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A new trypsin inhibitory phthalic acid ester from *Heliotropium strigosum*

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Abstract A new phthalic acid ester has been isolated from the chloroform soluble fraction of *Heliotropium strigosum* and assigned the structure *o*-phthalic acid bis-(2-ethyl decyl)-ester **1**. In addition, 4-hydroxybenzoic acid **2** and *β*-sitosterole **3** have also been isolated. 4-Hydroxybenzoic acid **2** is reported for the first time from this species. The structures of these compounds were elucidated with the help of 1D and 2D NMR techniques and by comparison with the data reported in the literature. The new compound **1** indicated to be a competitive inhibitor of trypsin with the K_i value of 12.1 μm.

Keywords *Heliotropium strigosum* · Phthalic acid ester · Trypsine inhibition

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Introduction

Heliotropium strigosum belongs to family Boraginaceae. Traditionally, this plant is used as laxative and diuretic. The juice of the plant is used to treat gum boils, sore eyes and also in cure of stings of nettles, insects and snake bites (Nasir, 1970; Qureshi *et al.*, 2010; Khan *et al.*, 2013). Previously, we have reported antimicrobial and antioxidant activities of various fractions of *H. strigosum* (Sajid *et al.*, 2010). Herein, we report the isolation and structure elucidation of a new **1**, a new-source **2**, and a known compound **3** from the chloroform soluble fraction of *H. strigosum* and trypsin inhibition activity of the new compound **1**.

Results and discussion

Structure elucidation of the new compound **1**

The compound was isolated as yellowish oil. The molecular formula was determined as $C_{32}H_{54}O_4$ with the help of ^{1}H NMR, ^{13}C NMR and EIMS. In EIMS, the base peak at *m/z* 149 that arises from the prominent peak at *m/z* 167 due to loss of water molecule is characteristic for long chain phthalic acid ester (Narendra *et al.*, 2006; Solimabi *et al.*, 1998). The ^{13}C NMR spectrum was very helpful in elucidating the structure of the compound. It showed the presence of four methyl, eighteen methylene, six methine and four quaternary carbons. The resonances in the downfield region at σ 132.37, 130.91 and 128.79 were due to aromatic ring, and the signal at σ 167.81 was assigned to the carbonyl carbon of ester moiety. The signal at σ 68.13 was assigned to oxymethylene. The appearance of peak for an aliphatic methine at σ 38.70 clearly speaks of a branch in the molecule. This was further supported by the presence of

Table 1 ^{13}C and ^{1}H NMR spectral data of compounds **1** (150 and 600 MHz respectively)

Position	^{13}Cδ	^{1}Hδ	Position	^{13}Cδ	^{1}Hδ
1, 2	132.37		5′, 5″	29.70	1.22–1.46 (32H, *m*)
3, 6	128.79	7.70 (2H, *dd*, $J = 3$, 5.4)	6′, 6″	29.66	1.22–1.46 (32H, *m*)
4, 5	130.91	7.53 (2H, *dd*, $J = 3$, 5.4)	7′, 7″	28.89	1.22–1.46 (32H, *m*)
C=O	167.81		8′, 8″	31.92	1.22-1.46 (32H, *m*)
1′, 1″	68.13	4.24 (2H, *dd*, $J = 5.4$, 11.4), 4.19 (2H, *dd*, $J = 5.4$, 11.4)	9′, 9″	22.70	1.22–1.46 (32H, *m*)
2′, 2″	38.70	1.68 (2H, *m*)	10′, 10″	14.05	0.88 (6H, *t*, $J = 3$, 7.8)
3′, 3″	30.30	1.22–1.46 (32H, *m*)	3‴, 3⁗	22.99	1.22–1.46 (32H, *m*)
4′, 4″	23.68	1.22–1.46 (32H, *m*)	4‴, 4⁗	11.00	0.92 (6H, *t*, $J = 3$, 7.8)

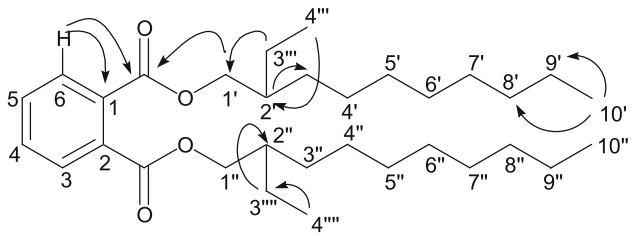

Fig. 1 Structure of compound **1** and its important HMBC correlations

doublets of doublets at σ 4.24 (2H, *dd*, $J = 5.4$, 11.4) and 4.19 (2H, *dd*, $J = 5.4$, 11.4) for oxymethylene in the ^{1}HNMR spectrum indicating that it is adjacent to methine and not to methylene. These two doublets of doublets could be attributed to oxymethylene protons. The existence of an aliphatic side chain was evident from the appearance of multiplets at σ 1.22–1.46 which integrated for 28 protons and the corresponding methylene resonances for 3′, 3″–5′, 5″ in ^{13}C NMR as shown in Table 1. Furthermore, the two triplets each integrating for six hydrogen in the up field regions at 0.88 ($J = 7.8$) and 0.92 ($J = 7.8$) were assigned to the terminal methyls of the side chain and methyls of the branched chain. On the basis of the above evidences, the compound was identified to be *o*-phthalic acid bis-(2-ethyl decyl)-ester. The HMBC correlations fully supported the assigned structure. Important HMBC correlations have been shown in Fig. 1.

Mechanism-based kinetic study revealed that compound **1** is a competitive inhibitor of trypsin. Lineweaver–Burk, Dixon plots and their re-plots indicated pure competitive type of inhibition for **1** against trypsin enzyme, as there was increase in V_{max} without affecting the affinity (K_m values) of the trypsin towards the *N*-succinyl-phenylalanine-*p*-nitroanilide. In other words, we can say that **1** and *N*-succinyl-phenylalanine-*p*-nitroanilide bind randomly and independently at the active sites of trypsin.

Collection and identification of plant

The plant material was collected in January–February, 2009 from Malakand, Pakistan, identified by a plant taxonomist, Zafar Iqbal, Department of Plant Sciences, Kohat University of Science and Technology, Kohat where a voucher specimen (No. KUST-1230-B) has been deposited.

Preparation of crude extract and fractions

The whole plant material was dried in shade, crushed into small pieces and powdered. The coarse powder (15 kg) was macerated in methanol for 15 days with frequent stirring at room temperature (Williamson *et al.*, 1998) and then filtered off. The filtrate was concentrated under vacuum at low temperature (40 °C) with the help of a rotary evaporator. A crude extract (150 g) was obtained from the filtrate. The crude extract (130 g) was suspended in distiled water (500 ml) and sequentially partitioned with *n*-hexane (3 × 500 ml), chloroform (3 × 500 ml) and ethyl acetate (3 × 500 ml), to yield the *n*-hexane (40 g), chloroform (30 g), ethyl acetate (25 g) and aqueous fractions (35 g), respectively. The chloroform soluble fraction was subjected to column chromatography over flash silica gel eluting with *n*-hexane/ethyl acetate (95:5, 90:10, 85:15, 75:25, 60:40) in increasing order of polarity. The fraction which eluted with *n*-hexane/ethyl acetate (85:15) was subjected to repeated column chromatography eluting with *n*-hexane/ethyl acetate (88:12) to afford compound **1** (15 mg) and **3** (30 mg). The fraction which was obtained from *n*-hexane/ethyl acetate (60:40) was then subjected to repeated column chromatography to afford the compound **2** (12 mg).

Trypsin inhibition assay

Trypsin inhibitory activity of compound **1** was assayed (Table 2) by the method of Cannell *et al.* (1988). Trypsin (9 units/ml in 50 mM tris–HCl buffer pH 7.6, Sigma Chemical Co., USA) was preincubated with compound (**1**) for 20 min at 25 °C. 100 µl of substrate solution (*N*-succinyl-phenylalanine-*p*-nitroanilide, 1 mg/ml of 50 mM tris–HCl buffer, pH 7.6) was added to start the enzyme reaction. The absorbance of released *p*-nitroaniline was continuously monitored at 410 nm until a significant colour

Table 2 In vitro inhibition of trypsin by compound **1**

S. no.	Name of substance	$K_i^a \pm SEM^b$ (μM)
1	Compound **1**	12.10 ± 0.9
2	PMSF(standard)	10.01 ± 0.6

[a] K_i is the mean of three values calculated by using the Dixon plot and Lineweaver–Burk secondary plots

[b] Standard mean error of 3–5 assays

change was achieved. The final DMSO concentration in the reaction mixture was 8 %.

Conclusion

The study reports the isolation of a novel compound i.e. *o*-phthalic acid ester from the chloroform soluble fraction of *H. strigosum* and trypsin inhibition activity of this compound. The preliminary findings highlight the importance of this class of secondary metabolites in drugs designing and as therapeutic agents. Study is underway in our laboratory to isolate and identify further active components of the plant.

Estimation of IC_{50} Values

The concentrations of test compounds (**1**) that inhibited the hydrolysis of substrates (trypsin) by 50 % (IC_{50}) were determined by monitoring the effect of increasing concentrations of these compounds in the assays on the inhibition values.

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