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Identification of a Tadalafil Analogue Adulterated in a Dietary Supplement

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ABSTRACT

A dietary supplement which was claimed on the treatment of male erectile dysfunction was firstly screened by TLC, and two suspected spots showed on the TLC plate under the detection of UV 254 nm. One of the spot (**1**) was determined as tadalafil by direct comparison of its TLC, UV, and LC/MS/MS data with the reference standard of tadalafil. The other suspected spot was identified by extraction, separation and purification from the sample to get a yellowish powder (compound **2**). The structure of compound **2** with an amino group instead of the *N*2-methyl group in the piperazinedione ring of tadalafil was determined to be (6*R*,12*aR*)-2-amino-6-(1,3-benzodioxol-5-yl)-2,3,6,7,12,12a-hexahydro-pyrazino[1',2':1,6]pyrido[3,4-*b*]indole-1,4-dione by NMR, circular dichroism, and mass spectroscopy and compound **2** was named as aminotadalafil which was first detected in our laboratory. These two compounds were illegally adulterated into the dietary supplement and compound **2** has been listed as a new item for the screening of aphrodisiac adulterants.

Key words: dietary supplements, NMR, LC/MS/MS, tadalafil, tadalafil analogue, aminotadalafil

INTRODUCTION

Dietary supplements have been gradually accepted by the public in recent years as people think that they are natural and safe. Despite the dietary supplements are commonly used for provision of nutrients, the labels of many marketed products often claim the ability to treat diseases. Our laboratory is in charge of monitoring marketed products for public health. Most of the dietary supplements sent by the local health bureaus or justice systems are products used for enhancing body energy. Synthetic chemicals adulterated into the dietary supplements have been detected in some products and those published adulterants[1-14] mainly include sildenafil, tadalafil, vardenafil and their analogues.

Tadalafil, as shown in Figure 1, a phosphodiesterase type 5 (PDE5) inhibitor[15-18], was approved by the EU in 2002 and US-FDA in 2003 for the treatment of erectile dysfunction and was sold under the brand name, Cialis. Before tadalafil, sildenafil was the first approved drug for the treatment of erectile dysfunction; however, it had the adverse effects on retinal disturbance. It was owing to the inhibition of PDE6, which was a retina-specific enzyme involved in visual signal transduction. Tadalafil displays an 85-fold greater selectivity to PDE6 than sildenafil[18].

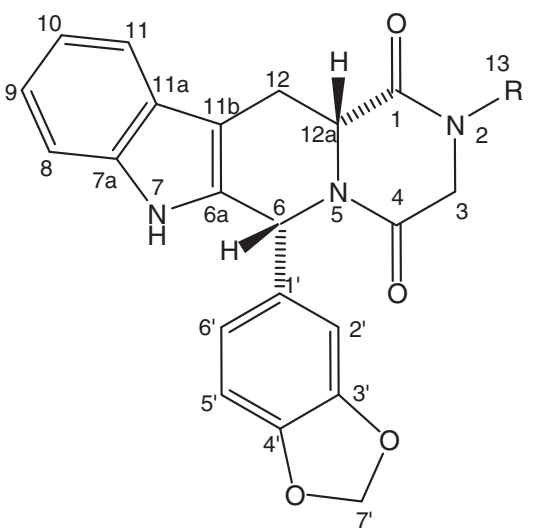

Figure 1. Chemical structures of tadalafil (R = CH_3) and aminotadalafil (compound **2**; R = NH_2) .

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Hence, tadalafil had rare occurrence of visual side effects. On the other hand, headache, flushing, and runny nose were more frequent. This paper reports that tadalafil (**1**) and its analogue (**2**) adulterated in a dietary supplement were detected by a routine screening method. This analogue has never been found in our laboratory. Therefore, it prompted us to elucidate its structure.

MATERIALS AND METHODS

I. *Sample and Chemicals*

The light yellow colored, encapsulated dietary supplement sample containing brown powder was submitted by the local health bureau during its routine inspection. Acetonitrile, methanol, chloroform, and ethyl acetate of LC grade were purchased from Labscan (Dublin, Ireland). Glacial acetic acid, potassium bromide, and dimethylsulfoxide-d_6 (DMSO-d_6) were purchased from E. Merck (Darmstadt, Germany). Ethanol (95%) was produced by Taiwan Tobacco and Liquor Corporation (Taipei, Taiwan). Tadalafil, provided by Lily ISCO Company for product registration, was used as reference standard.

II. *Instrumentations*

The NMR spectra were determined by a Bruker AV400 BBO-Z spectrometer (Ettlingen, Germany). Suspected compound **2** and reference standard of tadalafil was dissolved in DMSO-d_6 and used for NMR analysis by 1D and 2D NMR spectroscopic techniques (^{1}H, ^{13}C, DEPT, homo-COSY, HMQC and HMBC). Mass spectra were analyzed by a Thermo, Trace GC Ultra, Polaris Q mass spectrometer (Austin, Texas, U.S.A.). IR spectra were monitored on a Jasco FT/IR-480 plus fourier transform infrared spectrometer (Tokyo, Japan). Circular dichroism (CD) spectra (in MeOH) were identified by a Jasco J-815 spectropolarimeter between 200-350 nm, using constant N_2 flushing at 25°C (Tokyo, Japan). A Jasco DIP-1000 polarimeter (Tokyo, Japan) analyzed optical rotations. Ultraviolet spectra were scanned by a Cary 300 UV-visible spectrophotometer (Mulgrave, Victoria, Australia). The melting point was determined on a Fisher-Johns melting point apparatus (Illinois, IL, U.S.A.). Gas chromatography-mass spectrometry coupled with positive electron impact (EI^+) and positive chemical impact (CI^+) mode was used to determine the molecular weight. The mass fragments were determined by ESI^+ of LC/MS/MS. The LC/MS/MS experiments were carried out on a Quattro Ultima tandem mass spectrometer coupled with a Waters 2690 Alliance LC & 996 PDA with an automatic liquid sampler and an injector (Manchester, United Kingdom). The ESI^+-MS/MS conditions were as follows: ion source temperature, 120°C; desolvation temperature, 350°C; cone voltage, 100 V; collision energy, 16 V; and capillary voltage, 3 kV.

III. *Methods*

(I) *Identification of Compound **1***

Powder of sample (3.0 g) was extracted with 95% ethanol (25 mL) by ultrasonic shaking at room temperature for 30 minutes. The filtrate and reference standard of tadalafil were simultaneously analyzed by TLC method. One spot, corresponding to the same R_f value of tadalafil, and the other suspected spots on the TLC plate were also scraped and extracted with 95% ethanol (3 mL) by ultrasonication for 5 minutes, respectively. The supernatant was measured on a Cary 300 UV-visible spectrophotometer and a Quattro Ultima LC/MS/MS, respectively.

(II) *Purification and Identification of Compound **2***

Powder of sample (25.0 g) was removed from twenty-five capsules and extracted with 95% ethanol (250 mL) by ultrasonic shaking as described above. After filtration, the filtrate was evaporated under reduced pressure to obtain the extract (3.6 g). The extract was redissolved in methanol (20 mL) at room temperature. The insoluble part was collected and recrystallized with methanol to yield a yellowish powder (compound **2**, 311 mg). The structure of compound **2** was determined by the measurements of NMR, CD and mass spectroscopy.

RESULTS AND DISCUSSION

Through TLC screening, two suspected spots were scraped and analyzed with UV and LC/MS/MS; one was identified as tadalafil by direct comparison with tadalafil reference standard; and the other spot showed UV spectrum similar to that of tadalafil, thus, it is suspected to be a tadalafil analogue. This unknown compound **2** was isolated and its structure was determined by comparing to the NMR, CD and mass spectra of tadalafil reference standard.

Compound **2** was obtained as a yellowish powder with a melting point at 265-268°C. The IR spectrum showed two apparent peaks at 1670 and 1654 cm^{-1} indicated the existence of carbonyl group**.** Compound **2** was optically active, $[\alpha]_D^{25}$ +85° (*c* 0.2, MeOH), compared with tadalafil, $[\alpha]_D^{25}$+65° (*c* 0.2, MeOH), indicating that both tadalafil and compound **2** were dextrorotation. UV spectrum (in ethanol) of compound **2** showed λ_{max} 291.00, 284.00, and 221.00 nm, which was very similar to that of tadalafil (Figure 2). As shown in Figure 3, the DIP-MS spectrum of compound **2** showed $[M^+]$ ion at m/z 390.0, whereas tadalafil showed its $[M^+]$ ion at *m/z* 389.0 ($C_{22}H_{19}N_3O_4$); except this, other ion peaks were almost the same. Also, their daughter ion spectra were almost the same, except for one amu difference. These aspects implied that their structures were similar. Figure 4 shows the positive daughter ion spectra of tadalafil

and compound **2**. The ESI^+-LC/MS/MS of compound **2** showed at *m/z* 391.6 ($[M+H]^+$) and its major mass fragment created by daughter ion scan was at *m/z* 269.5 ($[M+H-122]^+$), 122 amu equivalent to benzodioxole molecule ($C_7H_6O_2$), while a daughter ion from a negative collision appeared at m/z 389.6 ($[M-H]^-$). In short, the mass data of compound **2** revealed a molecular ion m/z 390, correlated to its molecular formula: $C_{21}H_{18}N_4O_4$.

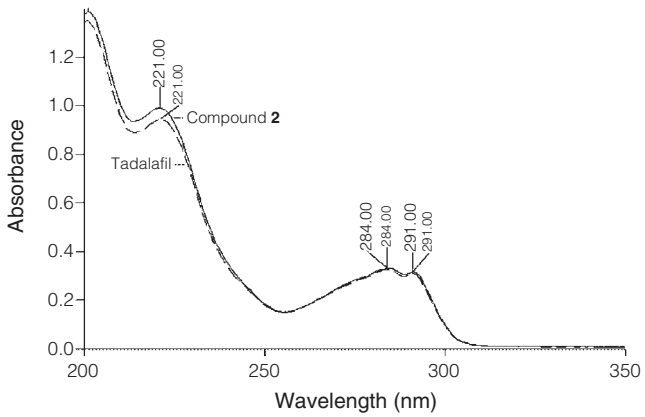

Figure 2. The UV spectra of tadalafil and compound **2**.
Tadalafil and compound **2** were dissolved in ethanol respectively, and diluted with ethanol to obtain the final concentration (10 g/mL).

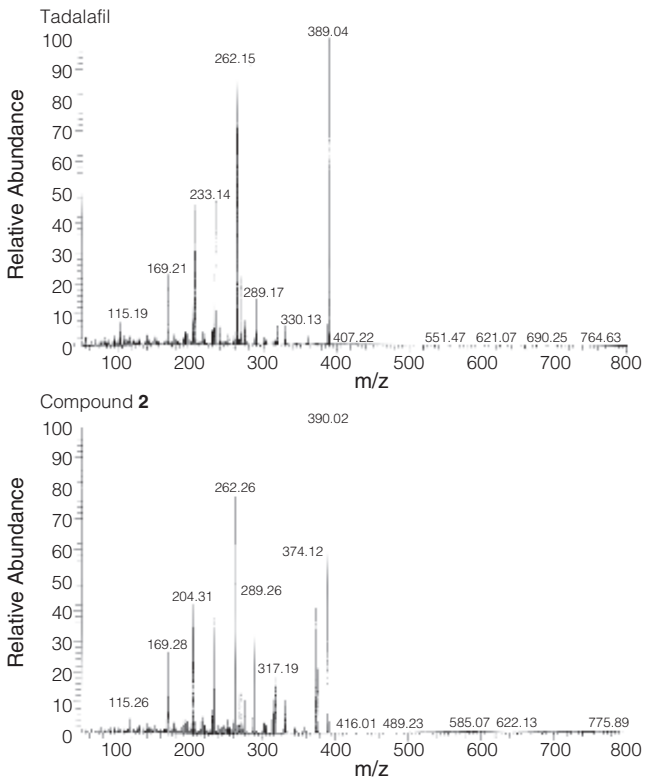

Figure 3. The mass spectra of tadalafil and compound **2**.

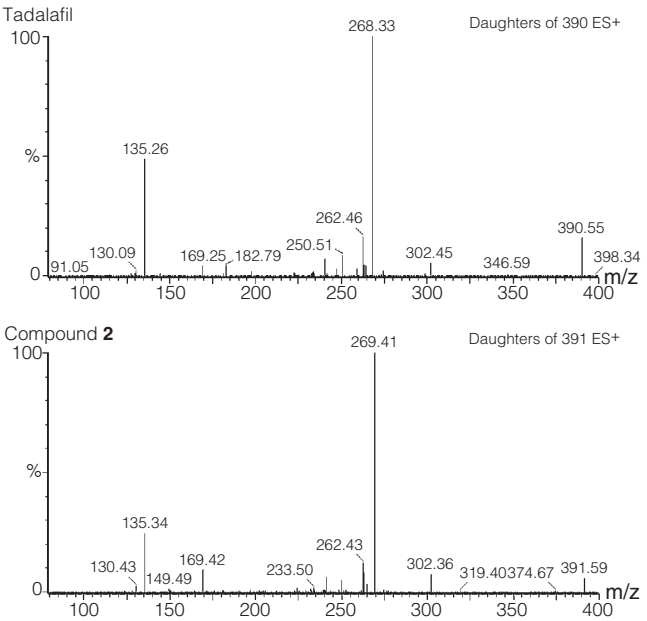

Figure 4. The LC/MS/MS fragmentation spectra of tadalafil and compound **2**.

The ^1H NMR spectrum of compound **2** showed a two-proton signal at δ_H 5.11 (2H, *s*) and the signal of methyl group of tadalafil at δ_H 2.92 (3H, *s*) disappeared (Figure 5)**,** together with the ^{13}C NMR spectrum of tadalafil showed a primary carbon signal at δ_C 32.8 which disappeared in that of compound **2,** those information indicated that the *N*-methyl group was replaced (Figure 6). The ^{13}C NMR and DEPT spectra (Figure 7) of compound **2** showed 21 carbons, including 3 secondary carbons, 9 tertiary and 9 quaternary carbons. This result also supported that the *N*-methyl group of tadalafil was replaced.

The homo-COSY data of compound **2** (Figure 8) showed the same correlations as tadalafil, except that a two-proton signal at δ_H 5.11 of compound **2** had no correlation peak, which implied that there was no hydrogen in the neighborhood of δ_H 5.11. With the correlation signals of δ_H 2.99/δ_H 3.57 and δ_H 3.57/δ_H 4.43, as well as δ_H 3.97/δ_H 4.26, coordinating with coupling constants, the chemical shifts of H-12, H-12a, and H-3 could be easily assigned. Besides, the chemical shift at δ_H 6.09 (1H, *s*) was assigned as H-6. The HMQC data (Figure 9) also indicated that the two-proton signal at δ_H 5.11 did not exist a correlation to carbon, supposing these two protons might be linked to a heteroatom-nitrogen. The HMBC data (Figure 10) showed one significant correlation of δ_C 164.6/δ_H 5.11, supporting the amino group being linked to the *N2*-position of the piperazinedione ring. From the above data, it was suggested that the structure of compound **2** possessed an amino group substitution in the piperazinedione ring.

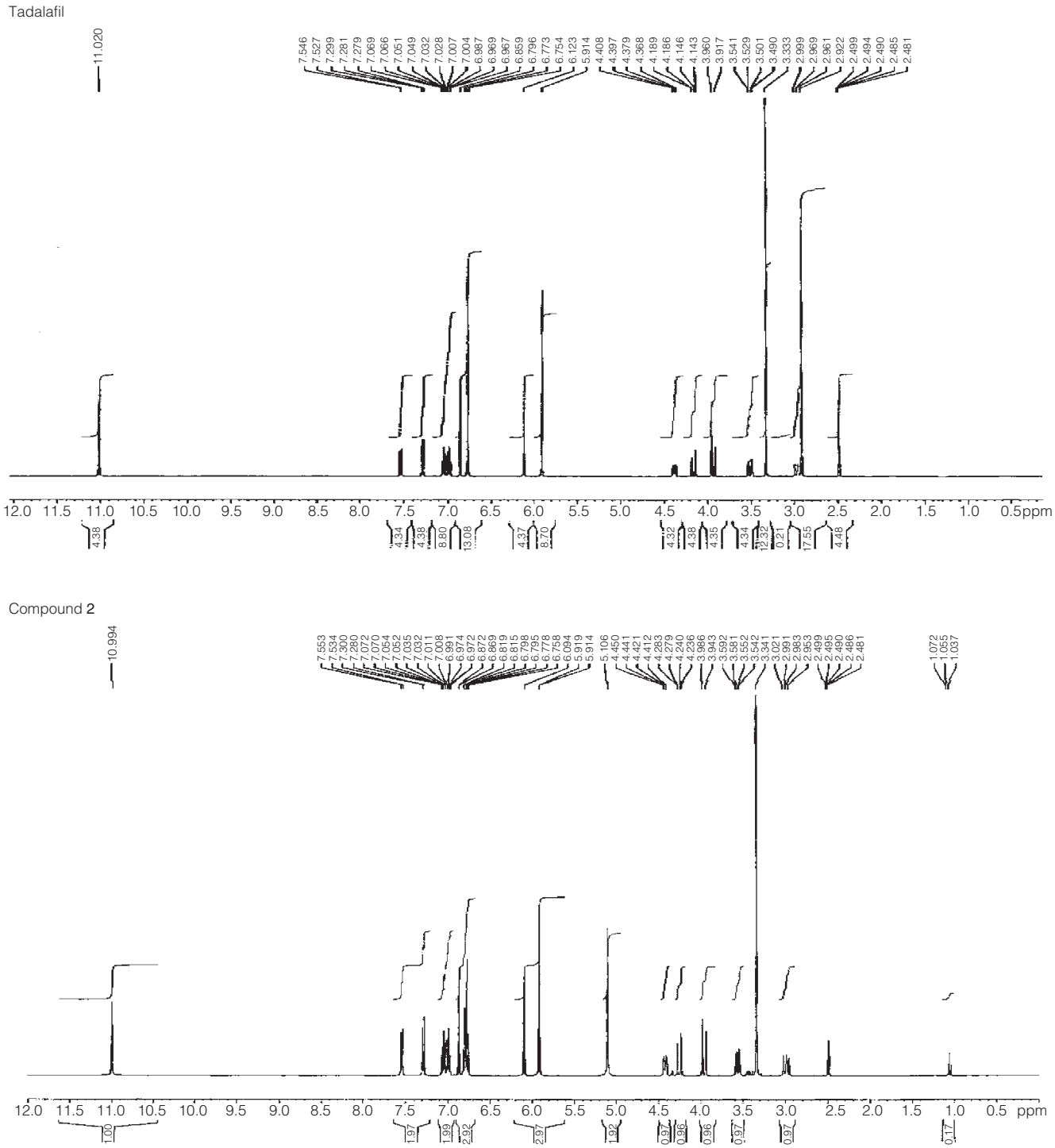

Figure 5. The ^{1}H NMR spectra of tadalafil and compound **2**.

The ^{1}H NMR spectrum showed that the signals at 5.91 (2H, *d, J* = 2 Hz), 6.78 (1H, *d*, *J* = 8.0 Hz), 6.81 (1H, *dd*, *J* = 8.0, 1.2 Hz), and 6.87 (1H, *d*, *J* = 8.0 Hz) were assigned to the methylene protons and the aromatic protons of the benzodioxole, respectively. The aromatic protons and carbons of indole moiety were further elucidated through the coupling constants, homo-COSY, and HMBC data. Hence, the signals of ^{1}H NMR and ^{13}C NMR spectra of compound **2** could be clearly assigned. Table 1 summarizes the NMR data of compound **2**. The absolute configuration of compound **2** was determined by CD spectrum. It showed negative

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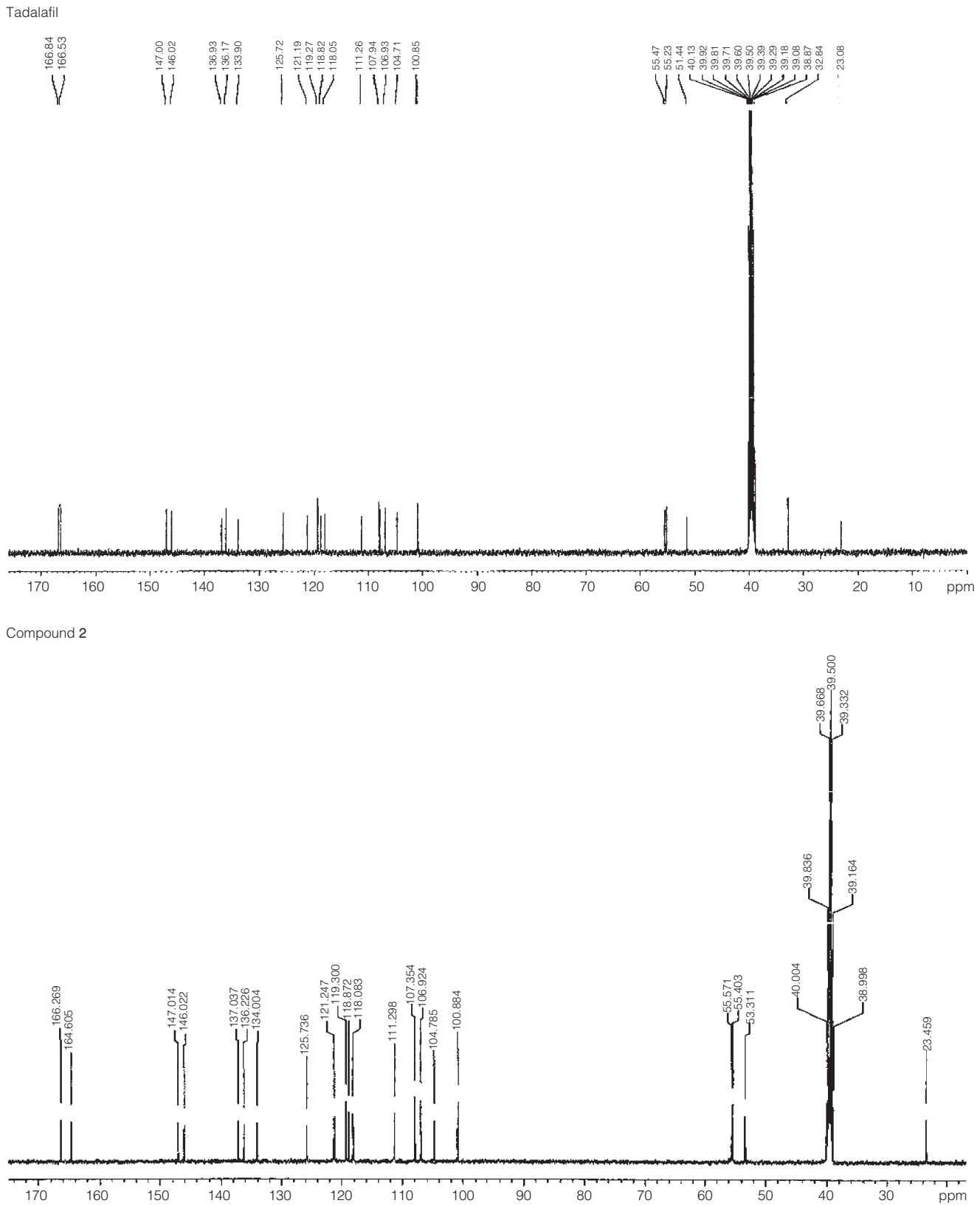

Figure 6. The ^{13}C NMR spectra of tadalafil and compound **2**.

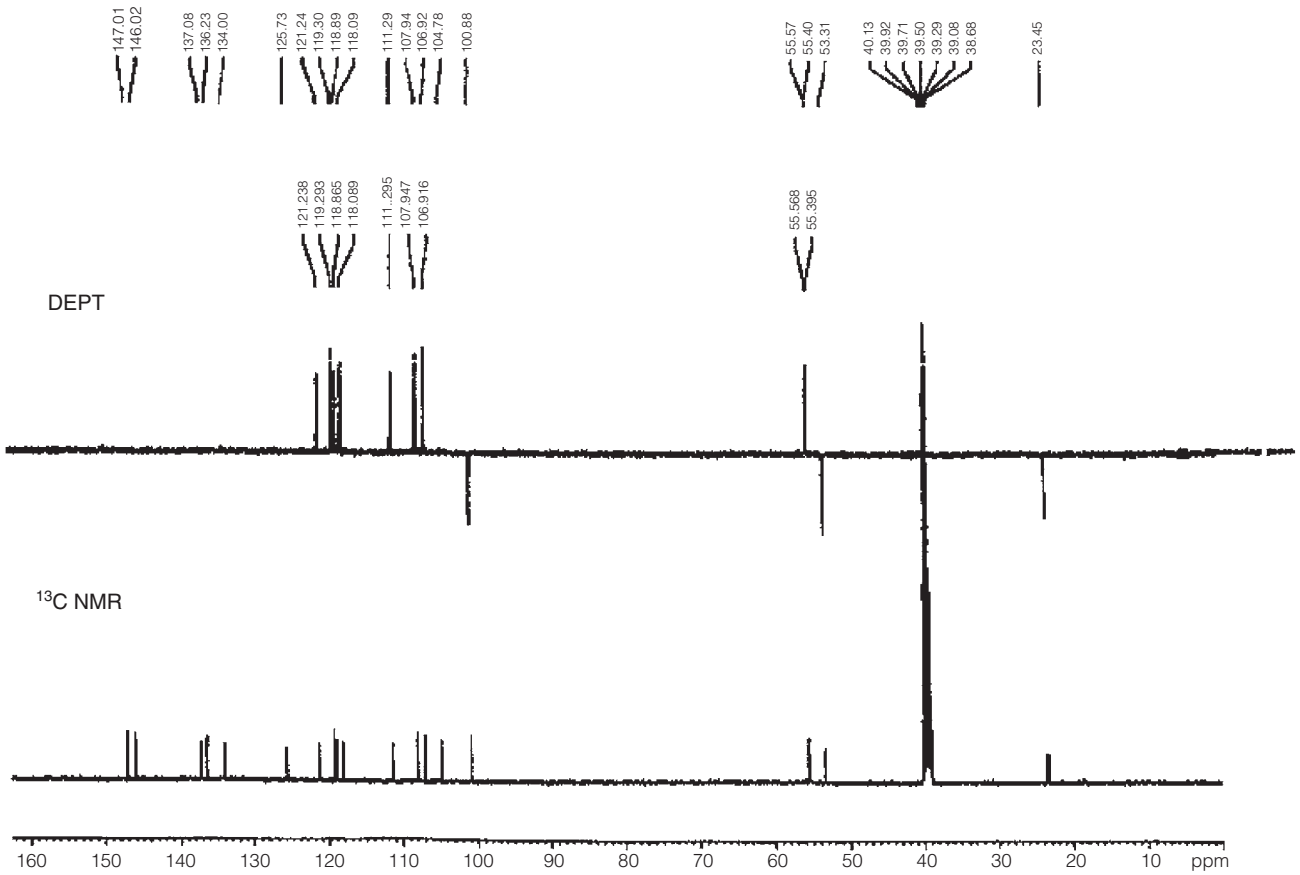

Figure 7. The ^{13}C NMR and DEPT spectra of compound **2**.

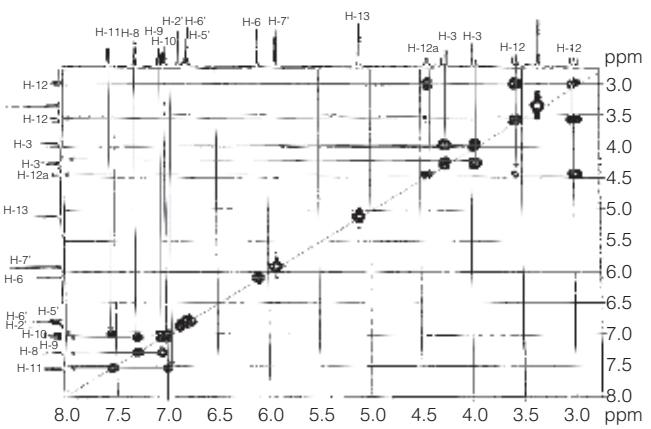

Figure 8. The COSY spectrum of compound **2.**

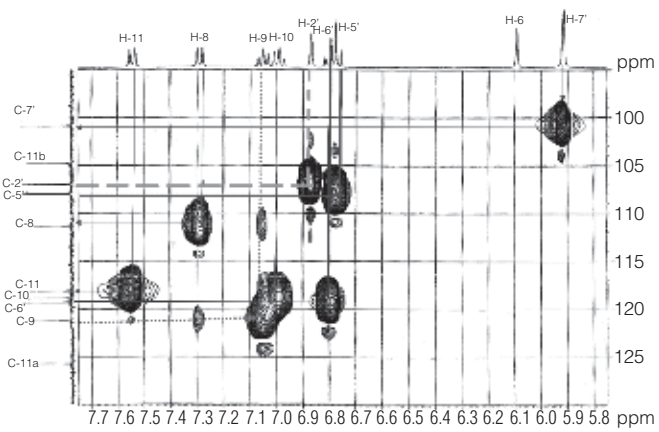

Figure 9. The HMQC spectrum of compound **2.**

cotton effect between 260 and 300 nm (289 nm △ε -9.3, 296 nm △ε -8.9), which was similar to that of tadalafil (289 nm △ε -15.0, 296 nm △ε -14.5). Hence, the H-6 and H-12a were R configuration according to the configuration of tadalafil.

Based on the mass spectrum and NMR spectra data, the structure of compound **2** was determined as (6*R*,12*aR*)-2-amino-6-(1,3-benzodioxol-5-yl)-2,3,6,7,12,12a-hexahydro-pyrazino[1',2':1,6]pyrido[3,4-*b*]indole-1,4-dione. This compound had been found in other countries and named as aminotadalafil[19-21].

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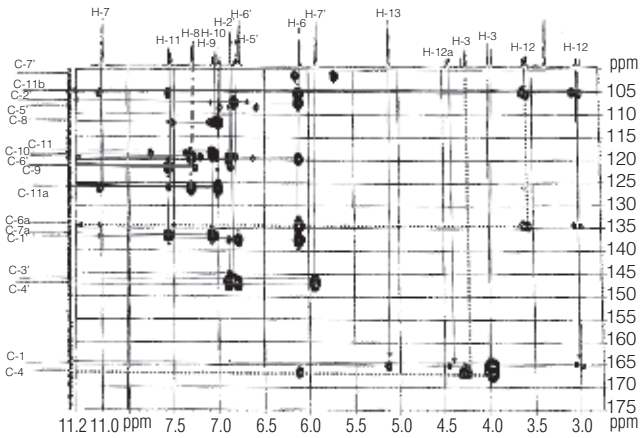

Figure 10. The HMBC spectrum of compound **2**.

CONCLUSION

In this paper, compound **2** was isolated by solvent extraction and recrystallization, and the structure was determined as aminotadalafil by elucidation of NMR correlation and mass spectrum. Compound **2** is a tadalafil analogue adulterated in the dietary supplements and reports about its biological activity are few. The basic structure of compound **2** is the same as the main structure of tadalafil. According to structure-activity relationship, its biological activity might be similar to that of tadalafil; therefore, it has been listed as a suspected item in the screening of aphrodisiacs for protection of public health. In the light of our recent survey, we suppose that more and more unknown analogues might be added to the dietary supplements and the possible consequences should not be overlooked.

Table 1. NMR data of compound **2** (DMSO-*d*6)

No.	^{1}H (ppm)	^{13}C (ppm)	COSY	HMBC
1		164.6		H-13/H-12a/H-3/H-12
3	3.97 (1H, *d,* J = 17.2 Hz)	53.3	H-3	
	4.26 (1H, *d,* J = 17.2 Hz)		H-3	
4		166.2		H-3
6	6.09 (1H, *s*)	55.6		
6a		134.0		H-6/H-12/H-7
7	10.99 (1H, *s*)			
7a		136.2		H-11/H-9
8	7.29 (1H, *d*, J = 8.0 Hz)	111.3	H-9	H-10/H-11
9	7.04 (1H, *ddd,* J = 7.3, 7.3, 0.9 Hz)	121.2	H-8/H-10	H-8/H-11
10	6.99 (1H, *ddd*, J = 7.3, 7.3, 0.9 Hz)	118.9	H-9/H-11	H-8
11	7.54 (1H, *d,* J = 7.6 Hz)	118.0	H-10	H-9
11a		125.7		H-7/H-11/H-8/H-10
11b		104.9		H-6/H-7/H-11/H-12
12	2.99 (1H, *dd,* J = 15.2, 12.0 Hz)	23.5	H-12/H-12a	
	3.57 (1H, *dd,* J = 15.8, 4.2 Hz)		H-12/H-12a	
12a	4.43 (1H, *dd,* J = 11.6, 3.6 Hz)	55.4	H-12	H-15
13	5.11 (2H, *s*)			
1’		137.1		H-6/H-2’/H-5’
2’	6.87 (1H, *d,* J = 1.2 Hz)	106.9		H-6/H-6’
3’		146.0		H-2’/H-5’/H-7’
4’		147.0	H-20	H-6’/H-7’
5’	6.78 (1H, *d,* J = 8.0 Hz)	107.9	H-6’	H-6’/H-6
6’	6.81 (1H, *dd*, J = 8.0, 1.2 Hz)	119.3	H-5’	H-6/H-2’
7’	5.91 (2H, *s*)	100.9		

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