75 MHz), see Table 2.

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