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Isolation and Identification of a Vardenafil Analogue in a Functional Food Marketed for Penile Erectile Dysfunction

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ABSTRACT

A vardenafil analogue was found to be added illegally to a dietary supplement marketed for erectile dysfunction. The structure was determined as 2-[2-ethoxy-5-(piperidine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3*H*-imidazo[5,1-*f*]-[1,2,4]triazin-4-one and named as piperidenafil. The sample was extracted with ethanol and isolated by column chromatography. The structure was identified by a series of 1-D and 2-D NMR techniques, mass and LC/MS/MS. As compared with the structure of vardenafil, it is shown that the ethylpiperazine was switched to piperidine. Since its structure was similar to that of vardenafil, side effects of vardenafil might be associated with this analogue. Basic on the law, foods using any illegal additive such as synthetic chemical compounds shall not be sold, manufactured, imported, processed, used, prepared, stored or transported. Therefore, this vardenafil analogue has been included in the inspection list of illegal health-related substances in Taiwan.

Key words: vardenafil analogue, piperidenafil, isolated, identified, NMR, LC/MS/MS

INTRODUCTION

Recently, sildenafil and vardenafil (Figure 1) are used popularly as an orally drug in the treatment of male erectile dysfunction[1,2], in spite of its association with several serious side effects[3].

In our laboratory we have found adulterants such as sildenafil[4], homosildenafil, acetildenafil[5], hydroxyhomosildenafil, tadalafil and vardenafil in many dietary supplements. 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[6]. Three sildenefil analogues, namely homosildenafil, acetildenafil and hydroxyhomosildenafil, were found from many functional foods marketed for penis erectile dysfunction in Korea[7] and Netherlands[8].

Another unknown suspicious compound related to vardenafil was found in a dietary supplement marketed for enhancing the male sex ability. Although its molecular weight and UV spectra were different from those of sildenafil, vardenafil and sildenafil analogues, the data of NMR inferred a vardenafil analogue.

This analogue was found from an herbal dietary supplement in USA in 2006 and was named as piperidenafil[9] or piperidino vardenafil[10].

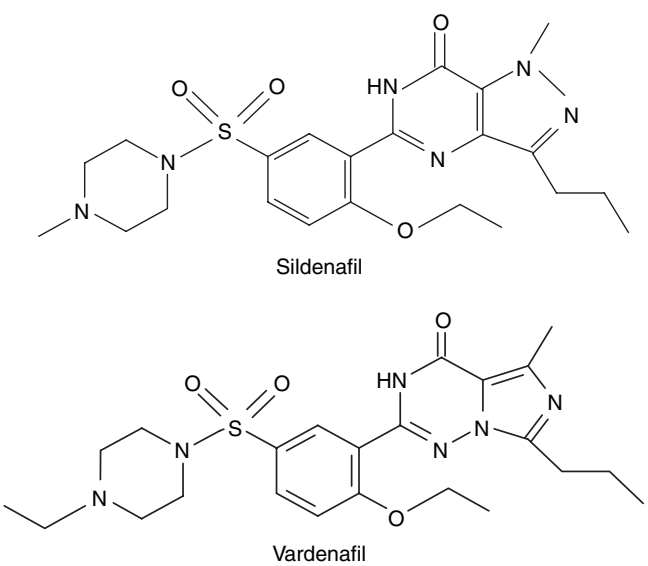

Figure 1. Structures of sildenafil and vardenafil.

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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. The infrared (IR) spectrum was recorded in the range

of 400~4000 cm^{-1} on a Jasco FT-IR-480 plus spectrometer using KBr pellets. The mass was acquired on a THERMO Polaris Q with a DIP module. All chemicals used were of analytical grade.

II. *Extraction and Isolation*

Test samples were randomly obtained from local markets and the consumer service centers of the local health bureaus by the health officers. Eight capsules of sample (4.2 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 silica gel column chromatography using chloroform containing methanol gradient. The fractions were collected and re-crystallized with methanol to yield the solid compound **A** (85 mg).

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

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

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

The HPLC of LC/MS/MS was performed out on a Cosmosil 5 C_{18}-AR column (4.6 × 150 mm, 5 μm) using acetonitrile/methanol/1% acetic acid (17:25:58) as the mobile phase. The flow rate was 0.5 mL/min, the injection volume was 10 μL and the running time was 35 min. The eluate was monitored by a photo-diode array detector and the scan range was 200 to 350 nm.

The analytical condition of tandem mass was as follows: positive ion electrospray (ES^{+}) modes; daughters ion: 460; capillary voltage: 3.0 kV; cone voltage: 80 V; collision energy: 25 eV; source temperature: 100°C; desolvation temperature: 300°C.

RESULTS AND DISCUSSION

Silica gel column chromatography using chloroform containing methanol gradient was employed to the separation and purification of compound **A** from a dietary supplement enhancing the male sex ability. The isolated compound **A** was obtained as colorless crystal from methanol. The melting point was between 185 and 186°C.

The UV spectrum of compound **A** by LC/MS/MS

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

No.	^{13}C (δ_C)[a]	^{1}H (δ_H)	DEPT[b]	COSY	HMBC
1	144.4	–	0	-	H-11/H-12
3	137.6	–	0	-	H-10
4	155.0	–	0	-	-
5	–	11.67 (1H, *s*)	-	-	-
6	146.1	–	0	-	H-15
9	113.7	–	0	-	H-10
10	14.2	2.48 (3H, *s*)	3	-	-
11	27.1	2.81 (2H, *t*, J = 7.6 H_Z)	2	H-12	H-12/H-13
12	20.2	1.71 (2H, *m*)	2	H-11/H-13	H-11/H-13
13	13.7	0.91 (3H, *t*, J = 7.4 H_Z)	3	H-12	H-11/H-12
14	127.0	–	0	-	H-15/H-18
15	129.9	7.83 (1H, *s*)	1	-	H-17
16	120.7	–	0	-	H-18
17	131.9	7.85 (1H, *d*, J = 7.6 H_Z)	1	H-18	H-15
18	113.2	7.36 (1H, *d*, J = 8.0 H_Z)	1	H-17	-
19	160.1	–	0	-	H-15/H-17/H-18/H-20
20	64.9	4.18 (2H, *q*, J = 6.8 H_Z)	2	H-21	H-21
21	14.2	1.32 (3H, *t*, J = 6.8 H_Z)	3	H-20	H-20
25	22.9	1.36 (2H, *m*)	2	H-24, H-26	H-23, H-27/ H-24, H-26
23,27	46.5	2.90 (4H, *br. m*)	2,2	H-24, H-26	H-27, H-23
24,26	24.6	1.53 (4H, *br. m*)	2,2	H-23, H-27	H-23, H-27

[a]δppm in DMSO-d_6 , J in H_Z, 100 MH_Z for ^{13}C, 400 MH_Z for ^{1}H.
[b]DEPT is the number of attached protons.

showed as λ_{max} at 213 nm (Figure 2). Both the molecular weight and absorption spectrum of UV/VIS were different from those of vardenafil.

The mass of compound **A** founded at m/z 459.11, corresponding to the molecular formula $C_{22}H_{29}O_4N_5S$, 29 a.m.u. less than vardenafil referred to $CH_3CH_2^+$. The fragmentation of compound **A** was shown in Figure 3. The proposed fragmentation pathways of compound **A** at the LC/MS/MS electrospray positive (ES^+) were illustrated in Figure 4.

The IR spectrum showed absorption bands with the characteristics of an amine at 3317 cm^{-1}, an aromatic ring at 1593 and 1470 cm^{-1}, an αβ-unsaturated lactam at 1695 cm^{-1} and an ether group at 1250 and 1031 cm^{-1}, all the same as those of vardenafil.

The ^{1}H-NMR, ^{13}C-NMR, DEPT, ^{1}H-^{1}H COSY and HMBC spectral data of compound **A** are shown in Table 1, which were similar to that of vardenafil, except the ethylpiperazine was switched to piperidine. The ^{1}H-^{1}H COSY, HMQC and HMBC spectra of vardenafil analogue (**A**) were shown in Figures 5, 6 and 7, respectively. The spectroscopic numbering used is given in Figure 8. All

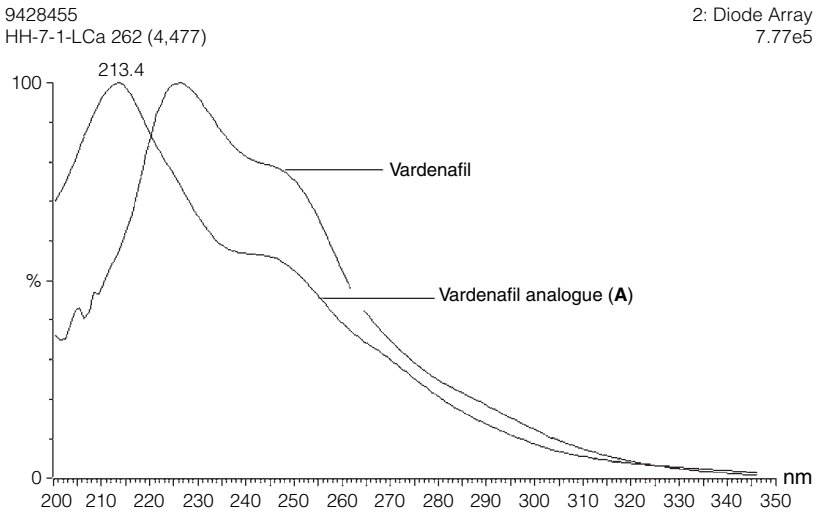

Figure 2. UV spectra of vardenafil and vardenafil analogue (**A**).

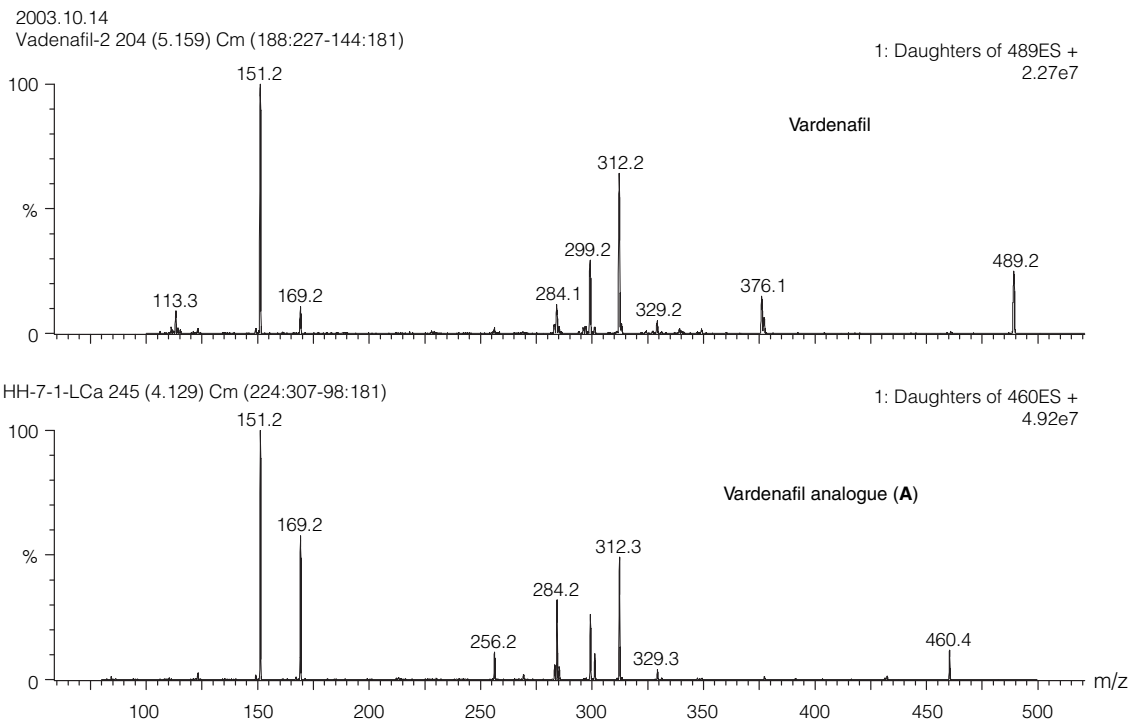

Figure 3. The LC/MS/MS fragmentations of vardenafil and vardenafil analogue (**A**).

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signals were assigned unequivocally according to various NMR spectroscopic data.

The ^{1}H-NMR spectrum showed characteristics of an amide at δ_H 11.67 (1H, *s*), three aromatic protons at δ_H 7.83 (1H, s), 7.85 (1H, *d*, J = 7.6 H_Z), 7.36 (1H, *d*, J = 8.0 H_Z). Two multiple peaks at δ_H 2.90 and δ_H 1.53 were assigned as the piperidine methylene for H_4-23,27 and H_4-24,26, respectively. One peaks at δ_H 1.36 (2H, *m*),

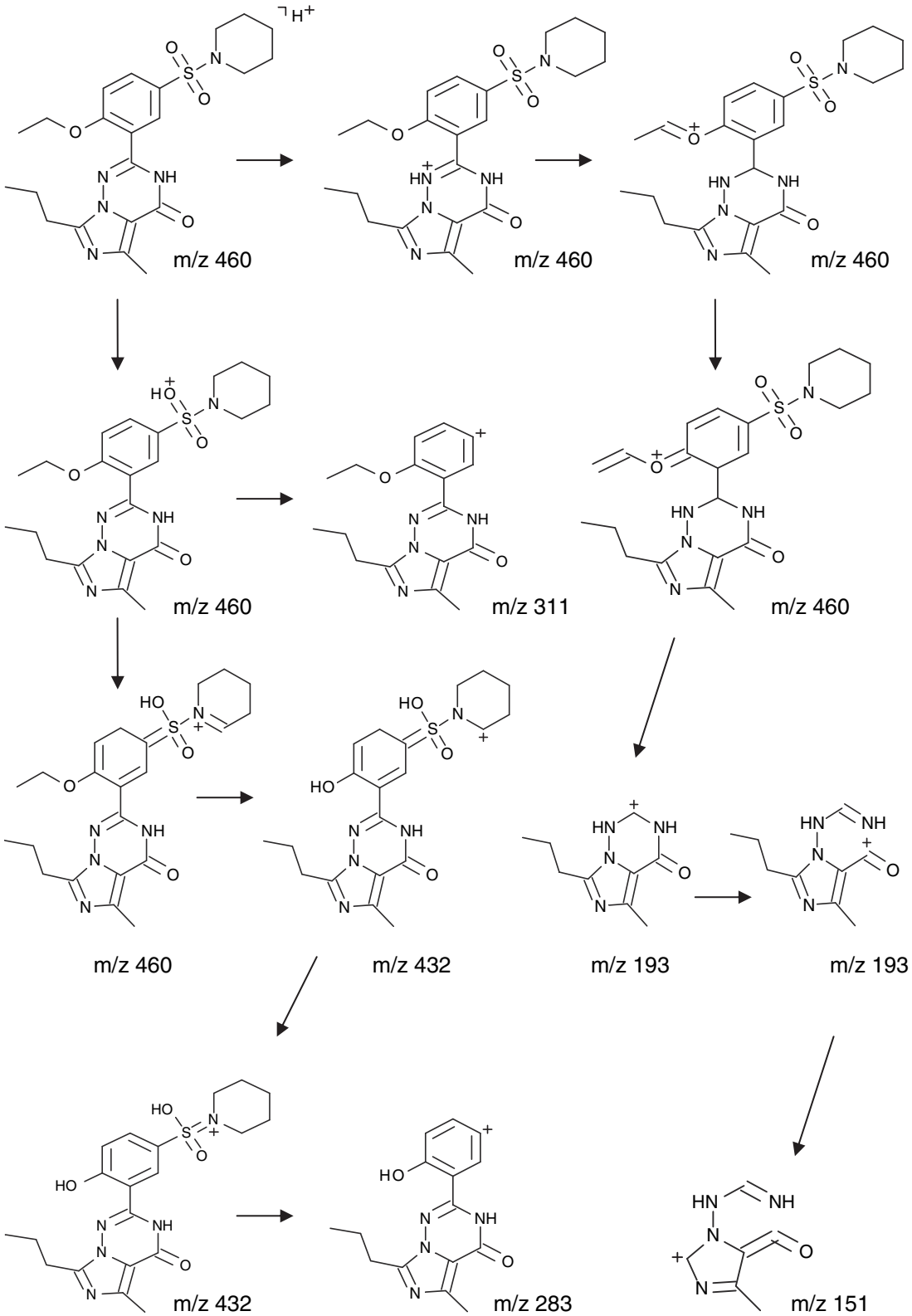

Figure 4. The possible fragmentation pathways of compound **A** at the LC/MS/MS.

further proved with 2D NMR data, was assigned as the piperidine methylene for H_2-25.

The ^{13}C-NMR and DEPT spectra indicated three primary carbons, which was one less than vardenafil, eight secondary carbons, three tertiary carbons and eight quaternary carbons, the same as that of vardenafil. One carbon peak at δ_C 155.0 belonged to lactam. One methylene signal at δ_C 22.9 was obviously different from vardenafil (Figure 1).

In the HMQC (Figure 6) and HMBC (Figure 7) spectra, the correlation of H-24,26/C-25 indicated that two methylenyl groups (δ_H 1.53) attached to a methylenyl carbon (C-25). The correlation of H-20/C-19 exhibited the attachment of the ethoxy group (δ_H 4.18, 1.32) to the phenolic carbon (δ_C 160.1). The correlation of H-11, H-12/C-1 showed the linkage of the propyl group (δ_H 2.81, 1.71, 0.91) to pyrazolic carbon (δ_C 144.4). The correlation of H-10/C-9 exhibited the attachment of a methyl group (δ_H 2.48) to the pyrimidine ring (δ_C 113.7). The correlation between H-15(δ_H 7.83) and C-6 (δ_C 146.1) suggested that the triazin ring is linked to the phenolic ring.

Based on the mass, infrared spectrum and NMR spectroscopic data, the structure of compound **A** was determined as 2-[2-ethoxy-5-(piperidine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3*H*-imidazo[5,1-*f*]-[1,2,4]triazin-4-one. Compound **A** was a vardenafil analogue (Figure 8), in which a piperidine replaces ethylpiperazine in vardenafil. This analogue was found in an herbal dietary supplement in USA in 2006 and was named as piperidenafil or piperidino vardenafil. In this

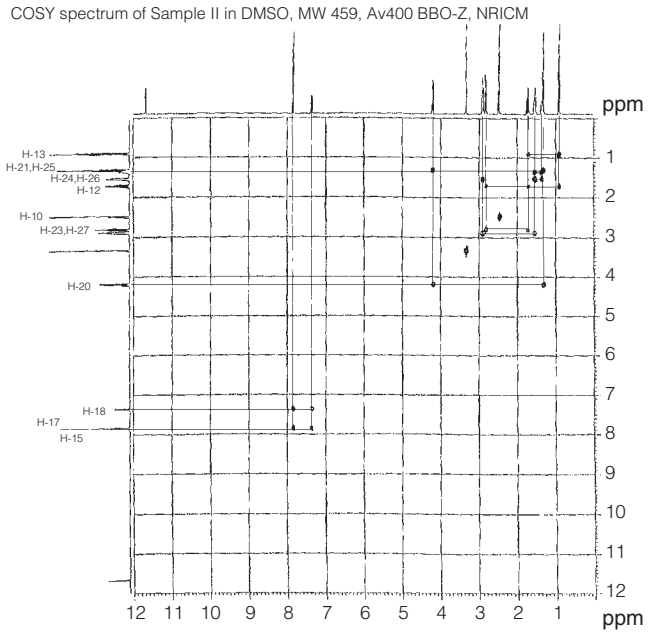

Figure 5. ^{1}H-^{1}H COSY spectrum of vardenafil analogue (**A**).

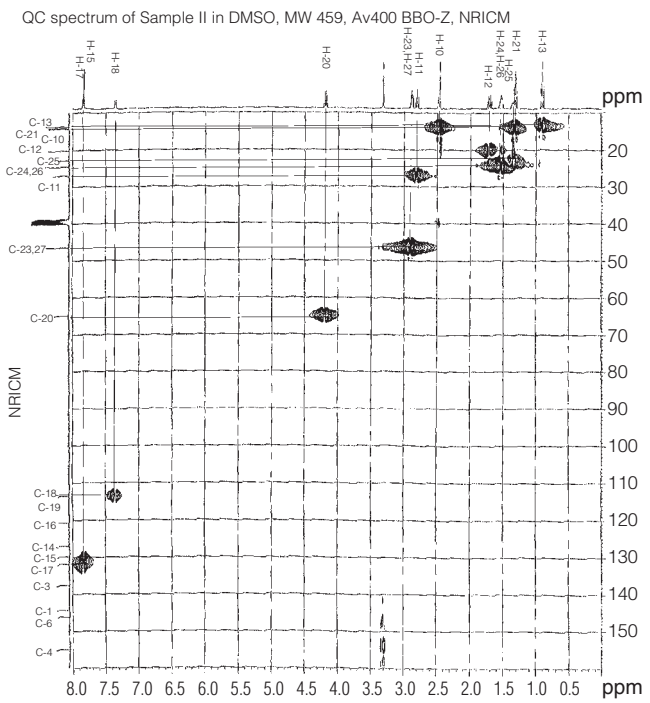

Figure 6. HMQC spectrum of vardenafil analogue (**A**).

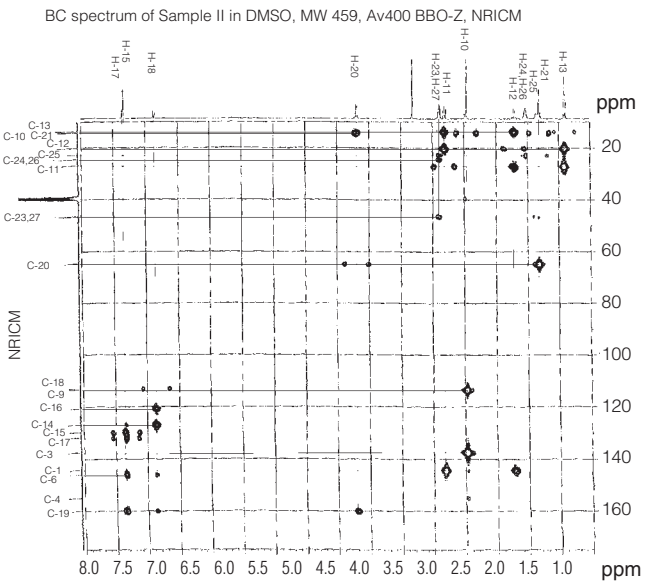

Figure 7. HMBC spectrum of vardenafil analogue (**A**).

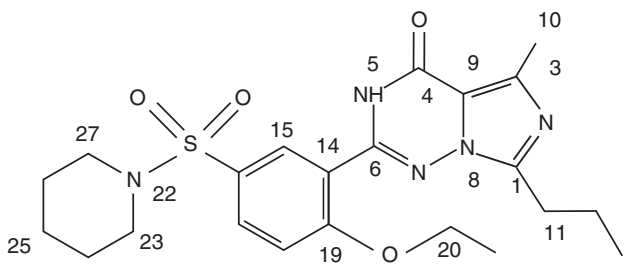

Figure 8. Structure of vardenafil analogue (**A**).

report, the isolation procedures along with a series of 1-D and 2-D NMR spectral data, the fragmentation of mass, the infrared spectrum, the absorption of UV spectra and the analytical condition of LC/MS/MS were established and elucidated.

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