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Isolation and Identification of a Novel Sildenafil Analogue Adulterated in Dietary Supplements

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ABSTRACT

A suspected sildenafil analogue (**1**) was firstly detected from dietary supplements, which claimed for energy enforcement, by TLC and UV method. The UV spectrum of this suspected compound showed close similar pattern to that of acetildenafil (**2**, sildenafil analogue). In order to identify this compound, separation and purification were carried out as well as structure determination was conducted by various NMR techniques, mass spectrum and IR. This new compound **1** had an acetyl group instead of sulfonyl-*N*-methylpiperazine moiety in sildenafil (**3**), and its structure was determined as 5-[2-ethoxy-5-acetyl-phenyl]-1-methyl-3-*n*-propyl-1,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one (molecular formula $C_{19}H_{22}N_4O_3$, molecular weight 354). So far no related report and literature to describe its pharmacological action and to provide related safety evaluation of this compound was published. The above results revealed that this compound was illegally adulterated into the dietary supplements, and has thus been included in our routine analysis list for public health.

Key words: dietary supplements, NMR, mass spectrum, IR, sildenafil, acetildenafil, sildenafil analogue

INTRODUCTION

A dietary supplement is a product that supplies one or more essential nutrients missing from the diet, the nutrients include vitamins, minerals, herb or other botanicals, amino acids and so on. Consumption of dietary supplements becomes more and more popular around the world. Unfortunately, the adulteration of undeclared synthetic chemical compounds in the dietary supplements increases gradually according to literatures and our recent surveys [(1-12)].

The adulteration of synthetic chemical compounds in dietary supplements is prohibited by law in Taiwan for public health and Bureau of Food and Drug Analysis (BFDA) is in charge of monitoring marketed products. The first rank of submission for investigation categorized by indication is so-called energy enforcement, the term of energy enforcement labeled on the product implicates that it can improve male sex ability after taking it.

Approved drugs for male erectile dysfunction (ED), such as sildenafil (**3**), tadalafil (**4**) and vardenafil (**5**), have been adulterated into the dietary supplements and their analogues have been detected, too. Figure 1 illustrates the structures of those approved drugs and their analogues. Although some adulterants were approved drugs, clinical data have indicated their adverse effects and contraindications[(13-15)]. In case a dietary supplement is adulterated, it might endanger users' health due to the lack of indication and quantity information of adulterants. Especially, consumers with cardiovascular disease who take nitrates and dietary supplements adulterated with sildenafil together might result in serious side effects.

In this case, a dietary supplement with certain suspected chemical drugs was submitted for analysis. A suspicious compound with similar UV spectrum pattern to that of acetildenafil (**2**, sildenafil analogue) was detected in the preliminary screening test by TLC and UV method followed by a series of extraction, separation, and purification to obtain a pure compound and to elucidate its structure. Meanwhile, a method for simultaneous analysis of sildenafil and its analogues by LC/MS/MS was developed, which can be afforded as routine analysis.

MATETIALS AND METHODS

I. *Sample and Chemicals*

The purple-light yellow capsules of a dietary supplement containing brown powder were submitted by the local health bureau for inspection. Acetonitrile, metha-

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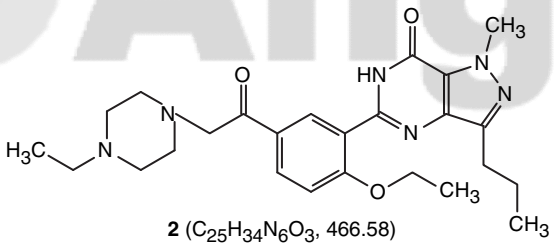
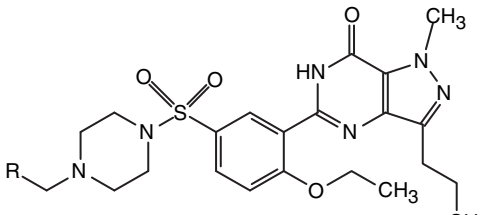
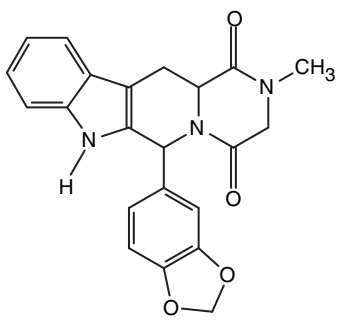


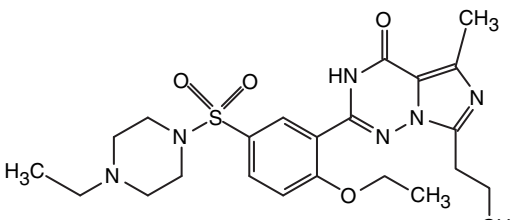
2 ($C_{25}H_{34}N_6O_3$, 466.58)



3 R=H ($C_{22}H_{30}N_6O_4S$, 474.59)
4 R=CH_3 ($C_{23}H_{32}N_6O_4S$, 488.60)
5 R=CH_2OH ($C_{23}H_{32}N_6O_5S$, 504.60)



6 ($C_{22}H_{19}N_3O_4$, 389.41)



7 ($C_{23}H_{32}N_6O_4S$, 488.60)

Figure 1. Structures of synthetic chemical drugs and analogues (with related molecular formulas and weights) adulterated in dietary supplements for energy enforcement had been identified in our laboratory. acetildenafil (**2**), sildenafil (**3**), homosildenafil (**4**), hydroxyhomosildenafil (**5**), tadalafil (**6**), vardenafil (**7**). See compound **1** in Figure 3.

nol, hexane 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 from E. Merck (Darmstadt, Germany). Ethanol (95%) was from Taiwan Tobacco and Liquor Corporation (Taipei, Taiwan).

II. *Instruments*

The NMR spectra were recorded on a Varian Unity Inova-500 spectrometer. The EIMS spectra were acquired using an Agilent 5973 GC/MS spectrometer at 70 eV. CIMS spectra were recorded on a Thermo Polaris Q with a DIP mass spectrometer. IR spectra were acquired on a Jasco FT/IR-480 plus Fourier Transform Infrared Spectrometer. The melting point was determined using a Fisher-Johns melting point apparatus. 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.

III. *Methods*

(I) *Extraction and Isolation*

Sample powder (4.8 g) was taken out from twelve capsules and extracted with 95% ethanol (75 mL) by ultrasonic shaking at room temperature for 30 minutes for three times. After filtration, the filtrate was combined and evaporated under reduced pressure to obtain the residue (710.8 mg) which was subjected to a open column (30 cm × 16 mm I.D.), packed with silica gel 60 (21 g, 0.063-0.2 mm, E. Merck), eluted with *n*-hexane and *n*-hexane-ethyl acetate (1:1) successively, each 12 mL eluate was collected into a tube. A mixture of *n*-hexane-ethyl acetate (1:1, v/v) was used as TLC mobile phase and the

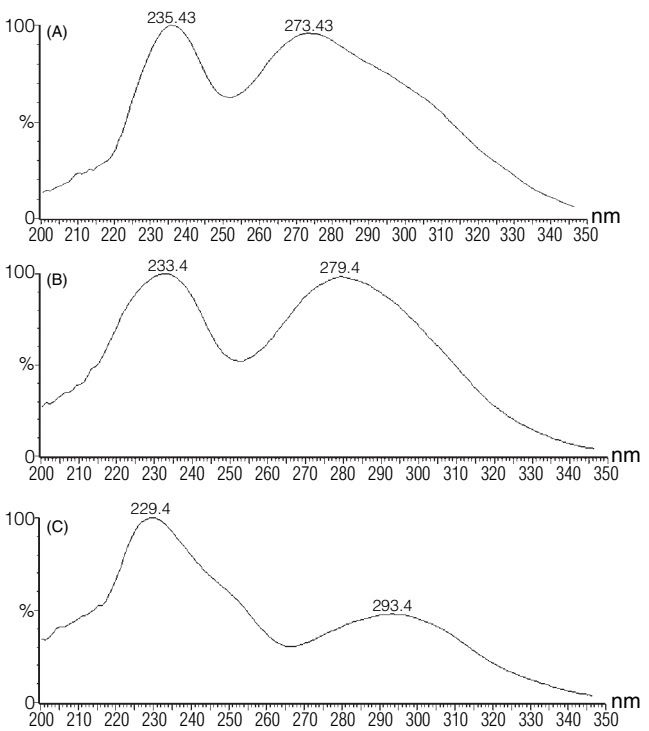


Figure 2. UV spectra of compound **1** (A), acetildenafil (**2**, B) and sildenafil (**3**, C). Compound **1**, **2** and **3** were dissolved in ethanol respectively, and diluted with ethanol to obtain the final concentration 10 μg/mL, which was injected into the LC/MS/MS. The LC/MS/MS conditions performed as describing in the section of materials and methods-Methods (III) Analysis of compound **1** by LC/MS/MS.

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target compound (R_f : 0.32) was visually detected using Dragendorff's reagent.

Three fractions of *n*-hexane-ethyl acetate (1:1, v/v) were collected according to TLC profiles. Fraction II (tube number 40-58) was separated into two sub-fractions (II-1, II-2). Sub-fraction II-2 was further purified by standing for overnight and then filtration to get white powder (Compound **1**, 16.7 mg).

(II) *Structure Identification*

The obtained compound **1** (Figure 3) was dissolved in DMSO-d_6 and subjected to 1D and 2D NMR spectroscopic analysis (^{1}H, ^{13}C, DEPT, homo-COSY, HMBC and HMQC). GC-MS coupled with positive electron impact (EI^+) and positive chemical impact (CI^+) modes was used to determine its molecular weight.

(III) *Analysis of Compound **1** by LC/MS/MS*

Compound **1** was dissolved in ethanol (1 mg/mL) and diluted to obtain the final concentration 10 μg/mL, which was subsequently analyzed by LC/MS/MS. The LC of LC/MS/MS was performed on a Cosmosil 5C18-AR (15 cm × 4.6 mm I.D., 5 μm) reverse phase column (Nacalai Tesque, Kyoto, Japan) with the injection volume 10 μL. The mobile phase was acetonitrile (A) and 1% acetic acid (B) with gradient elution (A was increased from 10% to 90% in 20 minutes and then A was reduced to 10% in next 5 minutes). The flow rate was 0.5 mL/min. The split ratio of column effluent to PDA detector and MS detector was about 4:1. An approximate 0.1 mL of column effluent was introduced into tandem mass spectrometer through the positive electrospray ionization (ESI^+) interface. The ESI^+-MS/MS conditions were as follows: ion source temperature, 120°C, desolvation temperature, 350°C, cone voltage, 100 V, collision energy, 25 V, and capillary voltage, 3KV.

RESULTS AND DISCUSSION

Through column chromatographic techniques, compound **1** was obtained as white powder, melting point: 200~201°C, UV spectrum: λ_{max} 273.4 and 235.4 nm. Its

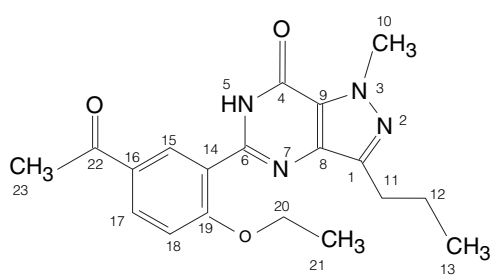

Figure 3. Structure of compound **1** with key atoms numbered for subsequent spectral analysis.

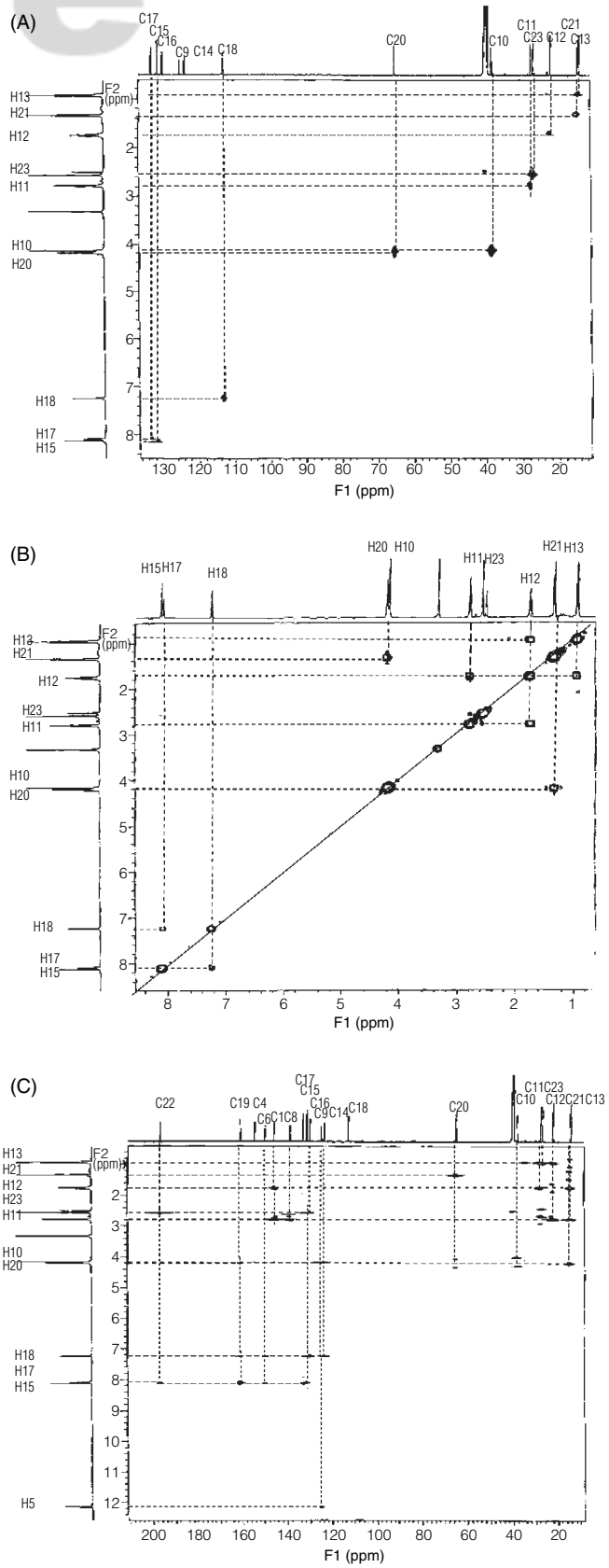

Figure 4. The HMQC (A), homo-COSY (B) and HMBC (C) spectra of compound **1**.

UV spectrum (Figure 2A) was similar to that of compound **2** (acetildenafil, Figure 2B), but obviously was different from that of compound **3** (sildenafil, Figure 2C).

Compound **1** showed its $[M+1]^+$ ion at m/z 355, accompanied with an adduct ion at m/z 383 ($[M+C_2H_5]^+$) by CI-MS. EI-MS analysis showed the base peak of **1** at m/z 354 and two major fragment ions compared to the intensity of the base peak were 80.3 and 50.5% at m/z 326 and 339, which revealed molecular ion m/z 354 as well as m/z 326 and 339 corresponding to $[M-28]^+$ and $[M-15]^+$. The above data revealed a molecular ion 354, and was referred to molecular formula $C_{19}H_{22}N_4O_3$ (Calculated molecular weight 354.4089).

Comparing to the IR spectrum of **3**, two apparent absorption bands at 1347 and 1169 cm^{-1} for a sulfonyl group were diminished. Instead, a band at 1699 cm^{-1} suggested the presence of an acetyl group in this compound.

The structure of **1** was identified by comparison of its data (^1H and ^{13}C NMR) with the NMR data of **2** and **3**[2,3,7-9]. Basically, ^1H-NMR and ^{13}C-NMR of them were similar. The major difference of ^{13}C-NMR spectra between **1** and **2** as well as **3** was that two secondary carbon signal during δ_C 45.2 to 53.1 of **2** and **3** disappeared, which indicated *N*-piperazine moiety of **2** and **3** was replaced. The characteristic ^{13}C-NMR spectrum of **1** contained one obvious carbonyl carbon at δ_C 196.1 which was similar to that of **2**, and one primary carbon signal at δ_C 26.5. The ^1H-NMR spectrum of **1** showed one singlet methyl group at δ_H 2.56, instead of methylene protons at δ_H 2.37, 2.50 of compound **3** and δ_H 2.64, 2.96 of compound **2**. The HMQC spectroscopy data (Figure 4A) of **1** showed a significant correlation of δ_C 26.5/δ_H 2.56 (C23/H23). The homo-COSY (Figure 4B) and HMBC spectra (Figure 4C) showed the similar correlation as that of **2** and **3** except the *N*-piperazine moiety. The HMBC data provided the similar correlation of δ_H 2.56/δ_C 196.1, 129.3 (H23/C23, C16); δ_H 8.12, 8.08/δ_C 196.1 (H15, H17/C23). Based on these 2D data and compared with the data of **2** and **3**, the structure of **1** was confirmed of bearing a substitution of acetyl group at phenyl moiety. Table 1 summarizes the NMR data of **1**.

According to the mass spectra, IR spectrum and NMR spectra data, the structure of compound **1** was concluded as 5-[2-ethoxy-5-acetyl-phenyl]-1-methyl-3-*n*-propyl-1,6-dihydro-*7H*-pyrazolo[4,3-*d*]pyrimidin-7-one. Compound **1**, a new sildenafil analogue, had an acetyl group instead

Table 1. NMR data of compound 1 (DMSO- d_6)

No.	^1H (ppm)	^{13}C (ppm)	COSY	HMBC
1		144.9		H-11/H-12
4		153.8		
5	12.20 (1H, *br.s*)			
6		148.9		H-15
8		137.9		H-11
9		124.3		H-10
10	4.16 (3H, *s*)	37.8		
11	2.78 (2H, *t*, J = 7.5 Hz)	27.1	H-12	H-12/H-13
12	1.74 (2H, *m*)	21.7	H-11/H-13	H-11/H-13
13	0.94 (3H, *t*, J= 7.3 Hz)	13.8	H-12	H-11/H-12
14		123.0		H-18
15	8.12 (1H, *d*, J=2.0 Hz)	130.7		H-17
16		129.3		H-23/H-18
17	8.08 (1H, *dd*, J=9.0, 2 Hz)	132.4	H-18	H-15
18	7.24 (1H, *d*, J= 8.5 Hz)	112.4	H-17	
19		160.1		H-15/H-17/H-18/H-20
20	4.20 (2H, *q*, J= 6.8 Hz)	64.6	H-21	H-21
21	1.34 (3H, *t*, J= 7.0 Hz)	14.3	H-20	H-20
22		196.1		H-15/H-17/H-23
23	2.56(3H, *S*)	26.5		

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of sulfonyl-*N*-methylpiperazine moiety in sildenafil.

In order to detect sildenafil and its analogues simultaneously, an LC condition was monitored to find that gradient elution could surpass longer retention of compound **1** in isocratic elution[3,11]. In this study, sildenafil, acetildenafil and compound **1** were resolved significantly in this LC/MS/MS condition within 20 min. At retention time 17.3 min compound **1** showed its positive and negative mass scanning spectra at m/z 355.7 ($[M+H]^+$) and 353.6 ($[M-H]^-$), to establish a possible formula $C_{19}H_{22}N_{46}O_3$ (M.W. 354.4), 112 amu less than that of compound **2**, which is equivalent to $C_6H_{12}N_2$. The positive daughter ion spectra of **1** showed at m/z 355 ($[M+H]^+$), 327, 285, 298 and 313; its speculative fragmentation pathway were depicted in Figure 5. The daughter ion from negative collision appeared at m/z 325 ($[M-H-28]^-$), which attributed to the breakdown of the *O*-ethyl group. The above obtained daughter ion spectra were established into homemade database as reference for the future screening and identification works.

CONCLUSIONS

In this paper, a new sildenafil analogue **1** was isolated by column chromatography and its structure was determined by NMR, MS spectrum and IR spectrum. Although compound **1** was not approved as a medicine and has not been reported on the literature, it has been listed as a suspicious adulterant in aphrodisiacs screening in our laboratory. Besides, we also developed a new LC/MS/MS method to detect and shorten the analytical time of this kind of compounds. Therefore, it is more suitable to increase the demand of detecting aphrodisiacs adulterants in dietary supplements.

While this new analogue was detected and identified, we supposed that more and more sildenafil analogues might be synthesized and distributed in the market. Hence, continuous monitoring and investigating the diverse compounds adulterated in herbal supplements together with risk assessments of analogues were requisite, in the view of public health.

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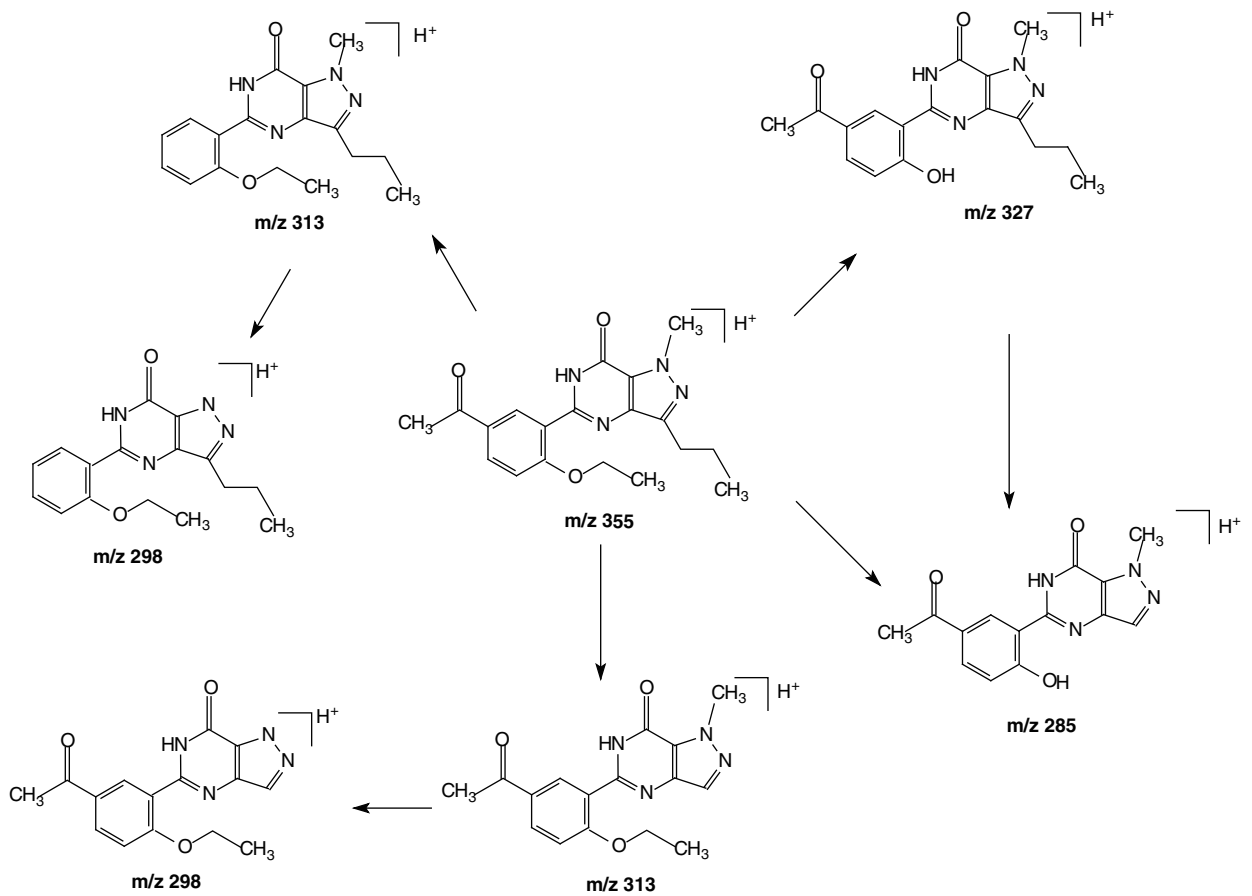

Figure 5. The speculative fragmentation pathways of compound **1** based on LC/MS/MS with positive electrospray mode.

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