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Isolation and Identification of a Vardenafil Analogue in a Dietary Supplement

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ABSTRACT

A vardenafil analogue was found to be added illegally into a dietary supplement marketed for erectile dysfunction. Its structure was determined as 2-(2-ethoxyphenyl)-5-methyl-7-propyl-imidazo[5,1-*f*][1,2,4]triazin-4(*3H*)-one. The sample was extracted with ethanol and isolated by column chromatography. The structure was identified with a series of 1-D and 2-D NMR techniques and LC/MS/MS. Having compared with structure of vardenafil, the data showed that the ethylpiperazinyl and sulfonyl of vardenafil were removed from the phenyl group. Since its structure was similar to that of vardenafil, side effects of vardenafil might associate with this analogue. This vardenafil analogue has been included in the inspection list of illegal adulterants in Taiwan.

Key words: vardenafil analogue, LC/MS/MS, NMR

INTRODUCTION

Sildenafil and vardenafil, two PDE (phosphodiesterase) inhibitors, are used popularly as orally effective drugs in the treatment of male erectile dysfunction[1,2], despite their association with several serious side effects[3]. A substantial identification system for sildenafil in health foods was reported using three different analytical methods, i.e. TLC, HPLC/MS and HPLC/PDA in Japan[4]. Four analogues, homosildenafil, acetildenafil, hydroxyhomosildenafil and piperidenafil were found from many functional foods marketed for penis erectile dysfunction in Korea[5], Netherlands[6] and USA[7].

In our laboratory we have found adulterants such as sildenafil[8], homosildenafil, acetildenafil[9], hydroxyhomosildenafil[10], tadalafil, vardenafil and piperidenafil in many dietary supplements (Figure 1). Another unknown compound **A** related to vardenafil was found in a dietary supplement marketed for enhancing male sex ability. Although its molecular weight and UV spectra were different from those of sildenafil, vardenafil and the above sildenafil analogues, the data of NMR indicated a vardenafil analogue was inferred.

MATERIALS AND METHODS

I. *Equipments*

The melting point was determined on a Fisher-Johns melting point apparatus. The LC/MS/MS was performed using a Waters 2690 Alliance LC module, equipped with 996 photodiode array detector and Micromass Quattro Ultima tandem mass. The NMR spectra were recorded on a Bruker AMX-400 spectrometer (400 MH_Z for ^1H, 100 MH_Z for ^{13}C) with dimethylsulfoxide-d_6 as solvent. All chemicals were of analytical grades.

II. *Extraction and Isolation*

Test samples were obtained from local markets and

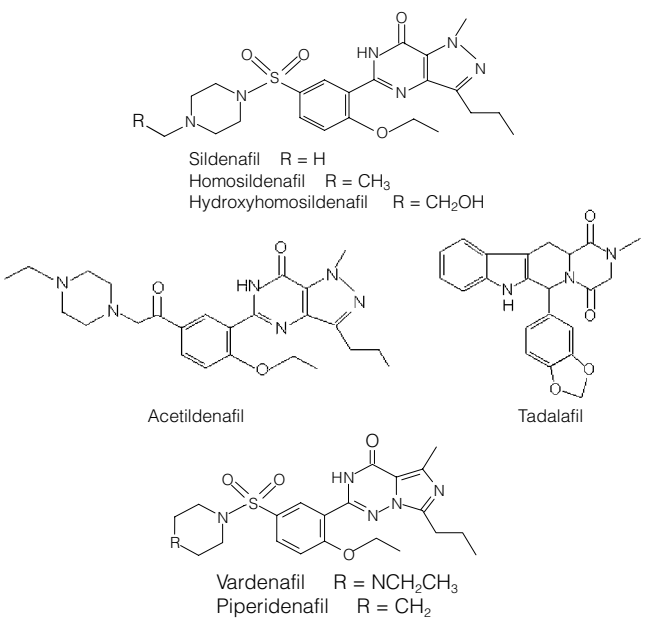

Figure 1. Structure of sildenafil, vardenafil, tadalafil, vardenafil analogue and sildenafil analogues.

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consumer service centers of the local health bureaus. Six capsules of sample (3.1 g) were extracted with ethanol, and the leftover was removed by filtration. The filtrate was dried under reduced pressure using a rotary evaporator at 40°C. The residue was subjected to a silica gel column chromatography using *n*-hexane with increasing amount of ethyl acetate as solvent. The target fractions were collected and recrystallized with methanol to yield the white crystal compound **A** (47.0 mg).

III. *NMR Correlation Data of Compound* **A**

The purified compound **A** was identified with a series of 1-D and 2-D NMR spectroscopic techniques, including ^{1}H, ^{13}C, DEPT, COSY, HMQC and HMBC. The data are shown in Table 1.

IV. *Analysis Condition of LC/MS/MS*

HPLC of LC/MS/MS was carried out on a column of Cosmosil $5C_{18}$-AR (4.6 × 150 mm, 5 μm) with methanol/0.25% formic acid (70:30) as mobile phase. The flow rate was 0.3 mL/min, the injection volume was 10 μL, and the running time was 40 min. The eluate was monitored by a photo-diode array detector and scan range was 200~350 nm.

The analytical condition of tandem mass was as follows: positive ion eletrospray (ES^{+}) modes, daughter ion: 313.2, capillary voltage: 3.0 kV, cone voltage: 60 V, collision energy: 20 eV, source temperature: 120°C, and desolvation temperature: 300°C.

RESULT AND DISCUSSION

A silica gel column chromatography using *n*-hexane containing increasing amounts of ethyl acetate as solvent was applied to the separation and purification of compound **A** from one dietary supplement which claimed to enhance male sex ability. The isolated compound **A** was obtained as white crystal from methanol. The melting point was between 144 and 145°C.

Table 1 shows the ^{1}H-NMR, ^{13}C-NMR, DEPT, ^{1}H-^{1}H COSY and HMBC spectral data of compound **A**.

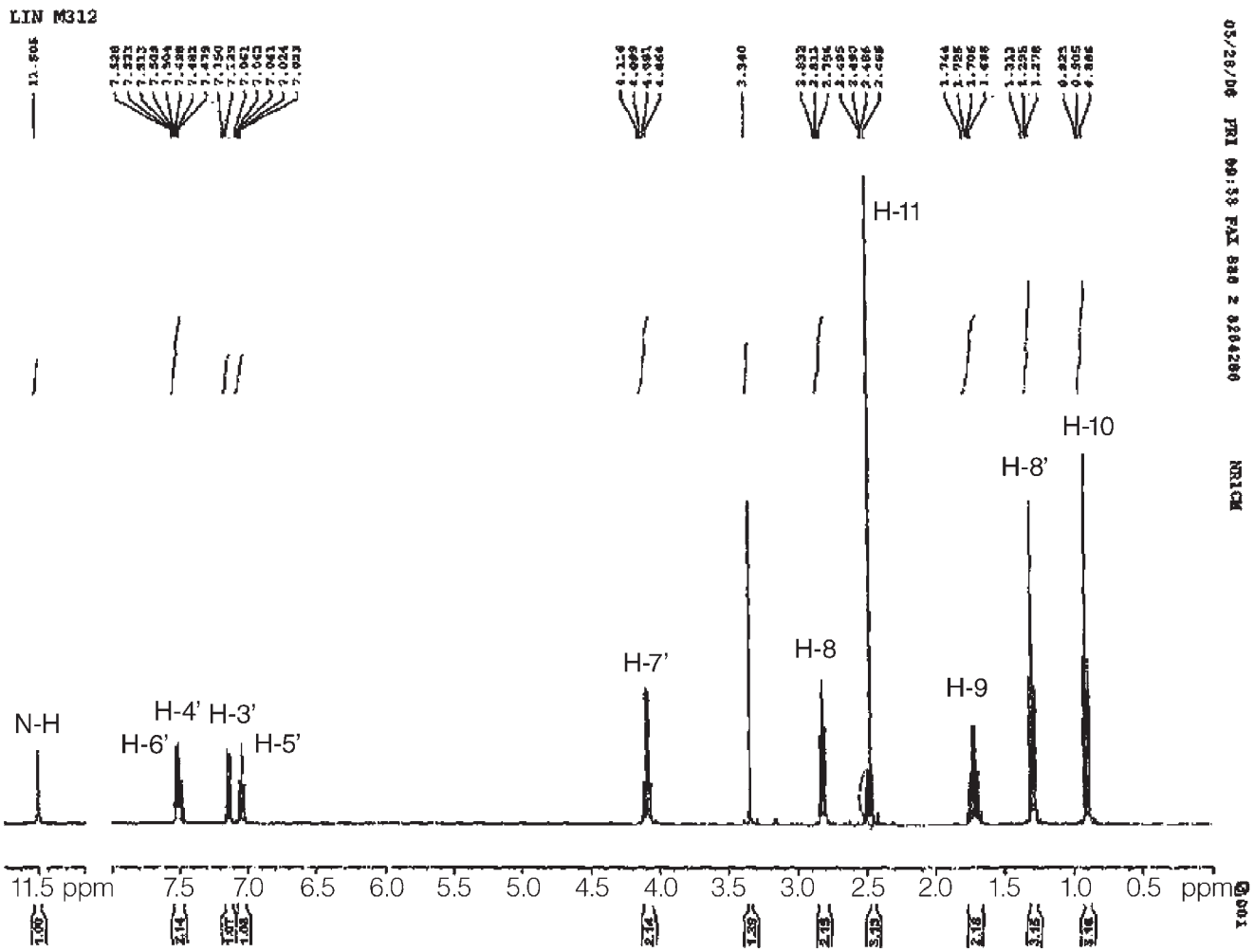

Figure 2. ^{1}H spectrum of vardenafil analogue **A**.

Table 1. NMR correlation of vardenafil analogue **A**

No.	^{13}C (δ_C)	^{1}H (δ_H)	DEPT	COSY	HMBC
2	147.5	-	0	-	H-6’
3	-	11.51 (1H, *s*)	-	-	-
4	155.1	-	0	-	-
4a	113.6	-	0	-	H-11
5	137.5	-	0	-	H-11
7	144.3	-	0	-	H-8/H-9
8	27.1	2.81 (2H, *t, J* = 7.6 H_Z)	2	H-9	H-9/H-10
9	20.3	1.71 (2H, *m*)	2	H-8/H-10	H-8/H-10
10	13.7	0.91 (3H, *t, J* = 7.4 H_Z)	3	H-9	H-8/H-9
11	14.2	2.49 (3H, *s*)	3	-	-
1’	120.1	-	0	-	H-6’/H-5’/H-3’
2’	156.8	-	0	-	H-6’/H-4’/H-3’/H-7’
3’	112.7	7.14 (1H, *d, J* = 8.4 H_Z)	1	H-4’	H-5’/H-4’
4’	132.4	7.50 (1H, *dd, J* = 7.6, 8.4 H_Z)	1	H-3’/H-5’	H-6’/H-5’/H-3’
5’	120.3	7.04 (1H, *dd, J* = 7.2, 6.8 H_Z)	1	H-6’/H-4’	H-6’/H-4’/H-3’
6’	130.3	7.52 (1H, *d, J* = 7.6 H_Z)	1	H-5’	H-5’/H-4’
7’	64.0	4.09 (2H, *q, J* = 7.0 H_Z)	2	H-8’	H-8’
8’	14.5	1.30 (3H, *t, J* = 7.0 H_Z)	3	H-7’	H-7’

$^{\delta}$ppm in DMSO-d_6 , *J* in H_Z, 100 MH_Z for ^{13}C, 400 MH_Z for ^{1}H
DEPT is the number of attached protons.

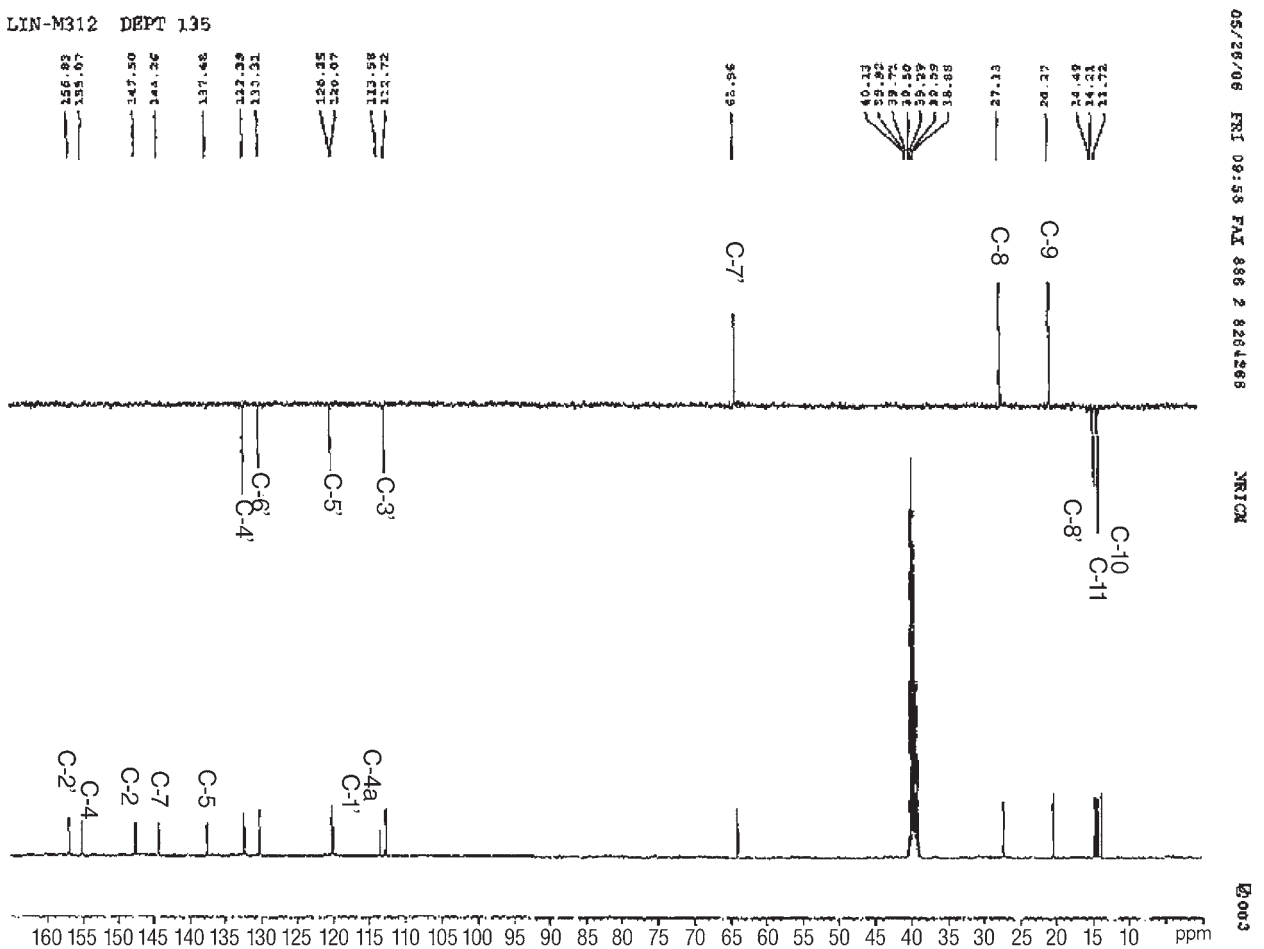

Figure 3. DEPT spectrum of vardenafil analogue **A**.

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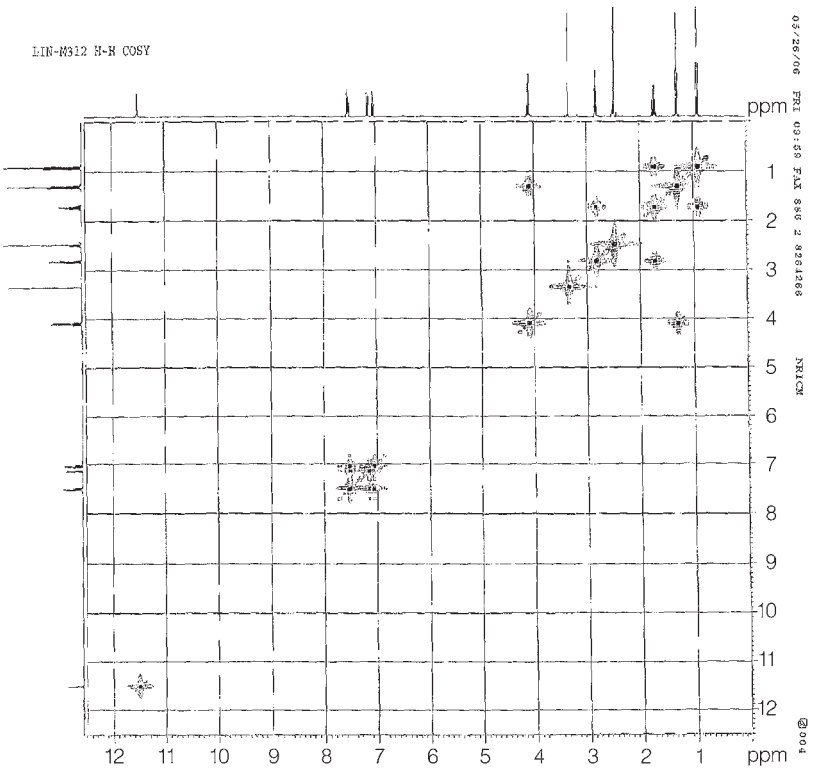

Figure 4. ^{1}H-^{1}H COSY spectrum of vardenafil analogue **A**.

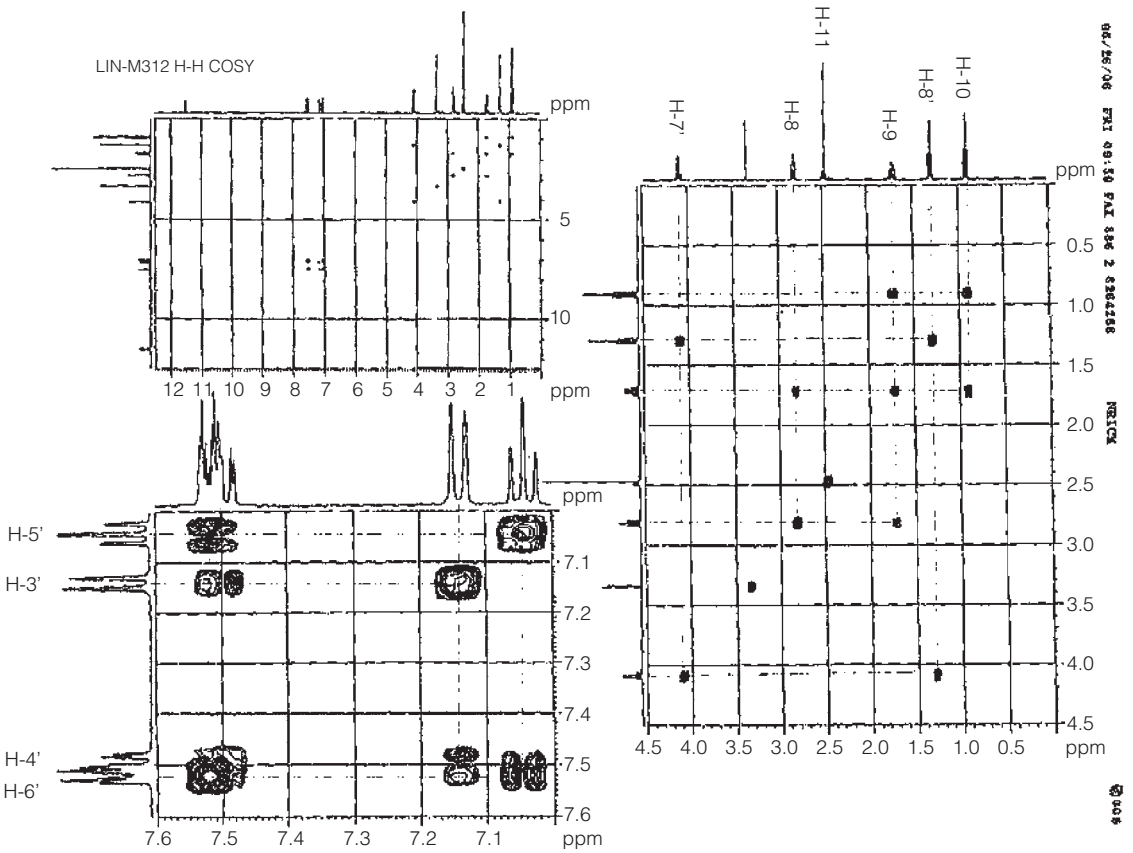

Figure 5. Partial ^{1}H-^{1}H COSY spectrum of vardenafil analogue **A**.

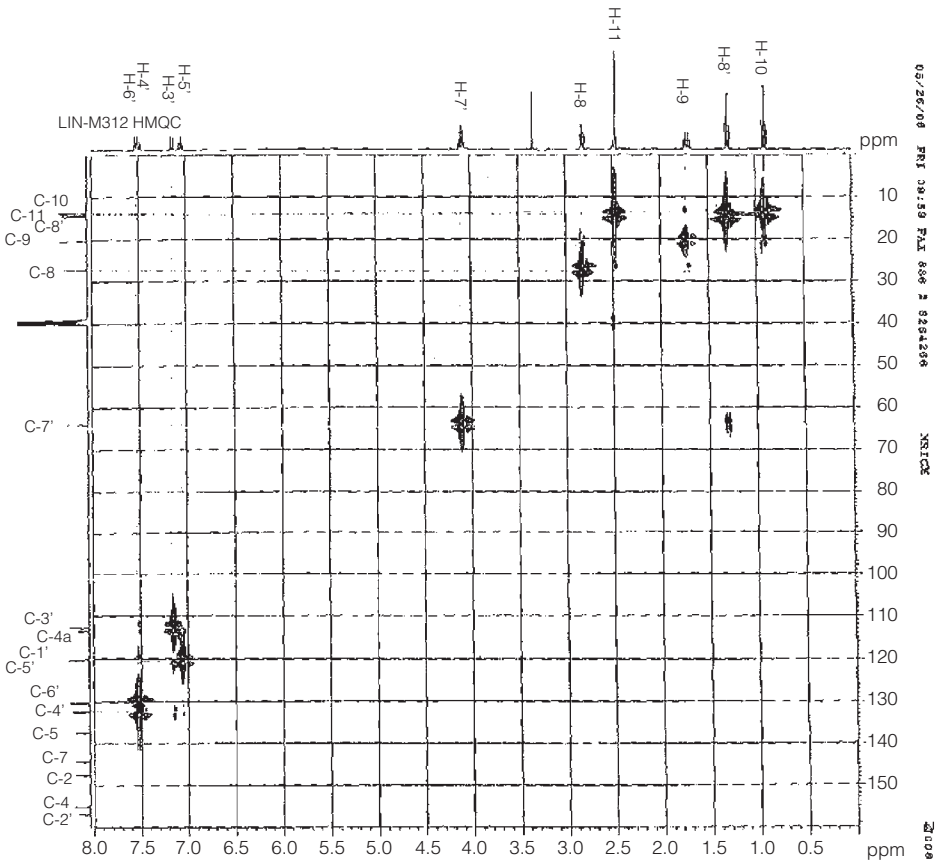

Figure 6. HMQC spectrum of vardenafil analogue **A**.

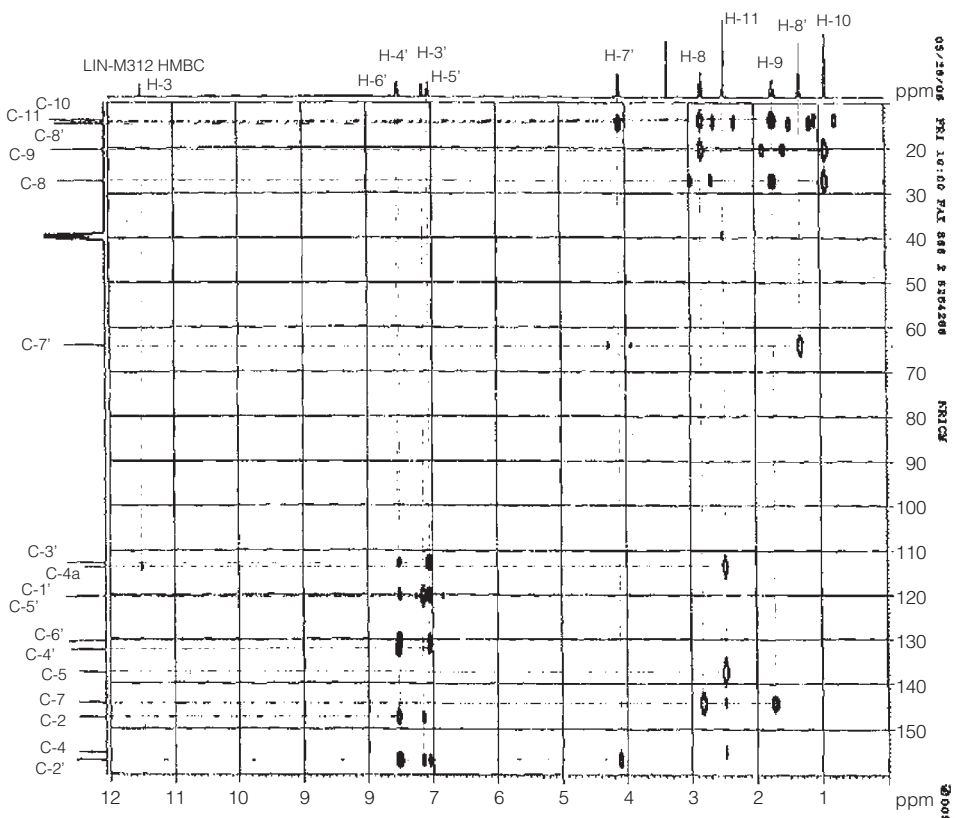

Figure 7. HMBC spectrum of vardenafil analogue **A**.

The spectra were similar to that of vardenafil, except the ethylpiperazinyl and sulfonyl group were removed from phenyl moiety, was named as vardenafil analogue **A**. The ^{1}H-NMR, DEPT, ^{1}H-^{1}H COSY, HMQC and HMBC spectra of vardenafil analogue **A** are shown in Figures 2, 3, 4, 5, 6 and 7, respectively. The spectroscopic numbering used is given in Figure 8. All signals were assigned unequivocally according to the various NMR spectroscopic data.

The ^{1}H-NMR (Figure 2) spectrum showed characteristics of an amide at δ_H 11.51 (1H, *s*), four aromatic protons at δ_H 7.52 (1H, *d, J* = 7.6 H_Z), 7.04 (1H, *d, d, J* = 6.8, 7.2 H_Z), 7.50 (1H, *d, d, J* = 7.6, 8.4 H_Z) and 7.14 (1H, *d, J* = 8.4 H_Z), respectively. Two triplet peaks at δ_H 0.91 and δ_H 1.30 were assigned as the methyl group for H_3-10 and H_3-8'. One singlet peak at δ_H 2.49 was assigned as the methyl group for H_3-11. A methine signal of phenyl at δ_H 7.04 in this vardenafil analogue **A** was significantly different from vardenafil, which lacked this signal. Three peaks at δ_H 2.81, δ_H 1.71 and δ_H 4.09 were assigned as the methylene for H_2-8, H_2-9 and H_2-7', respectively.

The ^{13}C-NMR and DEPT (Figure 3) spectra indicated three primary carbons, three secondary carbons, four tertiary carbons and seven quaternary carbons. One carbon peak at δ_C 155.1 belonged to lactam. Three methylene signals were shown at δ_C 27.1, δ_C 20.3 and δ_C 64.0 for C-8, C-9 and C-7'. Four methine peaks at δ_C 130.3, δ_C 120.3, δ_C 132.4 and δ_C 112.7 were assigned as the aromatic carbon for C-6', C-5', C-4' and C-3', respectively.

In the ^{1}H-^{1}H COSY (Figure 4) and Partial ^{1}H-^{1}H

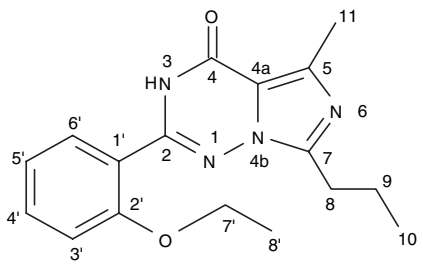

Figure 8. Structure of vardenafil analogue **A**.

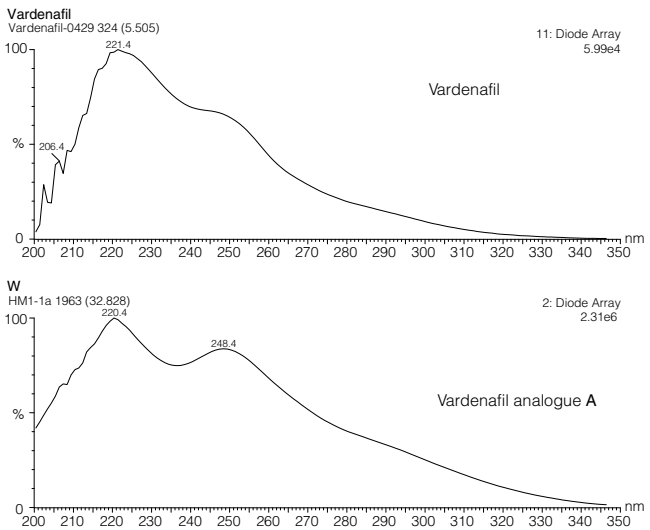

Figure 9. UV spectra of vardenafil and vardenafil analogue **A**.

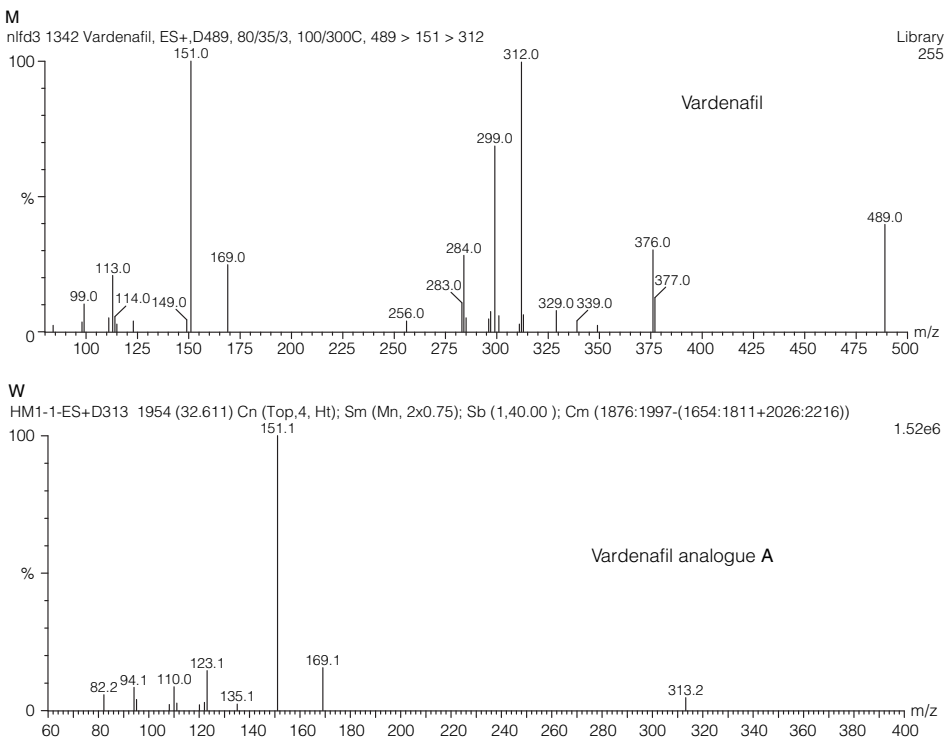

Figure 10. The fragmentation of vardenafil and vardenafil analogue **A**.

COSY (Figure 5) spectra, the correlation of H-6'/H-5', H-5'/H-6', H-4', H-4'/H-5', H-3' and H-3'/H-4' exhibited the ethylpiperazinyl and sulfonyl group of vardenafil were removed from phenyl moiety.

In the HMQC (Figure 6) and HMBC (Figure 7) spectra, the correlation of H-7'/C-2' exhibited the attachment of the ethoxy group (δ_H 4.09, 1.30) to phenolic carbon (δ_C 156.8). The correlation of H-8 and H-9/C-7 showed the linkage of the propyl group (δ_H 2.81, 1.71, 0.91) to pyrazolic carbon (δ_C 144.3). The correlation of H-11/C-4a exhibited the attachment of a methyl group (δ_H 2.49) to the pyrimidine ring (δ_C 113.6). The correlation between H-6' (δ_H 7.52) and C-2 (δ_C 147.5) suggested that the triazin ring linked to the benzene ring.

The UV spectrum of vardenafil analogue **A** was shown as λ_{max} at 248.4, 220.4 nm (Figure 9). Both the molecular weight and absorption of UV spectrum were different from those of vardenafil.

The [M+H] of vardenafil analogue **A** founded at m/z 313.2, corresponding to the molecular formula $C_{17}H_{20}O_2N_4$, 176 a.m.u. less than vardenafil ([M+H]: m/z 489.0) referred to ethylpiperazinyl and sulfonyl group. The fragmentation of vardenafil analogue **A** is shown in Figure 10. Possible fragmentation pathways of vardena-

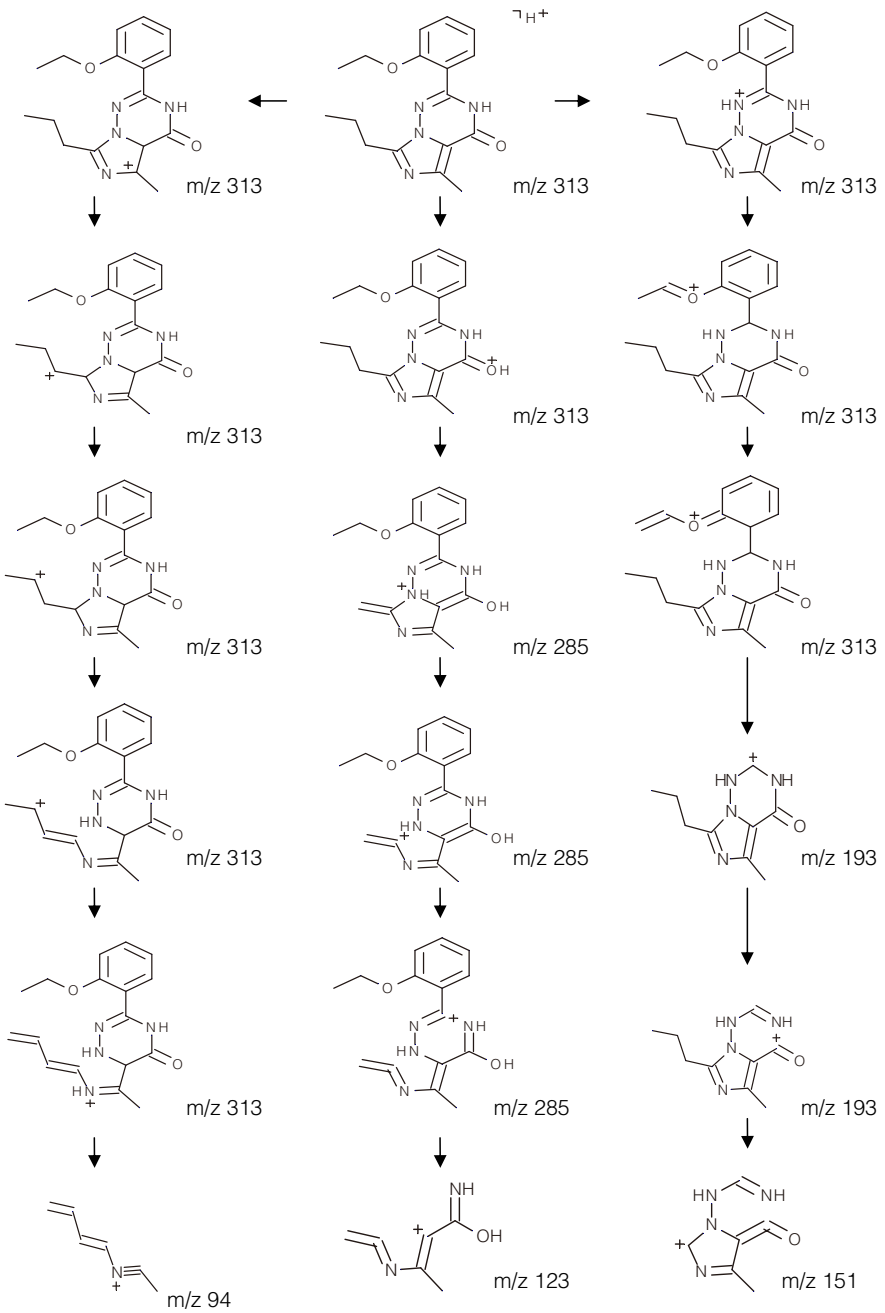

Figure 11. The possible fragmentation pathways of vardenafil analogue **A** at the LC/MS/MS.

fil analogue **A** at the LC/MS/MS electrospray positive (ES^+) are shown in Figure 11.

Based on the mass and NMR spectroscopic data, the structure of compound **A** was an analogue of vardenafil and was determined as 2-(2-ethoxyphenyl)-5-methyl-7-propyl-imidazo[5,1-*f*][1,2,4]triazin-4(3*H*)-one.

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