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Isolation and structure elucidation of an interaction product of aminotadalafil found in an illegal health food product

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ABSTRACT

An interaction product of aminotadalafil was isolated from an illegal health food product. The structure of the interaction product was elucidated by means of IR, NMR, and mass spectroscopy. The hitherto unknown compound was characterized as condensation product of aminotadalafil and hydroxymethylfuraldehyde and is probably the result of a drug-excipient incompatibility.

1. Introduction

Tadalafil (Cialis®) was approved in 2003 by the FDA as the third phosphodiesterase type 5 enzyme (PDE-5) inhibitor to treat erectile dysfunction (ED) [1]. Since then, different tadalafil analogues have been found as adulterants in illegal products. Hasegawa et al. detected an open chain molecule, probably the precursor of the synthesis of tadalafil, and *N*-octylnortadalafil in dietary supplements [2,3]. The highly reactive hydrazide aminotadalafil (**2**) was isolated by the laboratory of the Health Sciences Authority of Singapore from a herbal product [4]. Due to the lack of efficacy, toxicology and stability data, these illegal products are potentially dangerous for the consumers [5]. In this study, the isolation and structure elucidation of a so far unknown interaction product of aminotadalafil (Fig. 1) as well as its possible origin are described.

2. Experimental

2.1. Materials

Dimethylsulfoxide-d_6 (DMSO-d_6) was purchased from Aldrich. All solvents were HPLC grade. Water was purified to 18.2 mΩ cm using an ELGA Purelab Ultra system. The illegal product "Mentalk", which is sold worldwide as a purely natural health food consisted of boxes of individually wrapped dark brown candies of around 4 g weight, was seized by the Singaporean authorities.

2.2. LC-UV screening

By sonication, one candy was disintegrated in 50 ml of methanol. The suspension was filtered and the solution was analysed using an Agilent 1200 series HPLC system equipped with a Hypersil BDS C18 column (5 μm, 200 × 4.6 mm). Mobile phase A consisted of 25 mM sodium dihydrogen phosphate in water, adjusted to pH 3.2 with phosphoric acid. Mobile phase B was acetonitrile. The mobile phase composition A was linearly decreased from 90% to 30% over 30 min and kept at 30% for 5 min. The flow rate was 1.0 ml/min and the UV signals were monitored at 220, 254, and 280 nm [6].

2.3. Extraction

Five candies and 200 ml of water were stirred until the products were disintegrated. The suspension was sonicated for 30 min and 125 ml of ethyl acetate were added. The mixture was stirred for 15 min, sonicated for another 5 min and stirring was continued for 10 min. The organic layer was separated and centrifuged to remove remaining solids. The clear solution was evaporated to dryness.

2.4. Isolation of the unknown compound **3**

The extract was dissolved in 2 ml of DMSO. The unknown compound **3** was isolated by isocratic HPLC using a preparative

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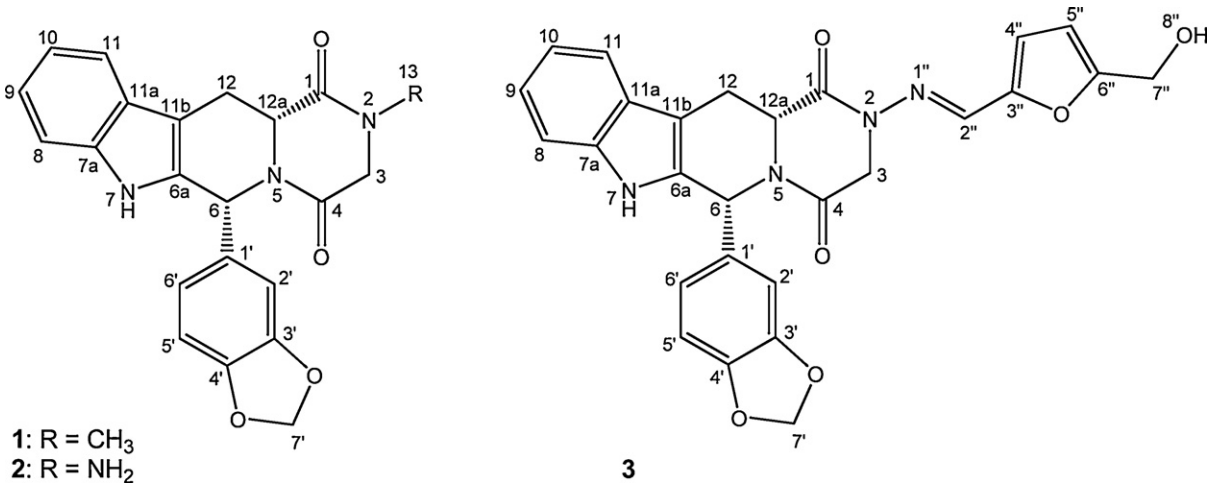


Fig. 1. Chemical structures of tadalafil (1, R = CH_3), aminotadalafil (2, R = NH_2) and the interaction product of aminotadalafil **3**.

Shimadzu system equipped with a Zorbax SB-C18 column (5 μm, 250 × 9.4 mm). The mobile phase was a mixture of methanol–water (65:35, v/v). The flow rate was 3.3 ml/min, and the injection volume was 50 μl. The column temperature was maintained at 40 °C. The photodiode array spectra were recorded from 200 to 400 nm. The fractions of the second main peak were combined and methanol was evaporated at 30 °C. The remaining aqueous suspension was stored at 4 °C overnight. After centrifugation at RCF 3026 × *g* (4 °C, 30 min), the clear supernatant was removed and the white solid was dried in high vacuum.

2.5. MS analysis of compound *3*

The MS^2 experiment was performed on the API 2000 triple quadrupole LC–MS/MS system (Applied Biosystems, 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 as 40 μl/min and 350 °C, respectively, the flow rates of nebulizer gas and turbo gas as 40 μl/min and 60 μl/min, respectively, ion spray voltage as 4500 V, declustering potential as 65 V, focusing potential as 400 V, and entrance potential as 10 V. The ion with *m/z* 499 was selected to produce

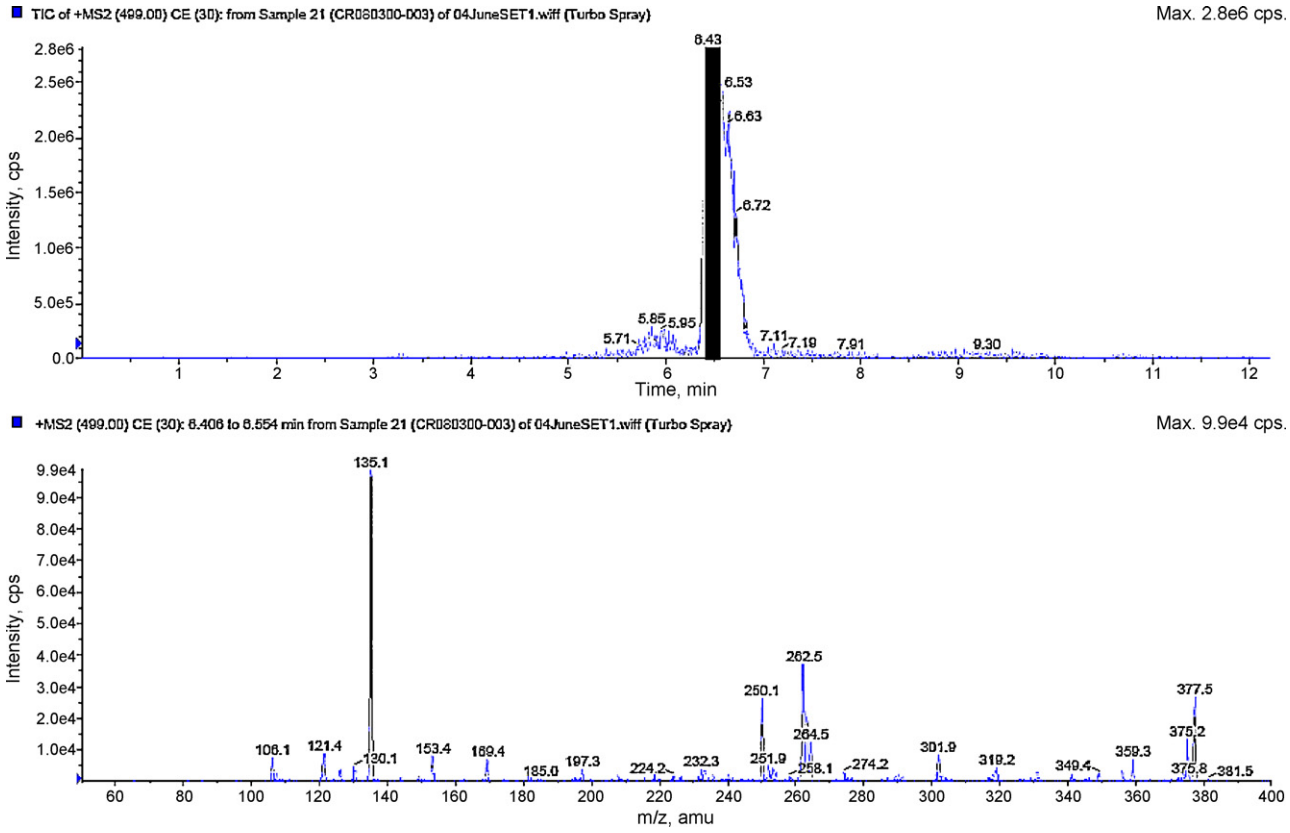
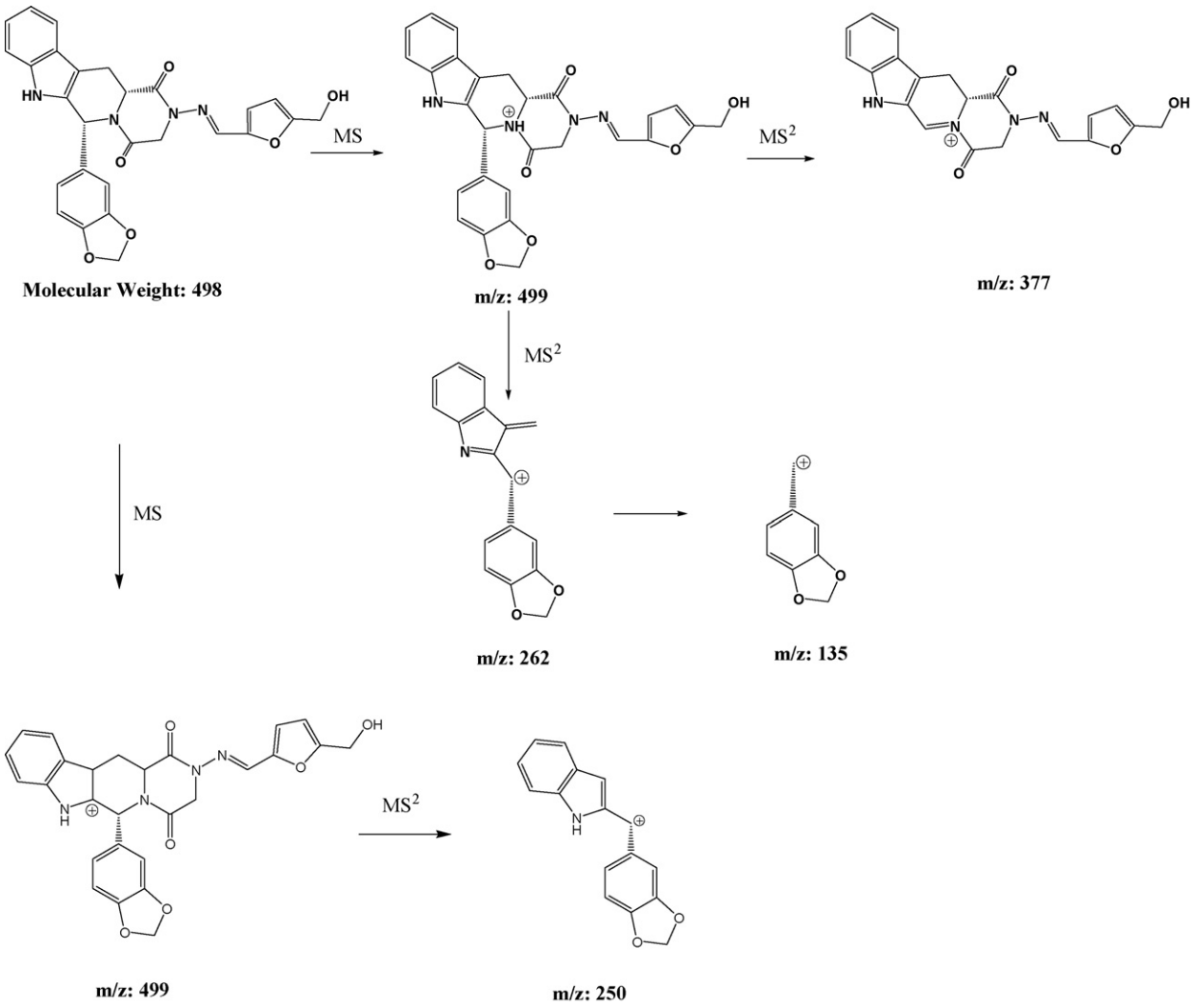


Fig. 2. MS^2 spectrum of compound **3** with parent ion *m/z* 499.



Scheme 1. The fragmentation process of the pseudo-molecular ion *m/z* 499.

MS^2 spectrum with collision energy of 30 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 Biosystems. The electrospray ionization (ESI) source was operated in the positive ionization mode with charging voltage 900 V, nebulizer gas pressure 45 PSI, drying gas flow rate 10.0 l/min, gas temperature 300 °C, and capillary voltage 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.

2.6. NMR and IR analyses of compound **3**

About 10 mg of **3** was dissolved in DMSO-d_6. ^1H, ^{13}C, DEPT 90, DEPT 135, COSY, ROESY, HMQC, ^1H–^{13}C HMBC, and ^1H–^{15}N HMBC spectra were recorded using a Bruker DRX500 NMR spectrometer. The solvent peak acted as the internal standard (DMSO-d_6, δ_H: 2.49 ppm, δ_C: 39.5 ppm). Coupling constants (*J*) were measured in Hertz (Hz) and chemical shifts in ppm. IR samples were prepared as KBr discs and spectra were recorded over the range of 4000–400 cm^{-1} on a Shimadzu FTIR-8400s instrument.

3. Results and discussion

3.1. LC-UV screening

The LC-UV screening of the methanolic extract at 254 nm showed two main peaks at 17.7 min and 20.3 min, respectively. The first peak was identified as aminotadalafil (**2**) by means of retention time matching, UV spectrum overlay, and spiking with the reference substance. The UV spectrum of the second peak had a similar pattern as **2** with a bathochromic shift of 9 nm. The screening method did not allow the identification of this peak.

3.2. Extraction and isolation of the unknown compound **3**

After disintegration of the product in water, extraction with ethyl acetate and isolation on a reversed phase C18 column, around 2–3 mg of the unknown compound **3** were isolated from each candy. During the whole process, the use of acid had to be avoided; it was noted, that when compound **3** was dissolved in aqueous acidic solution, the substance was completely converted into **2** after a few hours. This revealed that compound **3** had the same parent structure as **2**, plus an additional side chain, which was easily cleaved in acidic solution.

Table 1
^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data of aminotadaloafil (**2**) [4,7] and of the new compound **3** (δ ppm in DMSO-d_6, J in Hz in parentheses, number in DEPT is the number of attached protons).

	Aminotadalafil (**2**) [4,7]		New compound **3**			
	^{1}H (δ_H)	^{13}C (δ_C)	^{1}H (δ_H)	^{13}C (δ_C)	DEPT	COSY HH[e]
1	–	166.2	–	165.9	0	–
3x	3.96 (1H, d, J = 17.1)	53.2	4.45 (1H, d, J = 17.0)	50.5	2	H-3y
3y	4.26 (1H, d, J = 17.1)	–	4.50 (1H, d, J = 17.0)	–	–	H-3x
4	–	164.5	–	164.1	0	–
6	6.09 (1H, s)	55.5	6.15 (1H, s)	56.0	1	*H-2′*, *H-6′*
6a	–	136.1	–	136.7	0	–
7	11.01 (1H, s)	–	11.03 (1H, s)	–	–	–
7a	–	136.9	–	137.3	0	–
8	7.29 (1H, d, J = 7.9)	111.2	7.29 (1H, d, J = 8.0)[c]	111.8	1	H-9, *H-10*
9	6.99 (1H, dd, J = 7.9, 7.1)[a]	118.8	7.05 (1H, dd, J = 7.4, 7.4)[c]	121.8	1	H-8, H-10, *H-11*
10	7.06 (1H, dd, J = 7.5, 6.8)[a]	121.1	6.99 (1H, dd, J = 7.4, 7.4)[c]	119.4	1	*H-8*, H-9, H-11
11	7.55 (1H, d, J = 7.4)	118.0	7.56 (1H, d, J = 7.8)[c]	118.6	1	*H-9*, H-10
11a	–	125.6	–	126.2	0	–
11b	–	104.7	–	105.1	0	–
12x	2.98 (1H, dd, J = 15.6, 11.6)	23.3	3.09 (1H, dd, J = 15.5, 11.7)	23.8	2	H-12y, H-12a
12y	3.56 (1H, dd, J = 15.6, 4.0)	–	3.59 (1H, dd, J = 15.5, 4.4)	–	–	H-12x, H-12a
12a	4.43 (1H, dd, J = 11.6, 3.6)	55.3	4.61 (1H, dd, J = 11.7, 4.4)	56.5	1	H-12x, H-12y
13	5.11 (2H, s)	–	–	–	–	–
1′	–	133.9	–	134.3	0	–
2′	6.87 (1H, s)	106.8	6.91 (1H, d, J = 1.6)	107.4	1	*H-6*, *H-6′*
3′	–	146.9	–	147.6	0	–
4′	–	145.9	–	146.6[d]	0	–
5′	6.78 (2H, dd, J = 9.4, 8.3, 2.2)[b]	107.9	6.77 (1H, d, J = 8.2)	108.5	1	H-6′
6′	–	119.2	6.83 (1H, dd, J = 8.2, 1.6)	119.9	1	*H-2′*, H-5′, *H-6*
7′	5.92 (2H, s)	100.8	5.91 (2H, d, J = 3.1)	101.4	2	–
2″	–	–	8.29 (1H, s)	139.5	1	–
3″	–	–	–	148.9	0	–
4″	–	–	6.88 (1H, d, J = 3.3)	117.0	1	H-5″
5″	–	–	6.46 (1H, d, J = 3.3)	109.8	1	H-4″
6″	–	–	–	158.8	0	–
7″	–	–	4.46 (2H, d, J = 5.8)	56.2	2	H-8″
8″	–	–	5.44 (1H, t, J = 5.8)	–	-	H-7″

[a] the signals of H-9 and H-10 as well as their corresponding C-signals were most likely inverted in Ref. [4]
[b] signals of H-5′ and H-6′
[c] the signals of H-8, H-9, H-10 and H-11 and the corresponding C-signals were unambiguously assigned with the COSY HH, ROESY and HSQC data
[d] the signal of C-4′ was unambiguously assigned with to the strong cross peak between H-6′ and C-4′ in the HMBC experiment
[e] *in italics:* weak cross peaks due to four-bond correlations

3.3. MS analysis of compound *3*

The high resolution mass of compound **3** of 499.1625 $[M+H]^+$ corresponded to a molecular formula of $C_{27}H_{22}N_4O_6$ ($[M+H]^+$: 499.1612). The parent ion of m/z 499 was fragmented in the MS^2 system. The main fragments were observed as shown in Fig. 2. The spectrum showed the major fragments at m/z 135, 250, 262, and 377. The fragmentation process is indicated in Scheme 1.

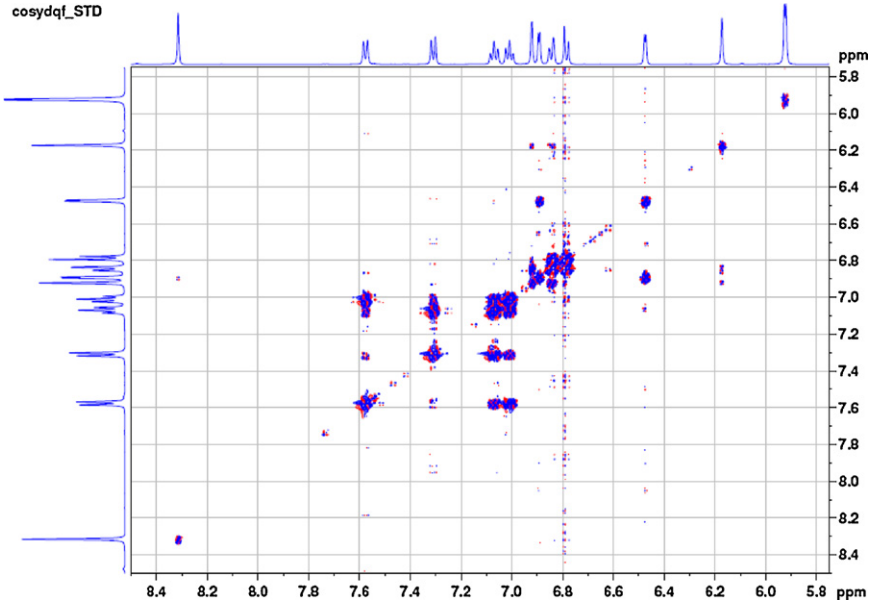
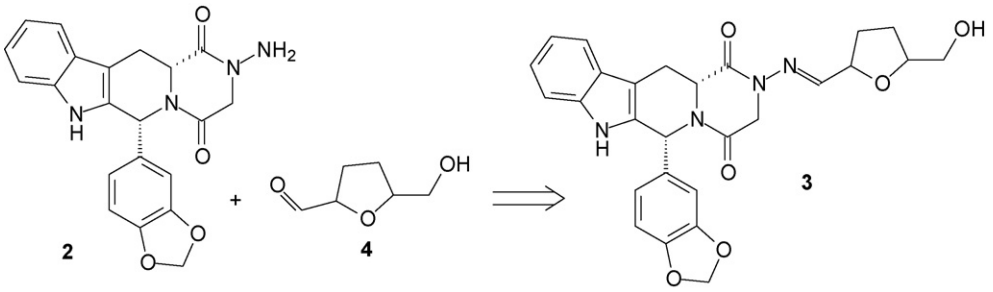


Fig. 3. ^{1}H–^{1}H COSY spectrum of compound **3**.



Scheme 2. Proposed origin of the new interaction product of aminotadalafil **3** (**2** = aminotadalafil, **4** = hydroxymethylfuraldehyde).

3.4. NMR analysis of *3*

The ^{1}H and ^{13}C NMR data of the new compound **3** were compared to the signals of aminotadalafil (**2**) [4,7] (Table 1). In the spectra of **3**, the protons of the hydrazide group of **2** (H_2-13) were missing, and some signals of the piperazinedione ring were slightly shifted (H-12a: +0.2 ppm, H_2-3: +0.2/+0.5 ppm, C-3: −3 ppm). All other ^{1}H and ^{13}C-signals of **2** were almost unchanged (±0.1 ppm for ^{1}H, and ±1 ppm for ^{13}C, respectively). This proved that **3** had the same parent structure as **2**, plus a side chain, which was attached via the hydrazide moiety. Five additional proton signals, among them a prominent singlet at 8.29 ppm, and six additional carbon signals were observed. DEPT 90 and DEPT 135 spectra indicated that these ^{13}C-signals were one methylene group, three methine groups and two quaternary carbons. Interpretation of the ^{1}H–^{1}H COSY (Fig. 3), and ^{1}H–^{13}C HMQC spectra of the new compound and ^{1}H–^{13}C HMBC correlations from H-2″ to C-3″, H-4″ to C-2″/C-3″/C-5″/C-6″, H-5″ to C-3″/C-4″/C-6″, and H-7″ to C-5″/C-6″ led to the structure of a hydroxymethylfuran moiety, which was connected to **2** via an imine bond. ROESY, and ^{1}H–^{15}N HMBC correlation experiments were completely consistent with the proposed structure of **3**. The strong cross peak between H-2″ and H-3 in the ROESY spectrum showed the E-configuration of the new double bond.

3.5. IR analysis of compound *3*

Infrared spectra of **2** and **3** were recorded and compared. Both spectra exhibited a similar fingerprint. The double peak of the two amide groups at 1671 cm^{-1} and 1654 cm^{-1} in the spectrum of **2**, turned into one broader peak at 1667 cm^{-1} in the spectrum of **3**.

3.6. Proposed origin of compound *3*

According to the proposed origin, compound **3** is probably a condensation product of **2** and 5-hydroxymethylfuraldehyde **4** (Scheme 2). The hydrazide group of **2** is known to be highly reactive. The illegal health food product, presented as candy, consists mainly of sugars. 5-Hydroxymethylfuraldehyde is one of the main degradation products of sugars, formed in acidic solutions at high temperatures [8].

4. Conclusion

The interaction product of aminotadalafil **3** isolated from an illegal health food product was either intentionally synthesized by the counterfeiter, or was, most likely, a result of a drug-excipient incompatibility of **2** and 5-hydroxymethylfuraldehyde (**4**) during production and/or storage of the candies. The current findings underline once more, that the stability and other characteristics of illegal drug analogues, which are often ordered via internet channels, are poorly investigated and that these products present a serious health risk to the consumers.

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