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Identification of a new sildenafil analogue in a health supplement

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ABSTRACT

A sildenafil analogue was detected and isolated from a health supplement claimed for human use. The structure of this new analogue was elucidated using LC–UV, LC–Orbitrap-MS, IR spectroscopy, 1D and 2D NMR. It was characterized as dithio-desmethylcarbodenafil containing 2 thiocarbonyl groups instead of 2 carbonyl groups, and 4-methyl substitution, on the piperazine ring, rather than 4-ethyl substitution, when compared to sildenafil.

1. Introduction

Adulterants are frequently detected in health supplements, which are meant for sexual enhancement. The adulterants commonly employed are sildenafil (Viagra®), vardenafil (Levitra®), tadalafil (Cialis®) and their analogues. More than 20 analogues have been found and reported [1–15]. In 2010, the most popular adulterated analogue was sulfoaildenafil [16], which was detected in a few products submitted to Health Sciences Authority for testing. A new nitrosated prodrug of aildenafil was also reported recently [17].

In this study, a new sildenafil analogue named as dithio-desmethylcarbodenafil was isolated from a health supplement which was claimed to contain only red algae and intended to be introduced into South East Asia market. The structure was determined using NMR, MS and IR.

2. Experimental

2.1. Materials

Chloroform-d ($CDCl_3$) was purchased from Merck (Germany). HPLC grade methanol, HPLC grade acetonitrile, AR grade ethyl acetate and diethyl ether were obtained from Labscan Asia Co. Ltd. Water was purified to 18.2 mΩ cm using a ELGA Purelab Ultra system (Vivendi Water System Ltd., USA). Sodium dihydrophosphate monohydrate ($NaH_2PO_4{\cdot}H_2O$) was supplied by Merck (Darmstadt, Germany). Formic acid was purchased from Sigma–Aldrich (Steinheim, Germany). Desethylacetildenafil and Carbodenafil reference standards were purchased from TLC PharmaChem Inc. (Canada). A health supplement sample in tablet form claimed to contain red alga was submitted by a client for testing.

2.2. Extraction and isolation of new analogue

The process used by Venhuis et al. [10] was followed to isolate the new analogue. A few tablets were pounded into powder. 3 g of the yellow powder were transferred to a glass sintered dropping funnel and subsequently washed with Et_2O (3 × 10 ml), EtOAc (3 × 10 ml) and ACN (3 × 10 ml). The combined washing of EtOAc and ACN was evaporated to dryness using Rotary Evaporator to yield yellow solid. The latter was recrystallized using hot EtOAc and about 50 mg faint yellow solid was obtained, mp 218.4–219.4 °C.

2.3. LC–UV analysis

The contents (0.4 g) of one capsule was dissolved in 10 ml methanol and ultrasonically extracted for 10 min followed by filtration. The filtrate was diluted 100 times with methanol and used for LC–UV analysis by Agilent 1100 series chromatograph with a diode array detector. The UV spectra from 200 nm to 400 nm were recorded on-line during the chromatographic run. The UV signals were monitored at 220 nm, 254 nm, and 280 nm. The LC analysis was carried out using a Hypersil BDS C_{18} column (200 mm × 4.6 mm, 5 μm particle size) with mobile phase A of 0.025 M sodium dihydrogenphosphate in water and mobile phase

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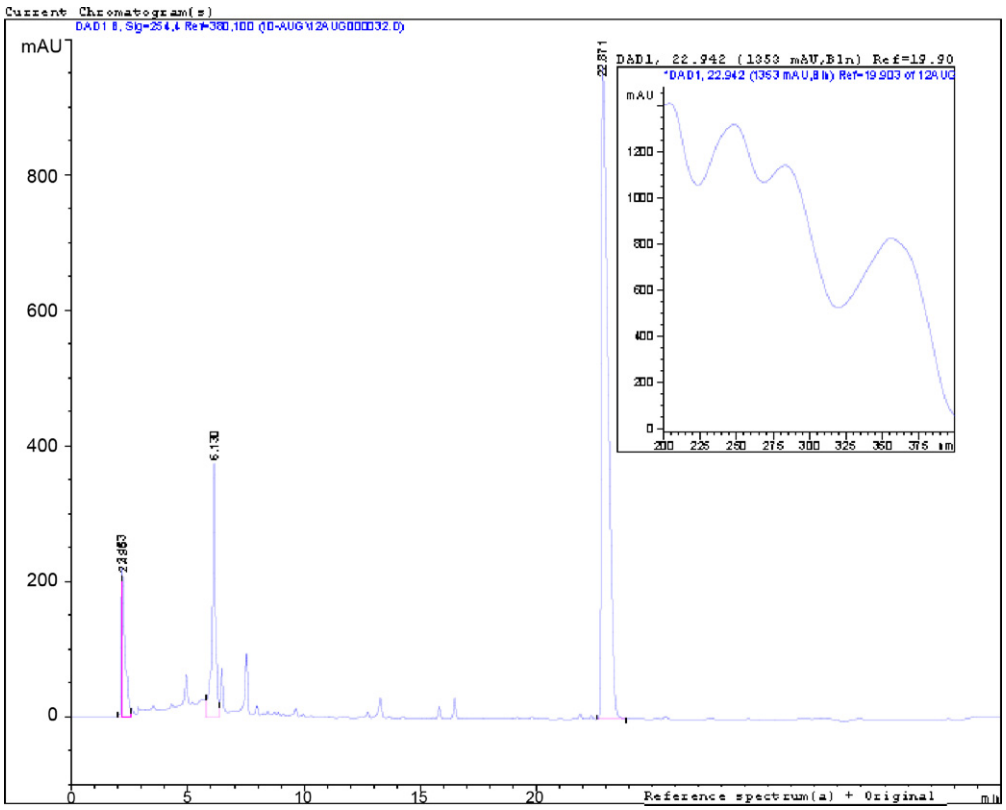


Fig. 1. HPLC chromatogram of a methanol extract of the health supplement at 254 nm. The insert shows the UV profile of the signal at 22.9 min.

B of acetonitrile. The mobile phase composition B was linearly ramped from 10% to 70% over 30 min and kept at 70% for 5 min. After that it was returned to 10% in 5 min and kept at 10% for another 5 min. The flow rate was 1 ml/min. The injection volume was 30 μl.

2.4. LC-LTQ Orbitrap FTMS analysis

The sample prepared for LC-DAD analysis was diluted 10 times with methanol for high resolution MS analysis. LC-LTQ Orbitrap XL FTMS included Accela HPLC, LTQ liner ion trap MS and Orbitrap FTMS, which was controlled by Xcalibur software (Version 2.0.7). The LC analysis was carried out using a Hypersil Gold ODS column (150 mm × 2.1 mm, 3 μm particle size) with mobile phase A of 0.1% formic acid in water and mobile phase B of 0.1% formic acid in acetonitrile. The mobile phase composition A was linearly ramped from 80% to 20% over 20 min and kept at 20% for 5 min. After that it was linearly returned to 80% in 5 min and kept at 80% for another 5 min. The flow rate was 0.2 ml/min.

The MS experiments were performed to get an accurate MS and MS^2 of the new analogue. The ionization source was operated in the positive ionization mode with the flow rates of the sheath gas and auxiliary gas at 60 and 10 arb. unit, respectively, capillary temperature at 250 °C, ion spray source voltage at 3 kV, source current at 100 μA, capillary voltage at 20 V, and tube lens at 70 V. The MS experiment had two scan events. Scan event 1 was used for full scan with scan range from 80 Da to 500 Da and resolution 30,000. Scan event 2 was used to produce MS^2 with scan range from 80 Da to 500 Da and the same resolution of 30,000. The ion with *m*/*z* 471.0 $[M+H]^+$ was selected as precursor. Collision energy was set at 35 V using High Energy Collision Dissociation (HCD).

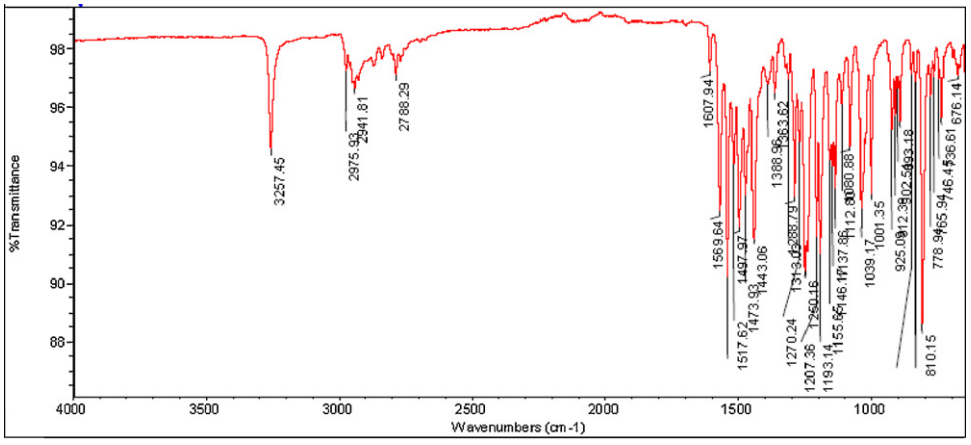


Fig. 2. Infrared spectrum of the new analogue.

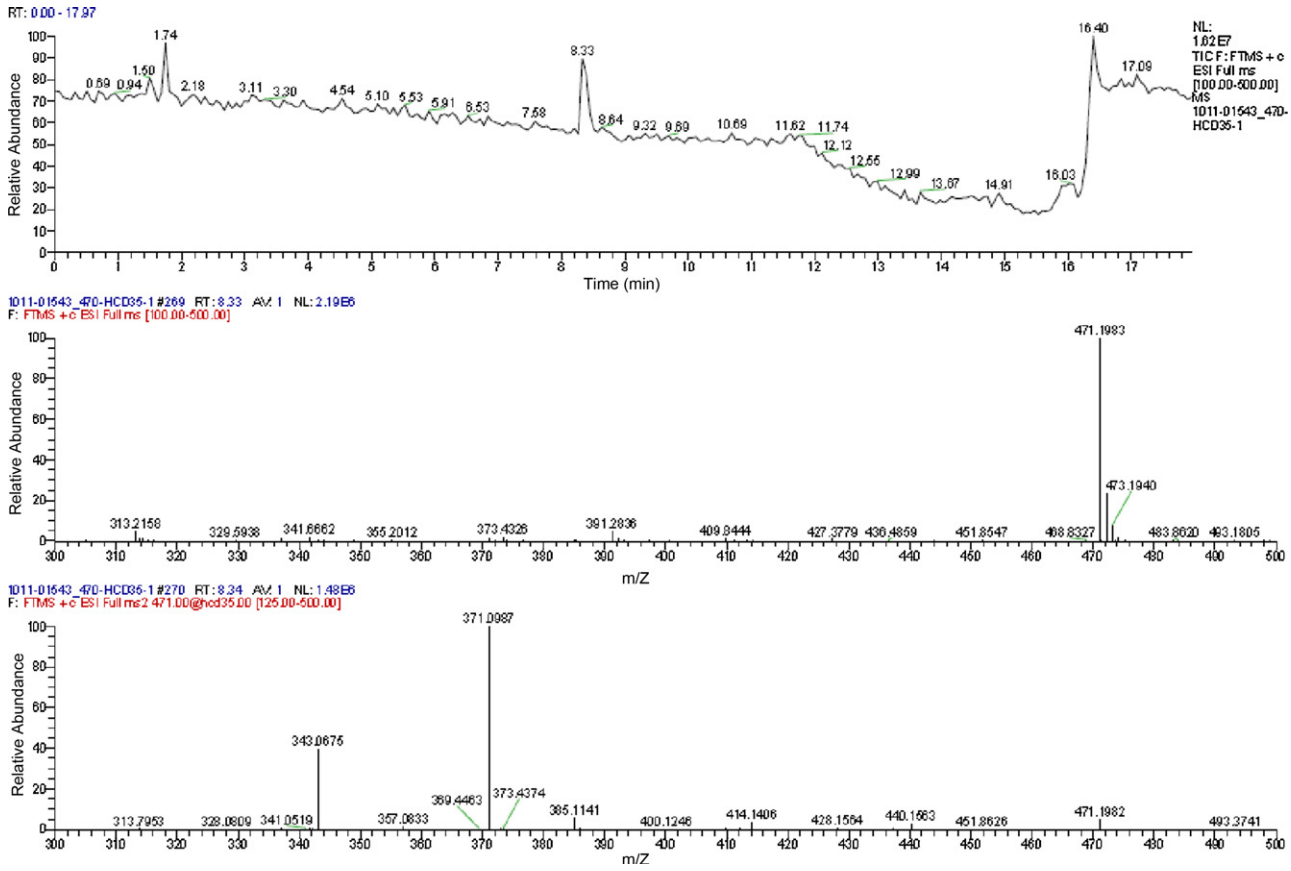


Fig. 3. MS^1 and MS^2 mass spectrum of new analogue in positive FTMS analysis.

2.5. NMR and IR analysis

About 15 mg of the isolated new analogue was dissolved in $CDCl_3$ for NMR analysis. ^1H, ^{13}C, COSY, HMQC, and HMBC spectra were obtained using Bruker DRX500 and AV500 [500 MHz (^1H) and 125 MHz (^{13}C)] (Rheinstetten, Germany) NMR spectrometers. DEPT-90 and DEPT-135 spectra were recorded on Bruker DPX300 [75 MHz (^{13}C)] NMR spectrometer. The solvent peak acted as the internal standard ($CDCl_3$, δ_H: 7.26 ppm, δ_C: 77.0 ppm). Coupling constants (J) were measured in Hertz (Hz) and chemical shifts were in ppm. IR spectrum was recorded over the spectral range 4000–600 cm^{-1} using Attenuated Total Reflection (ATR) in Thermo Scientific Nicolet 6700 FTIR spectrometer with a DTGS detector and OMNIC professional 7 software.

3. Results and discussion

3.1. LC–UV

The LC chromatogram of the health supplement and the UV spectrum are shown in Fig. 1. The chromatogram had few peaks with an outstanding one at 22.8 min. The UV spectrum of this new analogue had maximum absorbance at 258 nm, 285 nm and 356 nm. Library search showed that the UV spectrum of the new analogue was similar to that of desethylacetildenafil.

3.2. IR analysis

The infrared spectrum (Fig. 2) of the isolated compound was recorded. The IR spectrum revealed a pair of characteristic aromatic ring stretching absorptions ($\nu_{C=C}$) at 1569 cm^{-1} and 1498 cm^{-1}. The strong absorption at 810 cm^{-1} corresponded to the 1,2,4-trisubstituted aromatic ring. The stretching absorption of secondary amine (N–H) was at 3257 cm^{-1}. Aliphatic C–H stretches were detected from 2700 cm^{-1} to 3000 cm^{-1}. The absence of strong absorptions from 1600 cm^{-1} to 1800 cm^{-1} indicated that there was no carbonyl group in this new analogue. Meanwhile, the absorptions at 1250, 1240, and 1207 cm^{-1} suggested the presence of thiocarbonyl group C=S. The peak at 1207 cm^{-1} was characteristic of C=S absorption of thiobenzophenone and its derivatives [18]. The IR absorptions are tabulated in Table 1.

3.3. Structure elucidation of new analogue with NMR and Accurate MS

1D and 2D NMR data of the isolated compound are listed in Table 2. There were 23 signals in ^{13}C NMR spectrum. DEPT 90 and DEPT 135 NMR spectra indicated that there were four methyl groups, 7 methylene groups, 3 methine groups, and 9 quaternary carbons. The Orbitrap FTMS results revealed protonated molecule $[M+H]^+$ at m/z 471.1975, which suggested that molecular formula of this new analogue was $C_{23}H_{30}N_6OS_2$. This molecular formula was supported by the twenty-three carbon atoms in the ^{13}C NMR

Table 1
IR absorption range and types of vibration.

Absorption range ν (cm^{-1})	Types of vibration
3257	N–H stretching
2975–2788	sp^3 C–H stretching
1517	N–H bending
1569, 1498	Aromatic ring C=C stretching
1250, 1240, 1207	C=S stretching
810	1,2,4-Trisubstituted benzene ring=C–H bending

Table 2
^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data of new analogue (δ ppm in $CDCl_3$, J in Hz in parentheses, number in DEPT is the number of attached protons).

	Carbodenafil		New analogue					
	^{1}H (δ_H)	^{13}C (δ_C)	^{1}H (δ_H)	^{13}C (δ_C)	DEPT	COSY	HMBC	
							^2J	^3J
1								
2								
3		146.7		146.2	0			
4								
5		147.2		147.0	0			
6	11.00(1H,s)		12.59 (1H,s)					C-8
7		169.1		171.8	0			
8		120.0		132.3	0			
9		124.5		134.1	0			
10	4.27 (3H, s)	38.2	4.52 (3H, s)	39.2	3			C-8
11	2.92 (2H, t, 7.5)	27.8	2.93 (2H, t, 7.5)	27.6	2	H-12	C-3, C-12	C-9, C-13
12	1.85 (2H, Sextet, 7.5)	22.3	1.87 (2H, Sextet, 7.5)	22.3	2	H-11, H-13	C-11, C-13	C-3
13	1.02 (3H, t, 7.5)	14.1	1.01 (3H, t, 7.5)	14.0	3	H-12	C-12	C-11
14		138.5		136.3	0			
15	8.57 (1H, d, 2.5)	130.5	8.41 (1H, d, 2.5)	128.1	1	H-17	C-16	C-17, C-5, C-19, C-22
16		120.0		118.5	0			
17	7.57 (1H, dd, 2.5, 8.0)	131.9	7.55 (1H, dd, 2.5, 8.0)	131.7	1	H-15, H-18	C-16	C-15, C-19, C-22
18	7.08 (1H, d, 8.0)	113.0	7.06 (1H, d, 8.0)	113.0	1	H-17	C-19	C-14, C-16, C-5[a]
19		157.5		156.9	0			
20	4.33 (2H, q, 7.0)	65.7	4.34 (2H, q, 7.0)	66.0	2	H-21	C-21	C-19
21	1.62 (3H, t, 7.0)	14.6	1.69 (3H, t, 7.0)	14.8	3	H-20	C-20	
22		153.8		199.3	0			
23								
24	3.61 (2H, brs)	[b]	3.73 (2H, brs)	52.0	2	H-25		
25	2.52 (2H, brs)	52.5	2.50 (2H, brs)	55.2	2	H-24		C-27, C-29
26								
27	2.52 (2H, brs)	52.5	2.68 (2H, brs)	54.3	2			
28	3.80 (2H, brs)	[b]	4.48 (2H, brs)	49.5	2			
29	2.48 (2H, q, 8)	52.3	2.38 (3H, s)	45.5	3			C-25, C-27
30	1.18 (3H, t, 8)	11.4	NA					

[a] Long range ^{1}H–^{13}C coupling due to "W plan" [19].
[b] ^{13}C signals are missing.

spectrum. Comparison of this molecular formula with that of desethylacetildenafil ($C_{23}H_{30}N_6O_3$) suggested that 2 of the oxygen atoms were replaced by 2 sulphur atoms in the new analogue. The chemical shift of the much deshielded carbon at 171.8 ppm and 199.3 ppm suggested two carbonyl groups. The absence of strong C=O IR absorption and the presence of the characteristic C=S in the IR spectrum indicated that this compound contained two thiocarbonyl carbons. Therefore, the 2 carbonyl groups in desethylacetildenafil were indicated to be replaced by two thiocarbonyl groups in the new analogue.

In the ^{1}H NMR spectrum, the singlet at 12.59 ppm was H-6, which conjugated with adjacent oxygen atom to form intramolecule hydrogen bond. Therefore H-6 was much deshielded and had a characteristic chemical shift, which was about 12 ppm. Three aromatic protons H-18 [δ_H 7.06 (1H, d, 8.0)], H-17 [7.55 (1H, dd, 2.5, 8.0), and H-15 [8.41 (1H, d, 2.5)] revealed a 1,2,4-trisubstituted benzene ring.

HMQC correlations helped to establish the assignments of ^{1}H signals to their attached carbons. Further HMBC correlations (see Table 2) assisted in establishment of the skeleton of this new analogue. It could be established that H-10 [δ_H 4.52 (3H, s)] was connected to nitrogen, which withdrew electrons from this methyl group causing higher chemical shift. HMBC correlations from H-10 and H-6 to C-8 [δ_C 132.3] helped to assign this carbon C-8 as other carbons are all more than three bonds away from H-6 and H-10. HMBC correlations from H-11 [δ_H 2.92 (2H, t, 7.5)] to C-3, C-9, C-12, and C-13 and from H-12 [δ_H 1.86 (2H, Sextet, 7.5)] to C-3, C-11, and C-13 established the linkage of this propyl substituent and assignments of C-3 [δ_C 146.2] and C-9 [δ_C 134.1]. The chemical shift of C-19 [δ_C 156.9] revealed that it was an oxygenated aromatic proton. HMBC correlations from H-20 [δ_H (2H, q, 7.0)] to C-19 and C-21 confirmed the position of this ethoxy group. Four methylene groups with chemical shifts from 2.50 ppm to 4. 48 ppm indicated the existence of the piperazine group. HMBC correlations from H-15 and H-17 to thiocarbonyl carbon C-22 [δ_C 199.3] suggested that 1, 2, 4-trisubstituted benzene ring was linked to the piperazine moiety by a thiocarbonyl group. The chemical shift of H-29 [δ_H 2.38 (3H, s)] indicated that the methyl group was connected to the nitrogen atom. HMBC correlations from H-29 to C-25 [δ_C 55.2] and C-27 [δ_C 54.3] helped to assign these 2 carbons. COSY effects between H-24 [δ_H 3.61 (2H, brs)] and H-25 [δ_H 2.52 (2H, brs)] also helped in the assignment of H-24. H-28 [δ_H 3.80 (2H, brs)] was also assigned as it was the only methylene group left. Due to the diamagnetic anisotropy effect from thiocarbonyl group, H-24 and H-28 had higher chemical shifts than H-25 and H-27, which were far away from the thiocarbonyl group.

Therefore, this new analogue was revealed as 5-(5-(4-methylpiperazine-1-thioyl)-2-ethoxyphenyl)-1-methyl-3-propyl-1H-pyrazolo[4,3-d]pyrimidine-7(6H)-thione Fig. 4. It was named as dithio-desmethylcarbodenafil, as it had a similar skeleton to carbodenafil wherein 2 oxygen atoms in carbodenafil were replaced by 2 sulphur atoms. Also ethyl group in piperazine ring was replaced by methyl group. Further MS/MS analysis supported this structure.

3.4. MS/MS analysis

To confirm the structure, MS and MS/MS analyses were carried out in positive ionisation mode. The spectrum is shown in Fig. 3. MS analysis indicated the prontonated molecular ion $[M+H]^+$ to have a value of m/z as 471.1983. The parent ion m/z 471.0 was selected to be fragmented in the MS^2 system. It produced major fragments

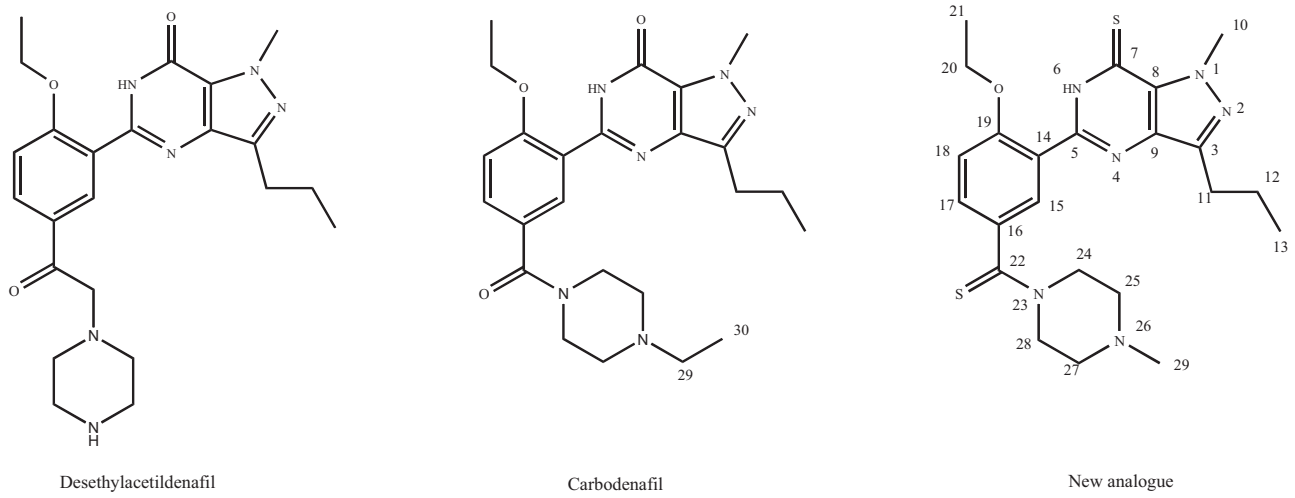
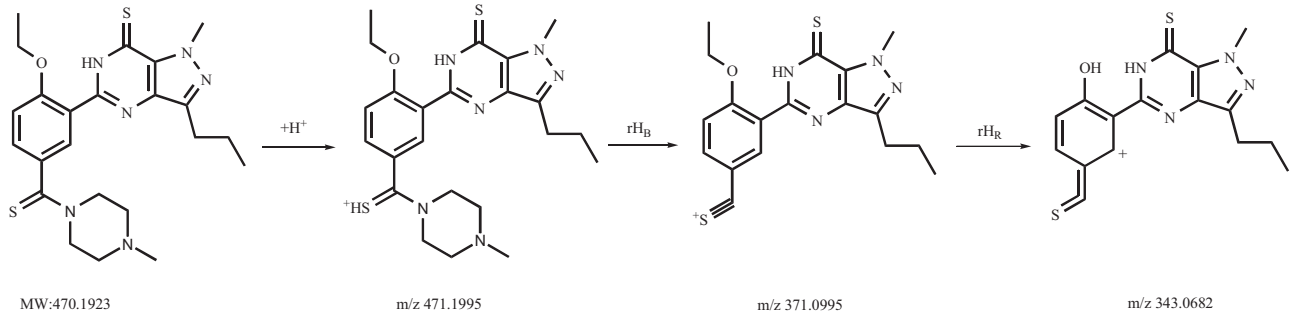


Fig. 4. Structures of desethylacetildenafil, carbodenafil and new analogue.



Scheme 1. The fragmentation process of protonated molecular ion *m*/*z* 471.0.

ion at *m*/*z* 371.0987 and *m*/*z* 343.0675. The ion of *m*/*z* 371.0987 could be achieved with the loss of piperazine ring. The further loss of the ethyl group produced a fragment ion of *m*/*z* 343.0675. The fragmentation process is indicated in Scheme 1. The accurate *m*/*z* value supported the proposed fragment structures.

4. Conclusion

A tablet formulation that was intended to be introduced into South East Asia market was found to contain a new sildenafil analogue, that after isolation and structure elucidation using NMR and MS was established to be 5-(5-(4-methylpiperazine-1-thioyl)-2-ethoxyphenyl)-1-methyl-3-propyl-1H-pyrazolo[4,3-d]pyrimidine-7(6H)-thione with trivial name dithio-desmethylcarbodenafil. As its structure has not been reported, it may not be easily identified in routine screening. The study is thus considered to be of value to help the regulatory authorities to safeguard the quality of the natural supplements and public health.

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