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Structural elucidation of a PDE-5 inhibitor detected as an adulterant in a health supplement

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ABSTRACT

A PDE-5 inhibitor was detected and isolated from a health supplement claimed to be a preparation of fresh oyster extracts and be able to promote and support healthy sexual function and endurance, etc. The structure of this PDE-5 inhibitor was elucidated using LC–UV, LC–TOF-MS, MS–MS, IR spectroscopy, and 2D NMR. It was characterized as 8-(2-(4-(hydroxymethyl)piperidin-1-yl)benzylamino)-3-ethyl-1H-imidazo[4,5-*g*]quinazoline-2(3H)-thione, a compound reported to be a PDE-5 inhibitor.

1. Introduction

Phosphodiesterase type 5 enzyme (PDE-5) inhibitors, namely sildenafil (Viagra®), vardenafil (Levitra®), and tadalafil (Cialis®) are prescription drugs approved by the U.S. Food and Drug Administration (FDA) to treat erectile dysfunction (ED). However, some analogues and other PDE-5 inhibitors have been found as adulterants in natural health supplement. In several laboratories as well as from the samples submitted to the Health Sciences Authority (HSA) of Singapore for testing, compounds such as homosildenafil [1,2], hydroxyhomosildenafil [2–4], acetildenafil [2,5], hydroxyacetildenafil [6,7], aminotadalafil [8], thiosildenafil [9] and thiohomosildenafil (homosildenafil thione) [9,10] had been detected. Other new analogues, such as piperidenafil [7,11], noracetildenafil [12], methisosildenafil [13], xanthoanthrafil [14] and imidazosagatriazinone [15], had also been reported. In this study, an adulterant with different UV and MS profiles from previously isolated PDE-5 analogues was found in a health supplement. 2D NMR, MS and IR were used to identify this compound.

2. Experimental

2.1. Materials

Dimethylsulfoxide-d_6 (DMSO-d_6) was purchased from Merck (Germany). HPLC grade methanol, AR grade ethyl acetate and hexane were obtained from Labscan Asia Co., Ltd. Water was purified to 18.2 mΩ cm using an ELGA Purelab Ultra system (Vivendi Water System Ltd., USA). A health supplement sample claimed to be a preparation of fresh oyster extracts and be able to promote and support healthy sexual function and endurance, etc. was submitted by a client for testing.

2.2. Extraction and isolation of unknown compound

The contents of the capsules in the form of yellow powder (14 g) were ultrasonically extracted with 200 ml methanol for 20 min. After filtration and solvent removal, 1 g of crude extract was produced. 240 mg of crude extract dissolved in 5 ml methanol and 1 ml dichloromethane was adsorbed on normal phase silica gel by removing the solvent. The pre-loaded sample was separated using Teledyne Isco CombiFlash® Companion™ 4× flash chromatography with a 4.3 g normal phase RediSep column. The eluant was ethyl acetate:*n*-hexane (95:5) and fractions were collected in 10 ml test tubes. Fractions with the same peak were combined

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and produced 20 mg unknown compound in the form of yellow powder after solvent removal.

2.3. LC–UV analysis

The contents (0.4 g) of one capsule were 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. LC–UV analysis was conducted on Agilent 1100 series chromatograph with a diode array detector in the wavelength range of 200–400 nm. The UV signals were monitored at 220 nm, 254 nm, and 280 nm. The LC analysis was carried out using a Hypersil C_{18} column (200 mm × 4.6 mm, 5 μm particle size) with mobile phase A of 0.025 M sodium phosphate in water and mobile phase B of acetonitrile. The mobile phase composition A was linearly ramped from 90% to 30% over 20 min and kept at 30% for 5 min. After that it was linearly returned to 90% in 5 min and kept at 90% for another 5 min. The flow rate was 1 ml/min.

2.4. LC–MS/MS and LC–TOF-MS analysis

Isolated compound was dissolved in methanol. The LC analysis was carried out using an Inertsil ODS-3 column (150 mm × 2.1 mm, 5 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 0.4 ml/min.

The MS/MS experiment was performed on the API 2000 triple quadrupole LC–MS/MS system (Applied Biosystem, USA) controlled by Analyst™ 1.4.2 software. The Turbo Spray ionization source was operated in the positive ion mode with the flow rate and temperature of the curtain gas at 40 μl/min and 350 °C, respectively, the flow rates of nebulizer gas and turbo gas at 40 μl/min and 60 μl/min, respectively, ion spray voltage at 4500 V, declustering potential at 65 V, focusing potential at 400 V, and entrance potential at 10 V. The ion with m/z 449.1 was selected to produce MS^2 spectrum with collision energy at 50 V. High resolution MS analysis was conducted using Agilent 6210 Time of Flight (TOF) LC/MS system controlled by Agilent Masshunter Workstation Console. The data was processed with Analyst™ QC version 1.1 from Applied Biosystem. The electrospray ionization (ESI) source was operated in the positive ionization mode with charging voltage 900 V, nebulizer gas 45 PSI, drying gas 10.0 l/min, gas temperature 300 °C, and capillary 4000 V. The fragmentor voltage was set to 200 V. All masses were corrected by the internal standards (Agilent G1969-85000) with m/z 121.0509 and 922.0098.

Table 1
^1H (500 MHz), HMBC and ^{13}C (75 MHz) NMR data in DMSO-d_6 (J in Hz in parentheses)

Position	δ_H (ppm)	HMBC		δ_C (ppm)
		^2J	^3J	
1				
2				172.9 C
3				
4	7.58 (1H, s)	C-11, C-12	C-10	102.8 CH
5				
6	8.45 (1H, s)		C-12	150.7 CH
7				
8				160.8 C
9	8.12 (1H, s)	C-10	C-8, C-11	99.3 CH
10				132.0 C
11				137.9 C
12				109.5 C
13				136.9 C
14	4.31 (2H, q, 6.5)	C-15	C-2, C-11	38.9 CH_2
15	1.27 (3H, m)	C-14		12.8 CH_3
16	9.08 Brs			
17	4.87 (2H, d, 4.5)	C-18	C-8, C-19, C-23	41.0 CH_2
18				131.9 C
19				152.1 C
20	7.12 (1H, d, 7.0)	C-21, C-19	C-18, C-22	120.3 CH
21	6.96 (1H, t, 7.0)	C-20	C-19, C-23	123.7 CH
22	7.24 (1H, t, 7.0)	C-21, C-23	C-18	128.4 CH
23	7.14 (1H, d, 7.0)	C-18, C-22	C-17, C-19, C-21	127.8 CH
24				
25	2.63 (1H, t, 10.9) 3.06 (1H, d, 10.9)	C-26	C-27, C-29	53.0 CH_2
26	1.27 (1H, m) 1.74 (1H, d, 11.3)	C-25, C-27	C-28, C-30	29.6 CH_2
27	1.47 (1H, m)	C-26, C-28, C-30	C-25, C-29	38.2 CH
28	1.27 (1H, m) 1.74 (1H, d, 11.3)	C-27, C-29	C-30	29.6 CH_2
29	2.63 (1H, t, 10.9) 3.06 (1H, d, 10.9)	C-28	C-25, C-27	53.0 CH_2
30	3.29 (2H, d, 6.0)	C-27	C-28, C-26	66.2 CH_2

2.5. NMR and IR analysis

About 10 mg of the isolated unknown compound was dissolved in DMSO-d_6 for NMR analysis. ^1H, ^{13}C, COSY, NOESY, 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 (DMSO-d_6, δ_H: 2.5 ppm, δ_C: 39.5 ppm). Coupling constants (J) were measured in Hertz (Hz) and

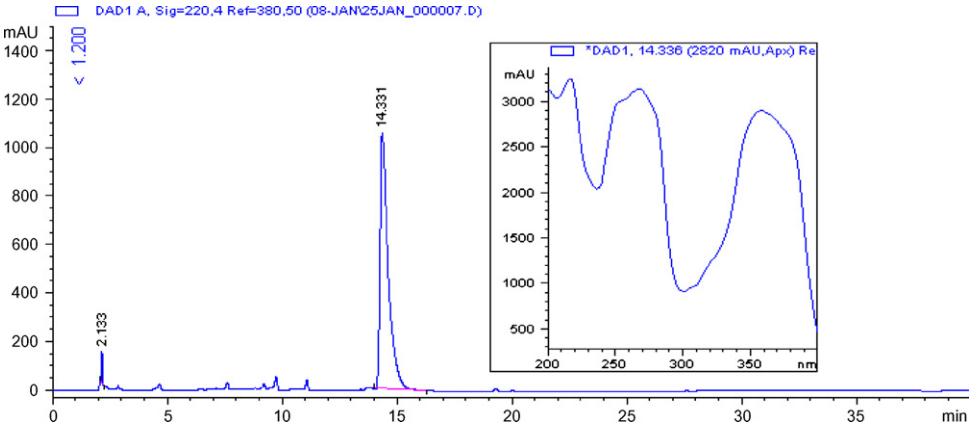


Fig. 1. HPLC chromatogram of a methanol extract of the health supplement at 220 nm. The insert shows the UV profile of the signal at 14.3 min.

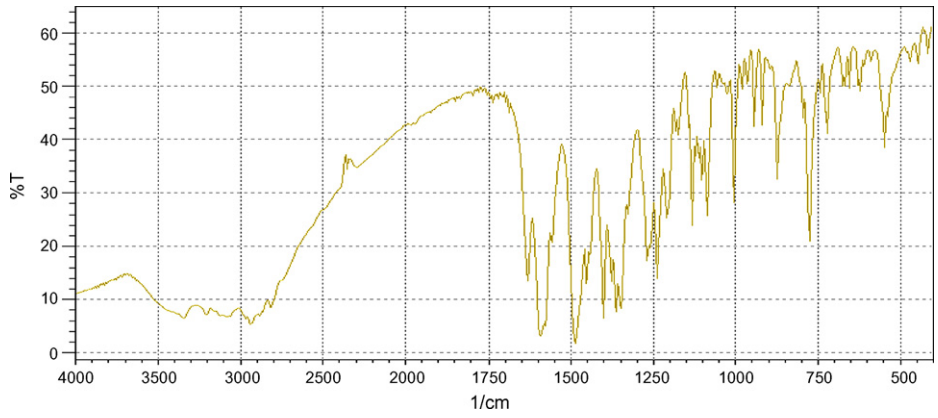


Fig. 2. Infrared spectrum of the unknown compound in KBr disk.

chemical shifts were in ppm. IR sample was prepared as a KBr disk and spectrum was measured over the range of 4000–400 cm^{-1} on Shimadzu FTIR-8400s spectrometer (Nakagyo, Japan).

3. Results and discussion

3.1. LC–UV

The LC chromatogram of the health supplement and the UV spectrum were shown in Fig. 1. The chromatogram had few peaks with an outstanding one at 14.3 min. The UV spectrum of this unknown compound was totally different from those of previously isolated PDE-5 inhibitor analogues (Fig. 1).

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 absorption ($\nu_{C=C}$) at 1593 cm^{-1} and 1486 cm^{-1}. The absorption at 773 cm^{-1} corresponded to the *ortho*-disubstituted aromatic ring. The stretching absorptions of sp^2 C–H, sp^3 C–H, N–H, and hydrogen bonded O–H overlapped in the region of 3000–3400 cm^{-1}. Aliphatic C–H stretches were detected from 2800 to 3000 cm^{-1}. The absence of strong absorptions from 1600 to 1800 cm^{-1} also indicated that there was no carbonyl group in this unknown compound. Meanwhile, the absorption in the range 1269–1201 cm^{-1} suggests the presence of thiocarbonyl group C=S. The IR absorptions are tabulated in Table 2.

Table 2
IR absorption range and types of vibration

Absorption range, υ (cm^{-1})	Types of vibration
3330–3060	O–H, N–H stretching
2936–2811	sp^3 C–H stretching
1629	N–H bending
1591, 1486	Aromatic ring C=C stretching
1400	C–H bending
1362, 1347	C–N stretching
1269–1201	C=S stretching
773	*Ortho*-disubstituted benzene ring = C–H bending

3.3. Structure elucidation of unknown compound with NMR

The unusual LC peak at 14.3 min and its different UV spectrum from those of other PDE-5 inhibitors led us to isolate and elucidate the structure of this unknown compound. There were twenty-two signals in ^{13}C NMR spectrum. DEPT 90 and DEPT 135 NMR spectra indicated that there were one methyl group, five methylene groups, eight methine groups, and eight quaternary carbons. However in the HMQC spectrum, each of the two methylenes at 29.6 ppm and 53.0 ppm corresponded to four protons, which meant two methylene carbon signals were overlapped at 29.0 ppm and 53.0 ppm separately. Therefore, there were seven methylene groups and the total number of carbon atoms was 24 with 25 attached protons. High resolution MS showed that the pseudo-molecular ion was m/z 449.2123 $[M+H]^+$ (see Fig. 3). The information from NMR and HR-MS suggested that the molecular formula of this unknown compound was $C_{24}H_{28}N_6OS$ (Fig. 4).

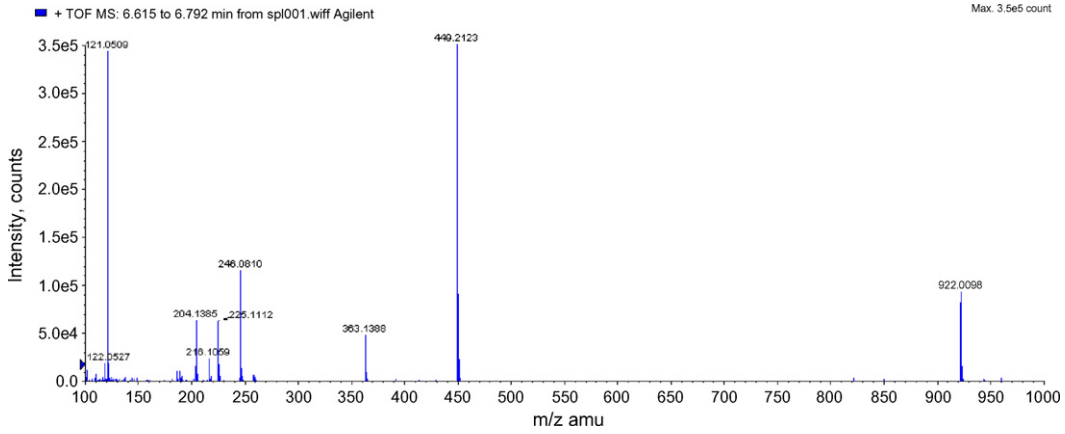


Fig. 3. High-resolution mass spectrum of unknown compound in positive ionisation mode.

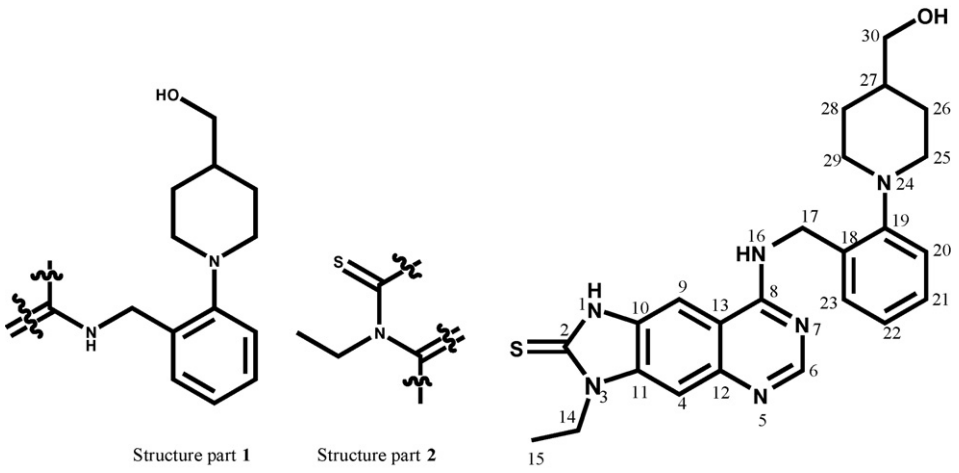


Fig. 4. Structure and substructures of KF31327.

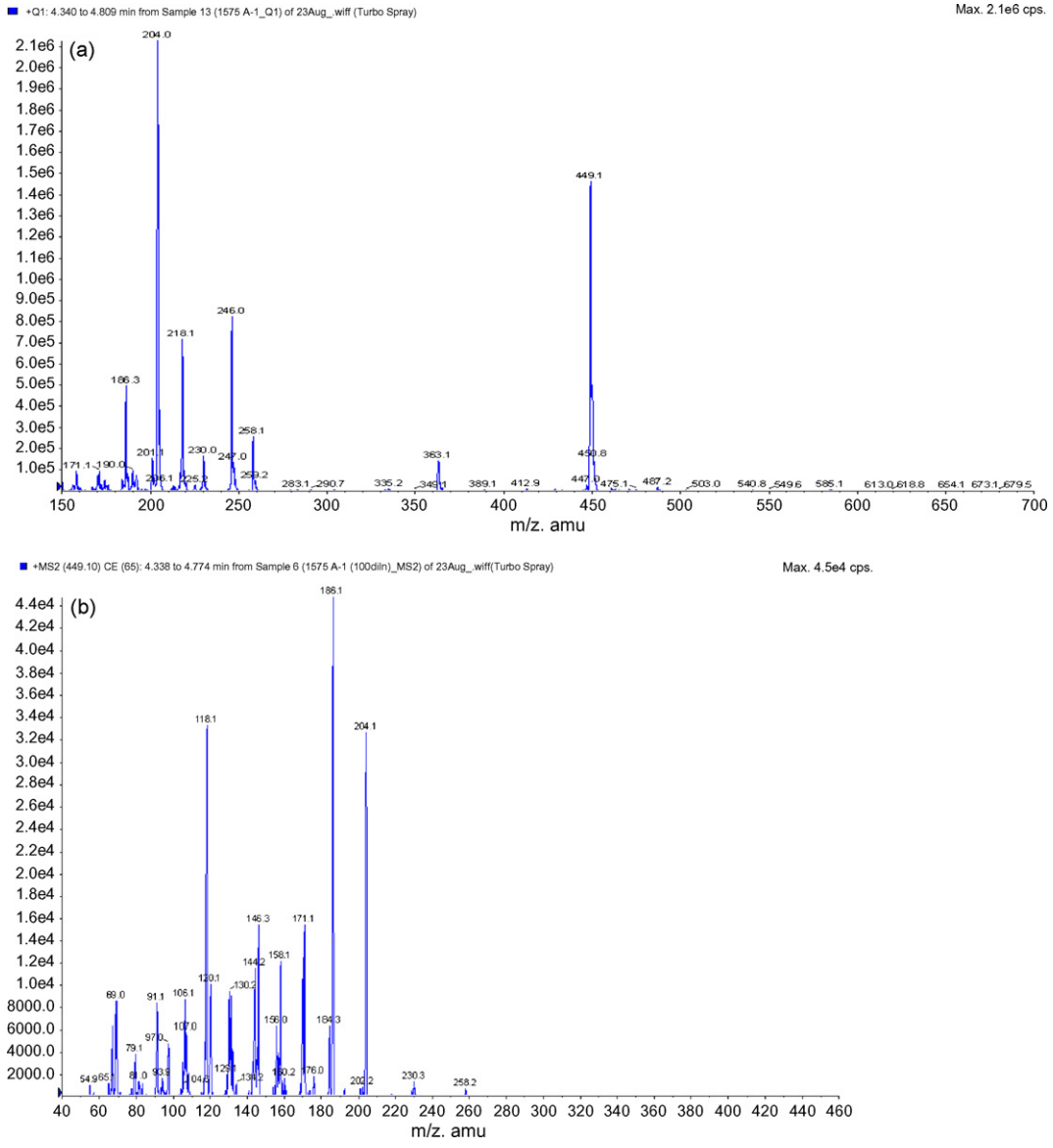
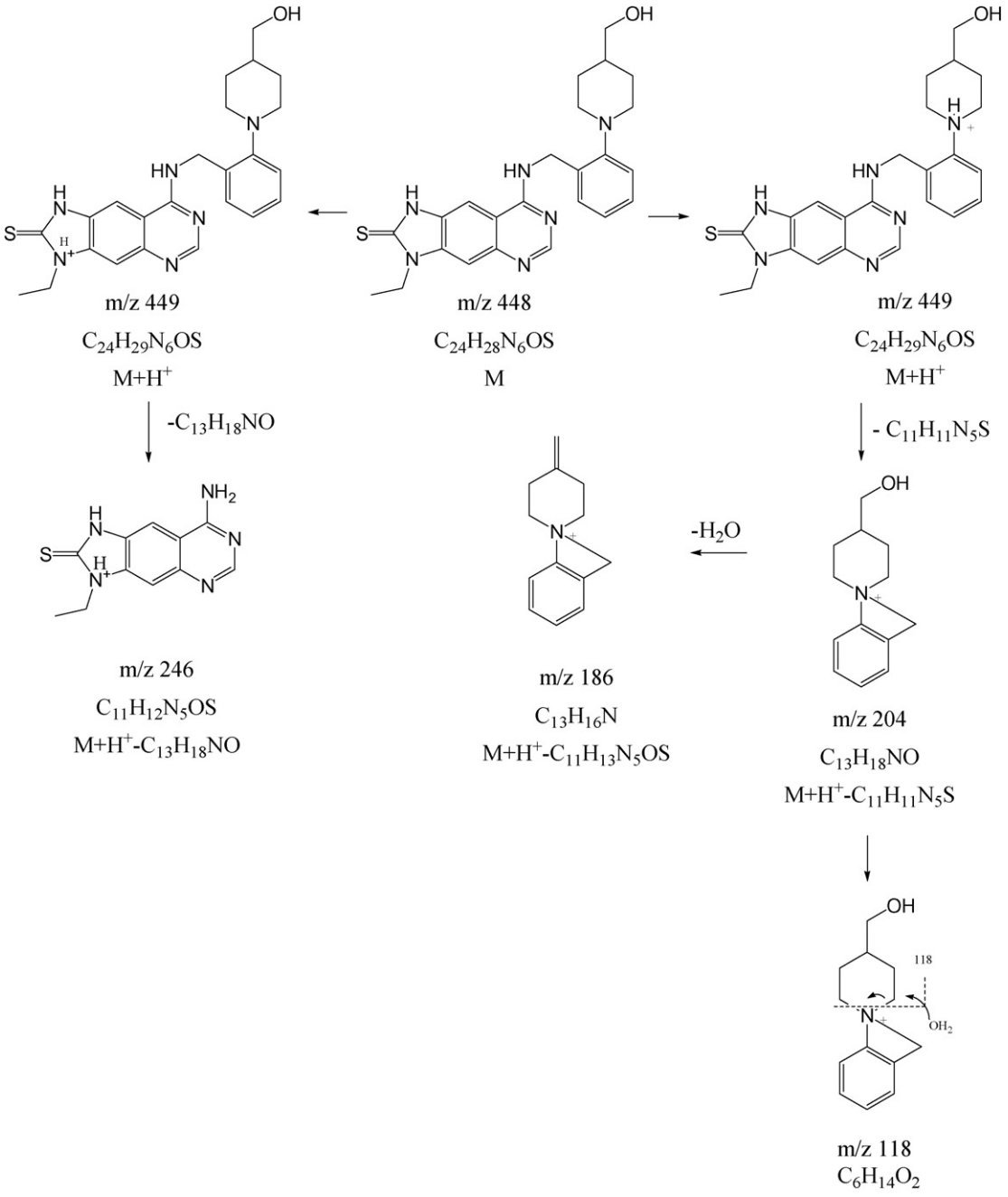


Fig. 5. ESI mass spectrum of unknown compound in positive MS analysis: (a) Q1 analysis; (b) MS^2 analysis with parent ion m/z 449.1.

HMQC correlations helped to establish the assignments of the ^{1}H signals to their attached carbons. In NMR experiments, if carbon atoms are connected to oxygen or nitrogen atoms, these carbons and the attached hydrogens will be much deshielded. The chemical shifts of oxygenated sp^3 carbons will be around 60 ppm in ^{13}C NMR spectrum and the attached hydrogens will appear at above 3 ppm in ^{1}H NMR spectrum. Therefore the chemical shifts of the C-30 and the attached ^{1}H [δ_H 3.29 (2H, d, 6.0) and δ_C 66.2] indicated this was an oxygenated methylene group. The chemical shift of the much deshielded carbon at 172.9 ppm suggested an ester or amide group. 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 a thiolamide group with the thiol carbonyl carbon at 172.9 ppm. In the ^{1}H NMR spectrum, there were four aromatic protons [δ_H 7.12 (1H, d, 7.0), 6.69 (1H, t, 7.0), 7.24 (1H, t, 7.0), and 7.14 (1H, d, 7.0)], which revealed a 1,2-disubstituted benzene ring. There was also a symmetric structure part because of two pairs of methylenes [δ_C 29.6 ($2CH_2$) and 53.0 ($2CH_2$)]. The chemical shifts of two methylenes at 53.0 ppm indicated they were connected to a nitrogen atom. HMBC correlations (see Table 1) helped to establish the skeleton of this unknown compound. HMBC correlations from H-25 to C-26 and C-27 gave the structure of piperidine. The position of the hydroxymethyl group was determined by the correlations from H-30 to C-26, C-27 and C-28. The chemical shift of C-19 [δ_C 152.1] and the NOESY effects between H-20 and H-25 suggested that C-19 was connected to the nitrogen atom of the piperidine ring. The position of C-17 was determined by the HMBC correlations from H-17 to C-18, C-19, and C-23.



Scheme 1. The fragmentation process of pseudo-molecular ion *m/z* 449.1.

COSY effect from H-16 to H-17 helped to establish the connection between C-17 and N-16. HMBC also showed the correlation from H-17 to the much deshielded quaternary C-8, which may be connected to another nitrogen atom because of its chemical shift [δ_C 160.8]. Structure part **1** Fig. 4 was deduced from above information. The chemical shifts of C-14 and its attached ^{1}H indicated it was connected to a nitrogen atom. HMBC correlations from methyl protons H-15 to C-14 suggested an ethyl group. The correlations from H-14 to thiol carbonyl carbon C-2 and another quaternary carbon C-11 gave the structure part **2**. Up to now, there were three nitrogens, three methine carbons, and four quaternary carbons unassigned. The HMBC correlations could not provide further structural information. The literature search in Scifinder Scholar™ (CAS, American Chemical Society, Columbia, OH, US) with the molecular formula and known structural information led to the exclusive candidate, 8-(2-(4-(hydroxymethyl)piperidin-1-yl)benzyl amino)-3-ethyl-1H-imidazo[4,5-g]quinazoline-2(3H)-thione with trivial name KF31327 [16,17]. The HMBC correlations from H-6 to C-12, from H-4 to C-10, C-11, and C-12, from C-9 to C-8, C-10, and C-11 also supported this structure. Further MS/MS analysis also supported this structure. This compound was previously synthesized and found to be a PDE-5 inhibitor in 2001.

3.4. MS/MS analysis

To confirm the structure of our compound deduced from NMR and database searching. MS and MS/MS analysis was carried out in positive ionisation mode. The spectrum was shown in Fig. 5. MS analysis indicated the pseudo-molecular ion $[M+H]^+$ was at m/z 449.1. This result corresponded with the LC–TOF-MS result (m/z 449.2123). The parent ion m/z 449.1 was selected to be fragmented in the MS^2 system. It produced a fragment ion at m/z 204.1. The ion m/z 186.1 could be easily achieved with the neutral loss of one H_2O molecule. The fragmentation process was indicated at Scheme 1.

4. Conclusion

This product was intended to be introduced into Singapore market and sold over the Internet. Isolation and structural elucidation using NMR and MS revealed this to be a PDE-5 inhibitor 8-(2-(4-(hydroxymethyl)piperidin-1-yl)benzyl amino)-3-ethyl-1H-imidazo[4,5-*g*]quinazoline-2(3H)-thione with trivial name KF31327. As its structure is totally different from those of sildenafil, vardenafil, tadalafil and their analogues, it will not be easily identified in routine screening. The study will help the regulatory bodies to safeguard the quality of the natural supplements and public health more efficiently and effectively.

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References

[1] M.H. Shin, M.K. Hong, W.S. Kim, Y.J. Lee, Y.C. Jeoung, Food Addit. Contam. 20 (2003) 793–796.
[2] P. Zou, S.S.Y. Oh, P. Hou, M.Y. Low, H.L. Koh, J. Chromatogr. A 1104 (2006) 113–122.
[3] L. Blok-Tip, B. Zomer, F. Bakker, K.D. Hartog, M. Hamzink, J. Ten Hove, M. Vredenbregt, D. De Kaste, Food Addit. Contam. 21 (2004) 737–748.
[4] W.T. Poon, Y.H. Lam, C.K. Lai, A.Y. Chan, T.W. Mak, Hong Kong Med. J. 13 (2007) 359–363.
[5] C. Shin, M. Hong, D. Kim, Y. Lim, Magn. Reson. Chem. 42 (2004) 1060–1062.
[6] P. Hou, P. Zou, M.Y. Low, E. Chan, H.L. Koh, Food Addit. Contam. 23 (2006) 870–875.
[7] H.J. Park, H.K. Jeong, M.I. Chang, M.H. Im, J.Y. Jeong, D.M. Choi, K. Park, M.K. Hong, J. Youm, S.B. Han, D.J. Kim, J.H. Park, S.W. Kwon, Food Addit. Contam. 24 (2007) 122–129.
[8] P. Zou, P. Hou, M.Y. Low, H.L. Koh, Food Addit. Contam. 23 (2006) 446–451.
[9] P. Zou, P. Hou, S.S.Y. Oh, Y.M. Chong, B.C. Bloodworth, M.Y. Low, H.L. Koh, J. Pharm. Biomed. Anal. 47 (2008) 279–284.
[10] J. Venhuis, G. Zomer, D. de Kaste, J. Pharm. Biomed. Anal. 46 (2008) 814–817.
[11] J.C. Reepmeyer, J.T. Woodruff, J. Chromatogr. A 1125 (2006) 67–75.
[12] J.C. Reepmeyer, J.T. Woodruff, J. Pharm. Biomed. Anal. 44 (2007) 887–893.
[13] J.C. Reepmeyer, J.T. Woodruff, D.A. d'Avignon, J. Pharm. Biomed. Anal. 43 (2007) 1615–1621.
[14] K. Kumasaka, N. Kawahara, K. Doi, T. Kojima, Y. Goda, Chem. Pharm. Bull. (Tokyo) 56 (2008) 227–230.
[15] Y.H. Lam, W.T. Poon, C.K. Lai, A.Y. Chan, T.W. Mak, J. Pharm. Biomed. Anal. 46 (2008) 804–807.
[16] F. Kenji, T. Hitoshi, A. Toshiyuki, O. Takehiro, M. Shin-ichiro, K. Masai, Org. Process Res. Dev. 5 (2001) 426–433.
[17] H. Ryo, O. Hirose, Y. Akiko, I. Junko, O. Yasuo, N. Yuji, T. Haruki, O. Tetsuji, I. Michio, Eur. J. Pharmacol. 431 (2001) 17–24.