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The identification of a nitrosated prodrug of the PDE-5 inhibitor aildenafil in a dietary supplement: A Viagra with a pop

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ABSTRACT

A new unapproved analogue of sildenafil was detected in capsules of a herbal dietary supplement promoted as a libido enhancing product. Using LC–DAD–MS, MS–MS, HRMS, IR and NMR the analogue was shown to be a derivative of the PDE-5 inhibitor aildenafil with a nitrosamine moiety. A hydrolysis experiment showed that the new analogue was a prodrug of aildenafil and was therefore named nitroso-prodenafil. A capsule contained 108 mg of nitroso-prodenafil which is equivalent to 84 mg of aildenafil and 5.1 mg of nitrogen monoxide (NO). Although it is unknown how much NO can be usefully generated there is 3-fold more NO present than in a 10 mg isorbide nitrate tablet.

Both PDE-5 inhibitors and nitrosamines cause vasodilatation by increasing levels of NO. To their coincidental use is warned against because it may cause a fatal drop in blood pressure. In addition, nitrosamines are known carcinogens. This is the first time a PDE-5 inhibitor and a potential NO donor were identified in one molecule. The findings indicate the dangerous level of advancement in medicinal chemistry by producers of unapproved drugs.

1. Introduction

The erectile dysfunction (ED) drug Viagra (sildenafil citrate) or other PDE-5 inhibitors are not to be used coincidentally with NO-donors [1–3]. NO-donating drugs are used in the treatment of angina pectoris (e.g. glyceryltrinitrate, isorbide nitrate) and act by causing coronary vasodilatation. Originally, sildenafil was developed as an alternative treatment for angina pectoris because it raised NO-levels through PDE-5 inhibition. However, in clinical trials the vasodilatation caused in the corpus cavernosum of the penis was more effective. Though the efficacy of sildenafil in inducing coronary vasodilatation was limited, coincidental use of drugs that give off NO may cause a dangerous drop in blood pressure [4,5]. ED patients using PDE-5 inhibitors are warned against the concomitant use of NO-donating medicine and 'poppers' which are NO-releasing recreational (sex) drugs [6].

It has been estimated that for every two legitimate prescriptions of an ED drug in Europe one third is purchased from illegal sources [7]. This ratio is expected to increase when adulterated dietary supplements are also considered. The booming market for illegal ED drugs is illustrated by the identification of many unapproved PDE-5 inhibitors [8]. Most of the unapproved PDE-5 inhibitors identified are structural varieties (analogues) of the approved ED-drugs sildenafil, vardenafil and tadalafil. Although many of these analogues are disclosed in Pfizer, Bayer, or Lilly patents as PDE-5 inhibitors they were never selected for clinical development. As patent literature is publicly accessible it can easily be misused.

The analogue identified in the current study is a cleverly designed nitrosated prodrug of aildenafil (Fig. 1). An N-methyl-N-nitroso-1,3-thiazole-5-amine moiety is linked to aildenafil through a hydrolyzable bond. The finding described in this article shows that the producers of these pharmaceuticals evidently invest time, resources and knowledge in the development of novel analogues and are concomitantly prepared to take large health risks. Nitrosamines may generate NO and enhance the vasodilating effect of a PDE-5 inhibitor with the risk of a dangerous drop in blood pressure [9,10]. The potentially toxic nature of nitrosamines is illustrated by the slimming drug nitroso-fenfluramine causing liver failure on a large scale [11].

2. Materials and methods

2.1. General

Reference sildenafil citrate, vardenafil·HCl·3H_2O and tadalafil were obtained from Pfizer, Bayer and Lilly, respectively. Methanol

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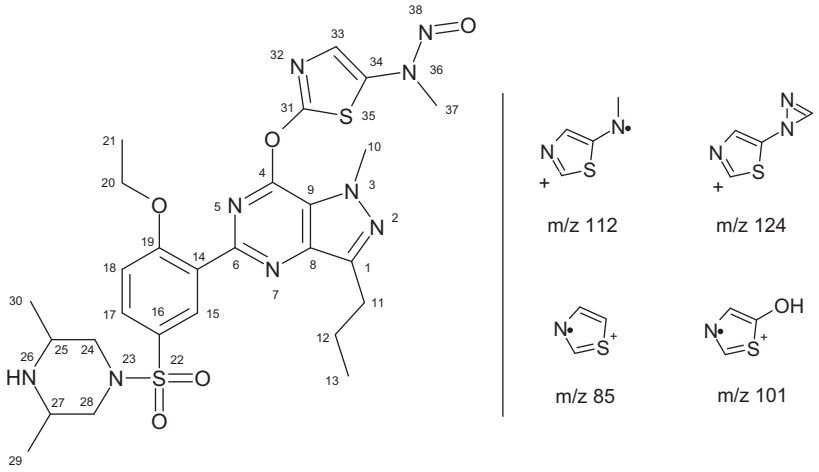

Fig. 1. The proposed structure of the new analogue prodrug and the suggested fragments for 'Moiety142'.

(HPLC supra-gradient; MeOH) was obtained from Biosolve (Valkenswaard, The Netherlands). Formic acid (p.a.), ammonium hydroxide (p.a.) and 2N NaOH were obtained from Merck (Darmstadt, Germany). Other chemicals were obtained from various commercial sources in the highest purity grade available. Water was demineralised and filtered using a Millipak® 200 0.22-μm filter from Millipore B.V. (Amsterdam, The Netherlands). Acidified water was prepared by addition of 2 ml of formic acid to 1000 ml of water and adjusting the pH to 4.0 using NH_4OH.

One package with two capsules of the herbal dietary supplement was brought in for analysis. The product label listed 9 herbal ingredients and a content of 560 mg per capsule. However, a capsule contained, on average, 386 mg of a white powder. A composite was prepared of the two capsules.

2.2. LC–DAD–MS

A stock solution of the sample was prepared by sonication of an amount of the composite equivalent to a quarter dosage unit in 25 ml of MeOH. An aliquot of the supernatant liquid was diluted 100× using MeOH/acidified water (v/v = 50/50), which solution was subsequently used for LC–DAD–MS^n analysis. All samples and solutions were filtered before use over a 0.45 μm Spartan 30 filter (Whatman GmbH, Dassel, Germany). For chromatography and UV detection, a Surveyor instrumentation (Thermo Finnigan, Breda, The Netherlands) was used under the following conditions: XTerra MS® C_{18} guard column (20 mm × 2.1 mm, 3.5 mm) and XTerra MS® C_{18} analytical column (100 mm × 2.1 mm, 3.5 mm; Waters Chromatography B.V., Etten-Leur, The Netherlands); elution using solvent A—MeOH and solvent B—acidified water/MeOH (v/v = 50/50): 0–15 min isocratic (B = 100), 15–17 min gradient to A/B = 60/40, 17–28 min isocratic A/B = 60/40, 28–33 min: isocratic B = 100; flow rate at 0.25 ml min^{-1}; column temperature 25 °C; injection volume 20 μl; ultraviolet light detection from 200 to 450 nm.

Mass spectrometry was carried out inline with LC–DAD using an ESI interface and an LCQ Advantage ion trap mass spectrometer, operated by Xcalibur software version 1.4 (Thermo Finnigan B.V). The ESI interface was used in the positive ion mode unless noted otherwise. Nitrogen was used as sheath gas (19 arbitrary units) and as auxiliary gas (10 arbitrary units). Source settings used: electrospray voltage 5.0 kV, capillary temperature 300 °C, capillary voltage 31 V, tube lens offset 55 V. Full scan: mass range *m/z* 80–1100 Da. MS/MS: relevant MS^1 ions were selected and fragmented using collision induced dissociation (CID) at an energy of 40.00 arbitrary units using an isolation width of 1 Da.

2.3. High resolution mass spectrometry (HRMS)

A small amount of the composite (20 mg) was dissolved in 1 ml of $CHCl_3$. The clear supernatant was collected after centrifugation and evaporated to dryness. The residue was reconstituted in 100 μl of water/acetonitrile (3:1, v/v) + 0.1% (v/v) formic acid and also containing 1 pmol/μl of the peptide Angiotensin-III as internal lock mass calibrant at the *m/z*-value of the doubly charged ion @ 466.26106 Da. A 10-μl aliquot of this mixture was pipetted into a gold-coated borosilicate glass nanospray needle (manufactured in-house, essentially as described by Wilm and Mann [12]) and positioned in a static nanoelectrospray ion source (built in-house) that was mounted onto an LTQ Orbitrap XL mass spectrometer (Thermo Scientific). The sample was continuously infused into the mass spectrometer operated in positive ion mode, using an electrospray voltage of 800 V and a heated capillary temperature of 200 °C. Automatic gain control was used to control the filling of the mass analyzer at a full MS target of 5×10^5 ions in the Orbitrap mass analyzer (operated at a mass resolution of 100,000 FWHM @ *m/z* 400 Da) or at a MS/MS target of 1×10^4 ions in the LTQ ion trap. Full scan MS spectra were acquired from *m/z* 150–1500 Da with 1 microscan and a maximum inject time of 150 ms. For structural elucidation, analyte ions of interest were fragmented in the ion trap with collision induced fragmentation (CID) and the resulting product ions were mass-analyzed in the Orbitrap analyzer at a mass resolution of 100,000 FWHM @ *m/z* 400 Da.

2.4. IR spectroscopy

The unknown compound was coated on KBr for IR analysis by mixing 20 mg KBr with 2 ml of the stock solution prepared under Section 2.2. After evaporation of the MeOH under a stream of N_2 a sample of the dry powder was used for IR analysis (KBr) in a Bruker IFS55 FT-IR spectrometer with a DTGS detector and OPUS software version 4.0.

2.5. Isolation of the unknown compound

A small amount of the composite (25 mg) was purified using a home-made preconditioned 3-cm silica-gel micro-column made in a Pasteur pipette. Using CH_2Cl_2/MeOH (9:1, v/v) mixture as an eluent fractions of about 1 ml each were collected. Fractions were analyzed using TLC (Merck, Silicagel 60 F_{254}). A pure fraction with the most intense spot under UV-light was evaporated under a stream of N_2 and was used for further identification.

2.6. Hydrolysis

To cleave the bond linking aildenafil and the unidentified group a hydrolysis experiment was set-up. The pure but less concentrated fractions isolated under Section 2.5 were mixed in a test-tube and evaporated under a stream of N_2. After adding 2 ml of 2N HCl the tube was closed with a plastic cap and was left at room temperature to avoid cleavage of the sulfonamide bond [13]. After 24 h a sample was taken for LC–DAD–MS^n analysis. After 7 days the mixture was evaporated under a stream of N_2 and was used for NMR analysis.

The original sample solution prepared in Section 2.2 was reanalyzed by LC–DAD–MS^n and by direct infusion MS^n after storage in the refrigerator for 6 weeks to investigate the hydrolysis. The sample was analyzed using the settings described earlier but now both in the ESI+ and ESI− mode with a range of *m/z* 50–2000 Da. Standard aildenafil was used a reference in the direct infusion MS experiments.

2.7. NMR

The purified unknown compound (or a chloroform dissolution of a crude extract) was dissolved in 0.55 ml of $CDCl_3$ for NMR analysis. One and two dimensional experiments were recorded on a JEOL JNM400 spectrometer and a Bruker AVANCE 700 equipped with a cryogenic probehead. Standard experiments such as DEPT-135, COSY, NOESY, ^1H,^{13}C-gradient-HMQC, ^1H,^{13}C-gradient-HMBC and ^1H,^{15}N-gradient-HMBC were recorded to assign all proton, carbon and most nitrogen resonances. For 2-D nuclear overhauser enhancement difference spectroscopy (NOEDIFF) 2 mg of the capsule content was dissolved in DMSO-d_6. Quantification of the sample was carried out by adding 3 mg of caffeine as an internal standard to a crude chloroform extract of 52 mg of the capsule content. Signal integration was performed on the well-resolved N-methyl resonances from the sample and the standard. A 30 degrees proton pulse and a 10 s relaxation delay were used to ensure complete relaxation of the signal and obtain reliable integrations.

3. Results and discussion

3.1. LC–DAD–MS

The total ion chromatograms for the standard mixture and the dietary supplement are shown in Fig. 2. The components in the standard mixture were well resolved and elute in the following order: sildenafil, vardenafil and tadalafil. The unknown component eluted at 10.78 min and had a UV_{max} at 241 and 301 nm which was unlike any of the components of the standard mixture or the known thiono-analogues of sildenafil [14] (Fig. 3). The unknown component generated an $[M+H]^+$ ion at *m/z* 630 and an $[M+Na]^+$ ion at *m/z* 652 (Fig. 4). Fragmentation of the $[M+H]^+$ ion showed product ions at *m/z* 600 (6%), 487 (19%), 377 (100%).

The fragment at *m/z* 377 is a characteristic ion for vardenafil, sildenafil and their analogues following the cleavage of the sulphonamide bond [15]. The fragment at *m/z* 487 was only two mass units below vardenafil, homosildenafil or aildenafil. The fragment at *m/z* 600 corresponds to a loss of 30 Da which can only be attributed to NO, CH_2O or C_2H_6. The possibility of the unknown compound being an NO-donor warranted further investigation.

The full scan mass chromatogram clearly showed peaks with *m/z* 142 and *m/z* 487 ions coinciding with the $[M+H]^+$ peak at *m/z* 630 (Fig. 5). In addition, in the MS^2 trace for *m/z* 489, two peaks were observed of which the second eluting peak coincided with the unknown compound (Fig. 5). The MS^2 spectra for the two peaks could not be distinguished from each other. Ions were observed at *m/z* 472 (20%), 432 (40%), 377 (100%), 313 (30%), 311 (30%) and 283 (12%), all of which are characteristic for aildenafil (=methisodenafil) [13]. Therefore, there is evidence for the presence of aildenafil (1st eluting peak) and an aildenafil derivative (2nd eluting peak). Aildenafil may be present as a synthesis impurity or as a degradation product.

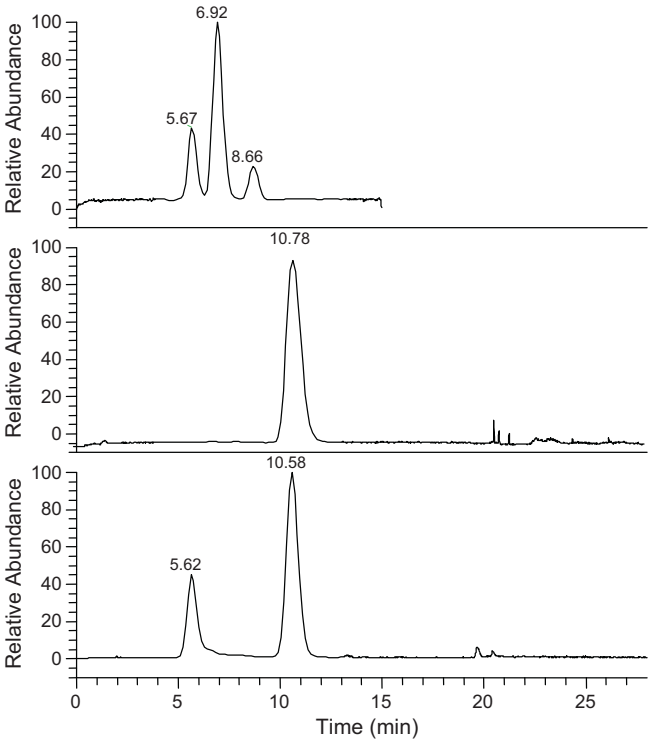

Fig. 2. Full scan ion chromatograms for the standard mixture (top) recorded up to 15 min, for the unknown compound (middle), and after partial hydrolysis of the unknown compound (below).

The MS data showed the unknown compound (*m/z* 630) was cleaved into two parts (*m/z* 489 and *m/z* 142). One of them being the protonated aildenafil part (*m/z* 489) and the other is an unknown group, designated as 'Moiety142'. The ion at *m/z* 487 Da suggested

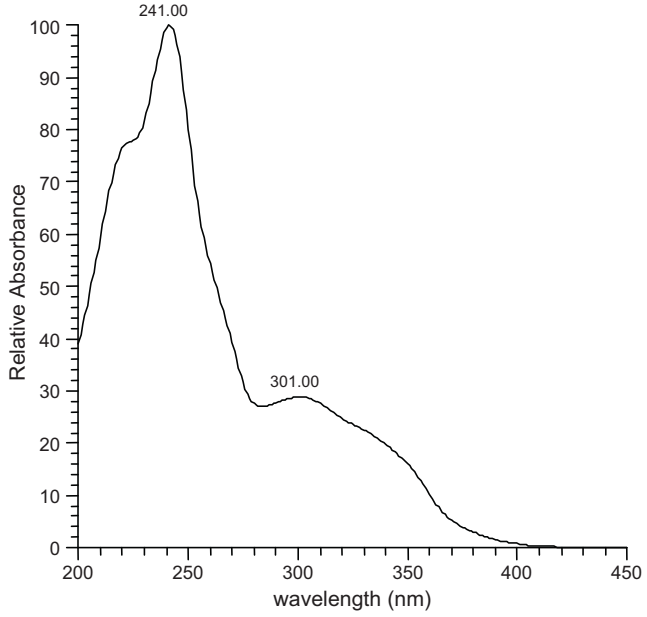

Fig. 3. UV absorbance spectrum of the unknown component.

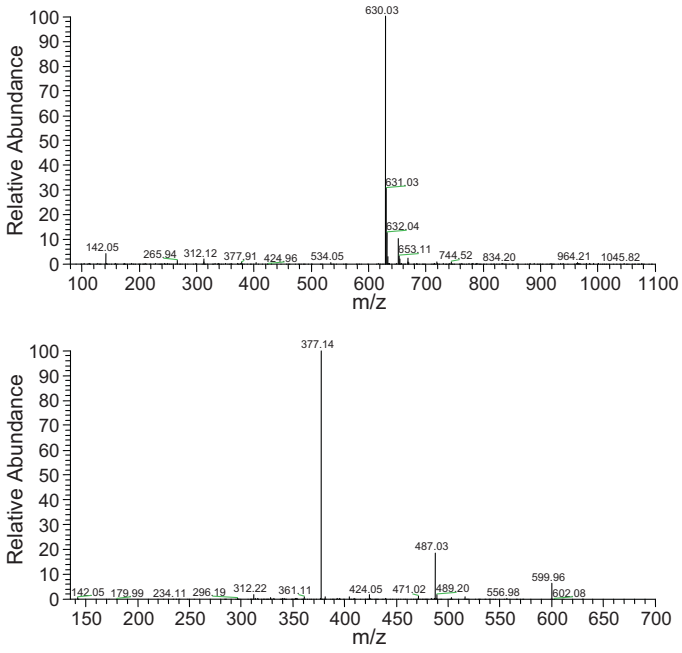

Fig. 4. The full scan (top) and MS^2 (middle) mass spectra for the unknown component.

'Moiety142' might located at the piperazine ring. Fragmentation of the ion at *m/z* 142 ('Moiety142') showed ions at *m/z* 124 (7%), 112 (15%), 101 (16%), *m/z* 85 (100%), *m/z* 74 (7%), and *m/z* 67 (10%).

3.2. HRMS

The accurate mass found for the $[M+H]^+$ ion of the unknown compound was 630.22786 Da. In addition, close examination of the molecular ion isotope cluster, showed 2 $[(M+2)+H]^+$ ions, one of them being the $^{13}C_2$-molecular ion and the other suggested as a

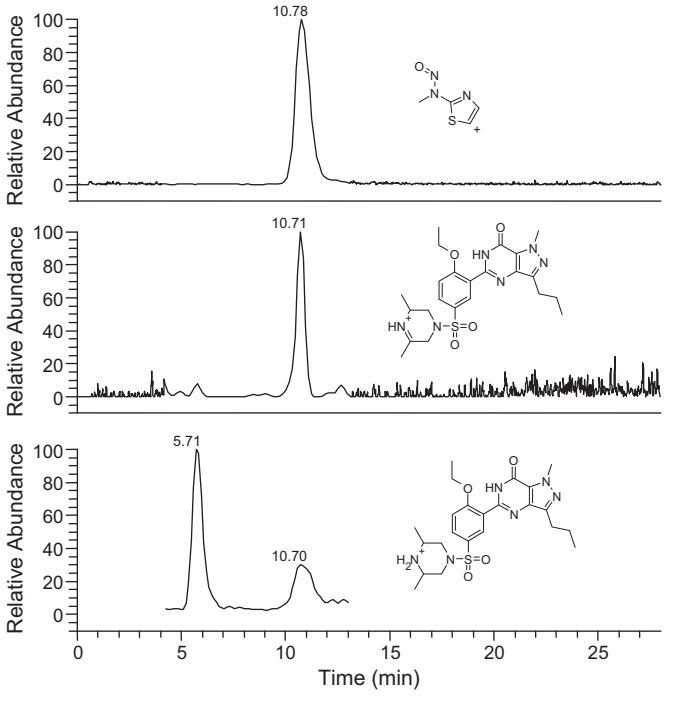

Fig. 5. Full scan ion chromatograms for the dietary supplement filtered for *m/z* 142 (top), *m/z* 487 (middle) and the MS^2 ion trace for *m/z* 489 (below).

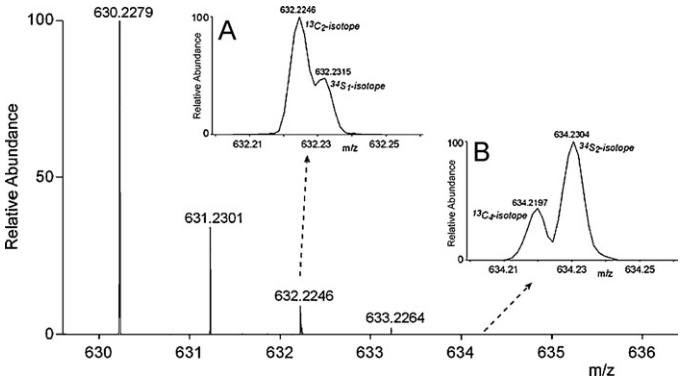

Fig. 6. The HRMS isotope pattern for the unknown compound showing the exact mass and the presence of 2 S-atoms.

^{34}S-isomer, based on its accurate mass. Furthermore, the presence of a significant $[(M+4)+H]^+$ ion was indicative for the presence of two ^{34}S-isomers in the molecule of interest (Fig. 6). The accurate mass of the native compound corresponds to a molecular formula of $C_{27}H_{35}N_9O_5S_2$ (mass error <1 ppm). Being an aildenafil derivative, the molecular formula therefore consists of $[C_{23}H_{31}N_6O_4S]^-$ part for aildenafil and a $[C_4H_4N_3OS]^+$ part for 'Moiety142'.

Fragmentation showed ions at *m/z* 600.23005 (loss of 29.99810 Da) which unambiguously confirmed the loss of one NO moiety from the molecule. Other fragments were observed at *m/z* 489.22779 ($C_{23}H_{33}N_6O_4S^+$ = [aildenafil+H]$^+$), 487.21226 ($C_{23}H_{31}N_6O_4S^+$ = [aildenafil−H]$^+$), 377.12793 (suggested molecular formula $C_{17}H_{21}N_4O_4S^+$), and 142.00735 (suggested molecular formula $C_4H_4N_3OS^+$) (errors < 1 ppm). The molecular formula of $C_{17}H_{21}N_4O_4S^+$ corresponds to the aromatic core of aildenafil after cleavage of the sulphonamide bond [15].

3.3. IR spectroscopy

The KBr recording of the unknown component showed a strong absorption band at 1458 cm^{-1} which is an absorbance characteristic for nitrosamines [16]. The IR spectrum lacked the characteristic strong lactam absorption band at 1695 cm^{-1} observed for sildenafil and its analogues [14]. Instead, a medium absorption band was observed at 1595 cm^{-1}.

3.4. Isolation of the unknown compound

Eight fractions were collected from the micro-column. TLC analysis showed fraction 2 to have a single and intense UV active spot at R_f = 0.8. Fractions 3 and 4 also had a single UV active spot but were less concentrated. Fraction 2 was used for NMR analysis (Section 3.6) and the mixed fractions 3 and 4 were used for the hydrolysis experiment.

3.5. Hydrolysis

A 20-μl sample from the hydrolysis experiment was taken after 24 h and was diluted with 1 ml MeOH/acidified water (50/50). LC–DAD–MS (ESI+) analysis of the hydrolyzed sample showed two distinct peaks (Fig. 2, below). The first eluting component could be positively identified as aildenafil based on RT, UV absorbance spectrum, MH^+, and MS^2 ions. The expected hydrolysis product (hydroxy-'Moiety142') could not be observed using this method.

Reanalysis of the original sample solution after 6 weeks showed a peak for aildenafil in the chromatogram of approximately 10% the height of the peak for the unknown compound. This shows the unknown compound slowly hydrolyzed in the solvent mixture

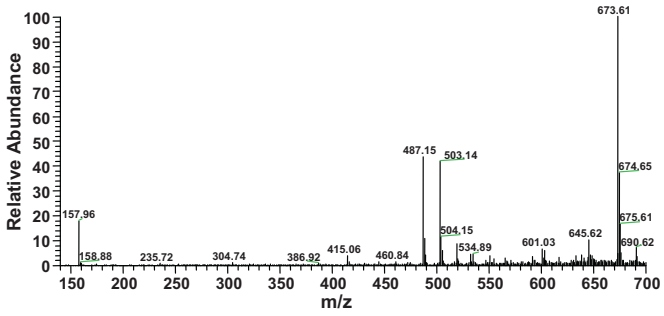

Fig. 7. The full scan mass spectra (ESI−) for the partially hydrolyzed sample obtained by direct infusion MS.

when kept in the refrigerator. Using LC–DAD–MS analysis in the negative ion ESI mode (ESI−) peaks the only peaks observed were for aildenafil (m/z 487, $[M-H]^-$) and for the unknown compound (m/z 503, $[M-126]^-$).

Direct infusion MS (ESI+) on the original 6 weeks old sample solution showed $[M+H]^+$ and $[M+Na]^+$ ions for aildenafil and the unknown compound. However, the other hydrolysis product expected, hydroxy-'Moiety142', was not observed under ESI+ conditions.

Using ESI− (full scan mode, capillary temperature at 200 °C) ions were observed at m/z 674 (100%), 503 (42%), 487 (43%) and 158 (18%) (Fig. 7). The ion at m/z 674 was attributed to the formiate adduct ($[M+HCO_2]^-$) and this ion was not observed at higher capillary temperatures. Fragmentation of the ion at m/z 674 gave a too poor signal despite several attempts to tune.

Fragmentation of the ion at m/z 158 showed ions at m/z 143 (20%), 128 (100%), 113 (20%), 74 (25%). The ions at m/z 143, m/z 128 and m/z 113 respectively indicate dehydroxylation, the loss of NO, and both of these processes, respectively. Therefore the ion at m/z 158 was attributed to hydroxy-'Moiety142'.

Fragmentation of the ion at m/z 487 showed ions at m/z 259 and 282 which is identical to the ions observed for reference aildenafil and can be explained by O-dealkylation of the EtO-group (−28 Da) and the sum of O-dealkylation and loss of the sulphonamide (−205 Da). Fragmentation of the ion at m/z 503 showed ions at m/z 475 and 298. The parent and the product ions are consistently 16 Da higher than those observed for aildenafil and were not observed for reference aildenafil. This similarity in fragmentation indicates 'Moiety142' is attached to the aromatic core of the aildenafil.

3.6. NMR

The ^{1}H- and ^{13}C-NMR spectra show a strong resemblance with aildenafil, indicating the unknown compound was an aildenafil derivative (Table 1). The quality of the spectra for the purified unknown compound and a crude extract were comparable. Initial analysis of the 1D-proton NMR spectrum revealed one extra proton at 7.95 ppm and the absence of the lactam proton usually observed around 10.7 ppm in sildenafil and aildenafil compounds [13,17]. In addition, the H15 signal had shifted upfield by about 1 ppm relative to the corresponding proton in aildenafil. To confirm the unknown compound was an aildenafil derivative the 7-day old hydrolysis mixture was analyzed by ^{1}H-NMR. Surprisingly, the unknown compound had completely degraded to aildenafil and the ^{1}H-NMR showed a relatively pure spectrum of aildenafil with its

Table 1
NMR data for nitroso-prodenafil (δ in ppm).

Atom #	δ ^{1}H	Multiplicity	δ ^{13}C	NOEDIF	δ ^{15}N[a]
1	–	–	147.08		–
2	–	–	–		131.4
3	–	–	–		167.9
4	–	–	149.13		–
5	–	–	–		n.o.[b]
6	–	–	126.78		–
7	–	–	–		n.o.
8	–	–	143.74		–
9	–	–	128.42		–
10	4.47	3H, s	39.27		–
11	3.05	2H, t, J = 7.7 Hz	27.92	H-12	–
12	1.94	2H, m	22.12	H-11, H-13	–
13	1.25	3H, t, J = 7.3 Hz	14.03	H-12	–
14	–		154.74		–
15	7.91	1H, d, J = 2.6 Hz	131.08	H-37	–
16	–		127.27		–
17	7.70	1H, dd, J = 2.6 Hz, J = 8.8 Hz	130.19		–
18	7.07	1H, d, J = 8.8 Hz	113.25		–
19	–		160.94		–
20	4.15	2H, q, J = 7.0 Hz	64.91	H-21	–
21	1.43	3H, t, J = 7.0 Hz	14.50	H-20	–
23	–		–		68.3
24, 28	1.763.57	2H, q, 10.2 Hz2H, t, J = 10.6 Hz	52.08	H-17, H-29, H-30, H-33	–
25, 27	2.98	2H, m	50.22		–
26	–		–		101.7
29, 30	1.04	6H, t	19.31	H-25, H-27	
31	–		115.34		–
32	–		–		54.4
33	7.95	1H, s	139.38	H-37	–
34	–		150.27		–
35	–		–		–
36	–		–		180.7
37	3.89	3H, s	33.25	H-15, H-33	–
38	–		–		n.o.

[a] The nitrogen chemical shifts were referenced indirectly to the proton chemical shifts. Under these conditions, the nitrogen chemical shifts of the N-CH_3 of the internal standard (caffeine) were resonating at 155.5, 151.2 and 151.8 ppm.
[b] Not observed.

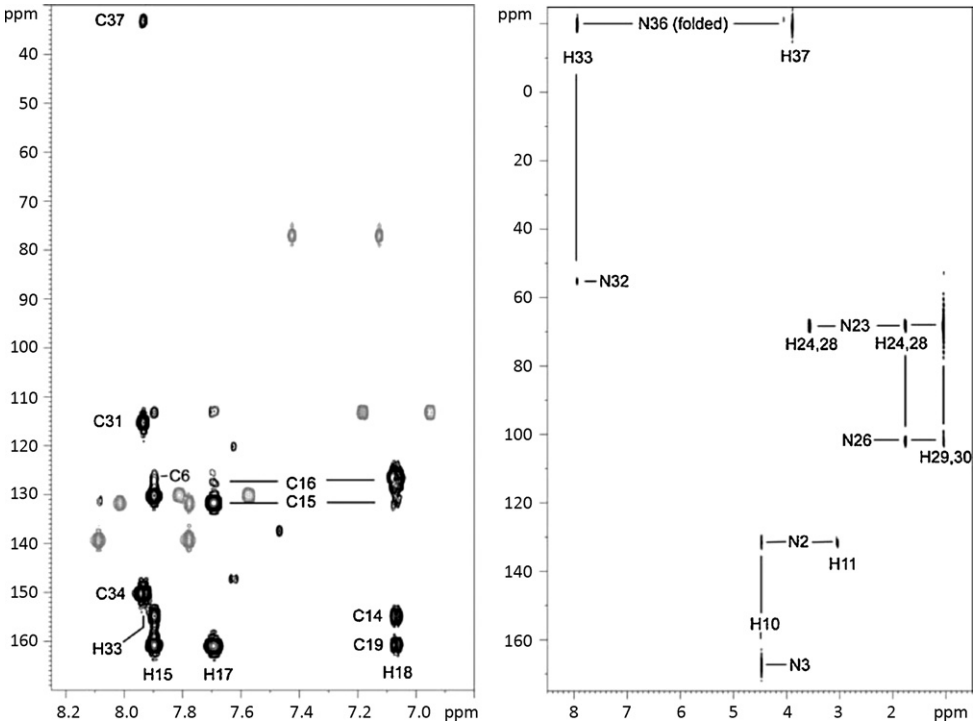

Fig. 8. Long-range ^{1}H-heteronuclear HMBC correlation. The left panel shows an expansion of the ^{1}H–^{13}C correlations in the aromatic region. The signals in grey are the one-bond correlation pairs that are incompletely filtered. The ^{1}H–^{15}N correlations are shown in the right panel. The N36 at 180.7 ppm resonance is folded to the top of the figure for clarity reasons.

chemical shift values in agreement with literature data [13]. Therefore, the unknown compound was confirmed to be an aildenafil derivative.

Relative to aildenafil, the ^{1}H-NMR for the unknown compound showed four additional protons and four additional carbon atoms which was in accordance with the molecular formula suggested by HRMS. The additional signal at 7.9 ppm (s, 1H, non-exchangeable) and 3.8 ppm (s, 3H) which were respectively interpreted as an aromatic proton (Ar-H) and a CH_3-group connected to a hetero atom. The additional protons showed a strong NOE interaction implying they were in each others proximity. The ^{13}C-NMR showed the presence of one aliphatic C-atom (33.25 ppm) and three aromatic C-atoms (115.34, 139.38 and 150.27 ppm). The ^{15}N-NMR showed the presence of two additional N-atoms which were interpreted as an aromatic (54.4 ppm) and an NCH_3-group (180.7 ppm). The ^{15}N signal for the NO-group was not observed. However, the observed ^{13}C and ^{15}N chemical shifts were in agreement with an N-methyl-nitrosamine attached to an aromatic group. Quantitative NMR showed the presence of 108 mg of the unknown compound per dose unit which is equivalent to 84 mg of aildenafil. The principle correlations for 'Moiety142' observed in 2D-NMR are presented in Fig. 8 and Table 2.

3.7. Structure elucidation

Although the fragment ion (ESI+) at *m*/*z* 487 points at the piperazine ring as the derivatization site of aildenafil, the majority of the evidence indicates that 'Moiety142' is located at the lactam. The formation of the *m*/*z* 487 ion may be the result of a gas-phase transfer reaction between the piperazine and the lactam similar to those described for (thio)-sildenafil analogues in literature [18,19].

^{1}H-, ^{13}C-NMR, MS, IR and the hydrolysis experiment indicate that the unknown compound consists of a 'Moiety142' ($C_4H_4N_3OS$) coupled to the lactam of aildenafil. Literature shows comparable lactams are easily derivatized on the O-atom in the enolic tautomeric form [20,21] which would explain the absence of the characteristic C=O absorption band in IR for sildenafil analogues [14] and the absence of an NH lactam proton in ^{1}H-NMR.

'Moiety142' was shown to contain three aromatic C-atoms, one aromatic N-atom, one aromatic H-atom, an aromatic NCH_3-group and an NO-group. Therefore, 'Moiety142' must have a (functionalized) aromatic nucleus of molecular formula C_3NS that is able to accommodate the identified substituents and is able to form a hydrolyzable bond to the lactam.

The only aromatic nuclei meeting these requirements were 1,2-thiazoles (=isothiazole) and 1,3-thiazoles (=thiazole). The observed chemical shift for the ^{15}N-atom in the aromatic nucleus at 54.4 ppm clearly showed that 'Moiety142' was a thiazole since ±80 ppm is expected for an isothiazole. In addition, NOE and HMBC data showed the aromatic proton and the NCH_3-group must be in adjacent positions which placed them at the thiazole-C4 and -C5 positions (Fig. 8). According to HMQC and HMBC data the aromatic proton was attached to the thiazole-C4 position (Table 2). Consequently, aildenafil

Table 2
Principle correlations in the gHMQC and gHMBC spectra of 'Moiety142'.

^{1}H (δ, ppm)	^{13}C (δ, ppm)		^{15}N (δ, ppm)
	gHMCQ	gHMBC	gHMBC
7.86 (H33)	139.39 (C33)	33.25 (C37)115.34 (C31)150.27 (C34)	54.4 (N32)180.7 (N36)
3.89 (H37)	33.25 (C37)	139.39 (C33)150.27 (C34)	180.7 (N36)

must be attached to the thiazole-C2 position and an N-methyl-nitrosamine must be attached to the thiazole-C5. The thiazole substituents being directed away from the bulk of the molecule would explain the absence of NOE interactions with H10. The presence of NOE interaction between H37 and H15 was in agreement with 'Moeity142' connected on the aildenafil oxygen on C4.

Based on the experimental data collected the unknown compound was identified as 2-({5-[5-(3,5-dimethylpiperazine-1-sulfonyl)-2-ethoxyphenyl]-1-methyl-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-7-yl}oxy)-N-methyl-N-nitroso-1,3-thiazol-5-amine. Many N-aryl-N-methyl-nitrosamines are known NO-donors and some have vasodilating properties [9,10]. Vasodilatation is the most probable intention for creating this derivative. However, some N-aryl-N-methyl-nitrosamines also generate the mutagenic diazomethane. The generation of diazomethane in the MS was in agreement with the fragments observed for 'Moiety142' at *m*/*z* 101 and 85 (Fig. 1). Structure activity relations for sildenafil show that it is unlikely this O-lactam derivative is a potent PDE-5 inhibitor [22–24]. This suggests the acid labile derivative of aildenafil was purposefully designed as a prodrug with dual action: (1) causing vasodilatation by generating NO and (2) causing vasodilatation with the hydrolysis to aildenafil. Because the unknown compound was shown to be a nitrosated prodrug of aildenafil it was named nitroso-prodenafil.

4. Conclusions

The dietary supplement investigated contained 108 mg of a new analogue of the potent PDE-5 inhibitor aildenafil. Based on MS, NMR and IR we propose this compound to be 2-({5-[5-(3,5-dimethylpiperazine-1-sulfonyl)-2-ethoxyphenyl]-1-methyl-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-7-yl}oxy)-N-methyl-N-nitroso-1,3-thiazol-5-amine. The analogue was named nitroso-prodenafil because it is probably developed to act as an aildenafil prodrug and to enhance its effect by generating NO.

The dosage of 108 mg nitroso-prodenafil is equivalent to 84 mg aildenafil and 5.1 mg of NO. Although it is unknown how much NO can be usefully generated there is 3-fold more NO present than in a 10 mg isorbide nitrate tablet.

This study shows that producers of these pharmaceuticals have ascended to a dangerous level of advancement in medicinal chemistry. This new analogue has been developed regardless of the risks that come with concomitant use of PDE-5 inhibitors and NO donors or with the use of potentially toxic nitrosamines. Considering the use of illegal erectogenics as lifestyle drugs is widespread medical practitioners should be on the lookout for signs of intoxication.

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