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## Isolation and identification of ten new sildenafil derivatives in an alleged herbal supplement for sexual enhancement

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1 **Isolation and identification of ten new sildenafil derivatives in an alleged**  
2 **herbal supplement for sexual enhancement**

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17 **Abstract**

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2 18 A sexual enhancer dietary supplement in pre-commercialization phase was analyzed. It  
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4 19 contained the two phosphodiesterase-5 inhibitors (PDE-5i) sildenafil and methisosildenafil as  
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6 20 major adulterants. Fourteen more sildenafil derivatives were detected and after isolation, their  
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8 21 structures were elucidated thanks to NMR, high resolution and tandem mass spectrometry, and  
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10 22 UV spectroscopy. Ten of them were never described. All these compounds are probably by-  
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12 23 products of different reaction steps during the synthesis of the two PDE-5i that were not  
13  
14 24 properly eliminated during the purification procedure. The total amount of sildenafil-related  
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16 25 compounds was estimated at 68 mg per capsule, sildenafil and methisosildenafil accounting for  
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18 26 20 mg and 38 mg respectively.  
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26 **Keywords:** SFC; HPLC; NMR; mass spectrometry; adulteration; dietary supplement;  
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## 29 **1. Introduction**

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2 30 Nowadays, consumers' keen interest in plant food supplements stems from their  
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4 31 sometimes inaccurate perception that these products are safe because of their natural origin. In  
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6 32 the absence of strict and uniform regulation and control of these products, unscrupulous  
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8 33 manufacturers may fraudulently add active substances to these herbal preparations to increase  
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10 34 the desired pharmacological effect. The phenomenon of adulteration of sexual enhancer dietary  
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12 35 supplements with synthetic phosphodiesterase-type 5 inhibitors (PDE-5i), whether approved  
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14 36 (sildenafil, tadalafil, vardenafil) or unapproved (their analogues), has been widely documented  
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16 37 [1-4]. Since the detection of homosildenafil, the first analogue reported in literature in 2003 as  
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18 38 adulterant in a dietary supplement [5], their number has regularly increased to 80 in March 2017  
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20 39 [6] and continues to increase, as evidenced by recent studies [see for example 7-9].  
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27 40 In the present study, sildenafil and fifteen analogues were isolated from a dietary  
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29 41 supplement intended for marketing, using Supercritical Fluid Chromatography (SFC) and  
30  
31 42 Liquid Chromatography (LC) purification methods. All of the compounds were then fully  
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33 43 identified by means of ultraviolet (UV) spectroscopy, mass spectrometry (MS), and <sup>1</sup>H and <sup>13</sup>C  
34  
35 44 nuclear magnetic resonance (NMR). To our knowledge, ten of these compounds are new and  
36  
37 45 have never been reported in literature.  
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## 43 47 **2. Experimental**

### 44 48 *2.1. Materials*

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46 49 One dietary supplement in pre-commercialization phase was submitted for analysis. Its  
47  
48 50 composition was claimed as follows: raspberry (*Rubus idaeus*) 25%, Solomon's seal  
49  
50 51 (*Polygonatum sibiricum*) rhizome 25%, common yam (*Dioscorea opposita*) rhizome 20%,  
51  
52 52 barbary wolfberry (*Lycium chinense*) fruit 15%, cassia (*Cinnamomum cassia*) bark 15%.  
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54 53 Twenty-four capsules containing each 360 ± 20 mg of brown powder were used for this study.  
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54 All chromatographic solvents (RS-HPLC-Preparative grade for purification steps with  
55 preparative HPLC and RS-HPLC-GOLD-Ultra gradient grade for analytical purposes) were  
56 purchased from Carlo Erba (27100 Val de Reuil, France). Authentic standards of sildenafil and  
57 methisosildenafil were supplied by TRC (North York, ON, Canada). All other chemicals and  
58 reagents used as well as the NMR reference for internal chemical shift and quantification  
59 (sodium 2,2,3,3-tetradeutero-3-(trimethylsilyl) propanoate (TSP)) were supplied by Sigma  
60 Aldrich (St. Louis, MO, USA). Deuterated solvents were obtained from Euriso-Top (91194  
61 Saint Aubin, France).

## 62 63 *2.2. Purification of active compounds*

### 64 *2.2.1. Sample preparation prior to SFC experiments*

65 A preliminary analysis showed that the sample contained a sort of tar which could  
66 prevent purification or complicate further purification steps. To get rid of it, a first pre-  
67 extraction procedure was implemented. The powder from 1 capsule was treated in two steps. It  
68 was first extracted with 3 mL of a CH<sub>3</sub>CN:H<sub>2</sub>O (80:20 v/v) mixture, vortexed for 15 s, sonicated  
69 for 10 min and centrifuged (3000 rpm) for 5 min at room temperature. The supernatant (2800  
70 µL) was collected and transferred into a glass tube. In a second step, the pellet was re-extracted  
71 with the same protocol using 1 mL of solvent. The supernatants were then pooled and the  
72 solution was evaporated to dryness. This procedure was repeated for 24 capsules. For SFC  
73 purification, 2.9 g of the residual powder were dissolved by sonication in 58 mL of methanol.  
74 Before injection, samples were filtered on 0.45 µm GHP membranes.

### 75 76 *2.2.2. Preparative SFC*

77 All preparative SFC separations were carried out on a BetaSil Diol-100 (250 x 21.2 mm,  
78 5 µm) column using a Berger Multigram II Preparative SFC system (Mettler Toledo, Viroflay,

79 France) and the SFC ProNT0 software. Preparative SFC was operated with an elution gradient  
80 at a back pressure of 100 bar, a temperature of 40°C, a flow rate of 42.5 mL min<sup>-1</sup> and a UV  
81 detection at 235 nm. The gradient started by an isocratic elution of CO<sub>2</sub> and 10% methanol  
82 containing 0.5% (v/v) isopropylamine during 0.5 min, and the organic modifier percentage was  
83 then increased to 28% at 8 min and remained at this value until 15 min. At last the column was  
84 re-equilibrated in the initial elution conditions from 16 to 20 min (Table 1). 116 injections of  
85 25 mg of raw extract dissolved in 500 µL of methanol were done. Seven fractions were collected  
86 as indicated in the chromatogram of Fig. 1A, i.e. 3-6 min (A), 6.05-6.45 min (B), 6.5-7.8 min  
87 (C), 7.85-8.75 min (D), 8.8-10.1 min (E), 10.15-11.45 min (F) and 11.5-13.2 min (G). All  
88 fractions were then evaporated to dryness.

### 90 2.2.3. Preparative LC

91 Each SFC fraction was dissolved either in the starting LC eluent (D, E, F, G) or in a  
92 mixture of the starting LC eluent and methanol (B, C) or acetonitrile (A) (Table 1). Purifications  
93 were performed on a Waters Prep 150 LC System instrument (Waters Corporation, Milford,  
94 MA, USA) equipped with a 2545 Binary Gradient pump, a 2707 autosampler, a 2998  
95 photodiode array (PDA) detector and a WFC III fraction collector. Data were processed using  
96 the Chromscope software. Columns and elution conditions for preparative LC are reported in  
97 Table 1 and chromatograms are shown in Fig. 1B. UV detection was set at 235 nm for all  
98 experiments. Two compounds (**15** and **16**) were collected in the same fraction. All fractions  
99 recovered were lyophilized.

### 101 2.2.4. Analytical Ultra High Performance Liquid Chromatography (UHPLC)

102 Each LC fraction was dissolved in methanol at a concentration of ≈0.5 mg/mL and its  
103 purity was controlled by UHPLC using a Waters Acquity UHPLC system equipped with a

104 binary solvent delivery pump, an auto sampler, a PDA detector, a QDa mass detector and the  
105 Empower 3 software. The analytical conditions are reported in [Table 1](#). UV spectra were  
106 obtained from these UHPLC analyses.

### 108 *2.3. NMR and MS characterization of the dietary supplement and the purified compounds*

#### 109 *2.3.1. NMR*

110 Two to 7 mg of each lyophilized final fraction were solubilized in 1 mL of a  
111 CD<sub>3</sub>CN:D<sub>2</sub>O (80:20 v/v) mixture, and the solution was vortexed for 15 s and sonicated for 10  
112 min. To achieve the solubilisation of compounds **9** and **14**, 5 µL of a 0.5 M NaOD solution  
113 were added. Thirty µL of a 5 mM solution of TSP in D<sub>2</sub>O as an internal reference for chemical  
114 shift ( $\delta$ ) measurement were added before the NMR analysis.

115 For the quantitative determination of sildenafil derivatives in the dietary supplement  
116 (done in triplicate), around 10 mg of powder were accurately weighed and 1 mL of deuterated  
117 methanol (CD<sub>3</sub>OD) was added. The suspension was submitted to vortex agitation for 15 s,  
118 sonication for 10 min, magnetic stirring for 20 min and it was then centrifuged (5 min, 3000  
119 rpm). Thirty µL of a 5 mM solution of TSP were added to 800 µL of supernatant and the solution  
120 was transferred into a 5 mm NMR tube.

121 NMR spectra were recorded on a Bruker Avance 500 spectrometer (Bruker Biospin AG,  
122 Fallanden, Switzerland) equipped with a 5 mm cryoprobe at 298 K. The structural elucidation  
123 of purified sildenafil derivatives was achieved thanks to one-dimensional (1D; <sup>1</sup>H and <sup>13</sup>C) and  
124 two-dimensional (2D; gCOSY, gHSQC, gHMBC and NOESY) experiments. The 1D <sup>1</sup>H NMR  
125 spectra of the dietary supplement were acquired with inverse gated decoupling for <sup>13</sup>C using a  
126 GARP sequence. Acquisition parameters were as follows: number of scans 32, pulse width 10.7  
127 µs (flip angle 90°), acquisition time 1.56 s, spectral width 10500 Hz, 32K data points, and  
128 relaxation delay 4 s; the recording time was thus  $\approx$ 4 min. The NMR assignment of sildenafil

129 derivatives in the dietary supplement was done by adding successively the previously purified  
1 and identified compounds. For quantitative NMR experiments, the relaxation delay was  
2 130 and identified compounds. For quantitative NMR experiments, the relaxation delay was  
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4 131 lengthened to 15 s for a full relaxation of the  $^1\text{H}$  resonances and the number of scans was raised  
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7 132 to 128. NMR data were processed using the TOPSPIN 3.1 software.  
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### 12 134 2.3.2. High-Resolution Mass Spectrometry (HRMS)

14 135 Each lyophilized final fraction was dissolved in  $\text{CH}_3\text{CN}:\text{H}_2\text{O}$  (80:20 v/v) and analyzed  
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17 136 after direct infusion using a Waters XEVO G2 QTOF mass spectrometer with electrospray  
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19 137 ionization in positive ( $\text{ESI}^+$ ) and negative ( $\text{ESI}^-$ ) modes. For both modes, the instrument  
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22 138 parameters were as follows: for MS analysis, cone voltage 30 V, source temperature  $110^\circ\text{C}$ ,  
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24 139 desolvation temperature  $300^\circ\text{C}$ , cone gas flow rate  $20\text{ L h}^{-1}$ , scan range  $m/z$  50-1200; for  
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26 140 MS/MS analysis, three different collision energies (15, 25 and 35 V) were applied with the cone  
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29 141 voltage maintained at 30 V and the spectra were acquired with a mass precursor ion selection  
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32 142 of 3 Da. All analyses were performed using the lockspray, which ensured accuracy and  
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34 143 reproducibility. Leucine enkephalin ( $1\text{ ng }\mu\text{L}^{-1}$ ) introduced by a lockspray at  $3\text{ }\mu\text{L min}^{-1}$  was  
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36 144 used as the lockmass generating reference ions at  $m/z$  556.2771 or 554.2615 in positive or  
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38  
39 145 negative mode respectively. The MassLynx software was used to calculate the most probable  
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41 146 chemical formula and the theoretical exact mass of the molecular and fragment ions by  
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44 147 comparison with their measured accurate ionic masses. The elemental formula of each ion was  
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46 148 confirmed by the agreement between the experimental and calculated values within a relative  
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49 149 mass error (RME)  $<3\text{ ppm}$  or between 3 and 5 ppm for respectively 93% and 7% of ions with  
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150 a molecular weight >100 Da and <5 ppm or between 5 and 10 ppm for 69% and 31% of ions  
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2 151 with a molecular weight <100 Da.

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#### 6 7 153 *2.4. Molecular modelling*

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9 154 The chemical structures were sketched (hybridization, hydrogenation, some geometry  
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11 optimizations) using ChemAxon Marvin 17.25 (2017, ChemAxon,  
12 155 <http://www.chemaxon.com>). In order to generate a coarse-grained conformational sampling,  
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14 156 compounds **1-5** were submitted to the calculation plugins (conformers, molecular dynamics) of  
15  
16 157 Marvin using standard parameter sets. The structures were merged in SDF libraries using in-  
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18 158 house software and imported in BIOVIA Discovery Studio Client (DSV) release 2016, Dassault  
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20 159 Systèmes (<https://www.3dsbiovia.com/>) software. DSV was used to align molecules using a  
21  
22 160 reference group and to measure interatomic distances for structures of interest (i.e. lowest  
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24 161 energy conformers or frames). Molecular graphics were also produced using DSV.  
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### 31 32 33 34 164 **3. Results**

#### 35 36 165 *3.1. Preliminary <sup>1</sup>H NMR analysis of the dietary supplement*

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38 166 As soon as the dietary supplement was received for control in our lab, its <sup>1</sup>H NMR  
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40 167 analysis was performed as usually [1] in order to detect a possible adulteration. The <sup>1</sup>H NMR  
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42 168 spectrum clearly showed that the herbal mixture was not natural as expected from its claimed  
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44 169 composition. The presence of two main contaminants could be deduced from the highest signals  
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46 170 that were assigned to sildenafil (**12**) and methisosildenafil (**1**) thanks to our in-house NMR  
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48 171 database. Nonetheless, our attention was caught by the numerous minor signals whose  
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50 172 multiplicity and chemical shift could be compatible with those of PDE-5i analogues. To get rid  
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52 173 of carbon satellites that increase spectrum complexity, a <sup>13</sup>C GARP broadband decoupled <sup>1</sup>H  
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54 174 NMR spectrum was acquired, which confirmed the presence of minor adulterants (Fig. 2).  
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175 In order to determine their structures, the dietary supplement was purified and the  
176 compounds detected were isolated. The main issue encountered in the purification of this  
177 complex mixture was the huge concentration differences of the various analytes as illustrated  
178 in the NMR spectrum where large and small signals co-exist. To overcome this difficulty and  
179 after some unsuccessful attempts using both reverse and normal LC (results not shown), we  
180 chose to work in two steps using a first SFC pre-fractionation followed by LC purifications.

### 181 3.2. Purification of the dietary supplement

182 The SFC purification was done using a BetaSil diol phase. This polar stationary phase  
183 and the chosen elution mode provided sharp enough peaks and satisfactory chromatographic  
184 resolution and retention times. The use of basic elution conditions allowed the reduction of peak  
185 tailing for molecules containing amine groups. Seven fractions were collected from 3 to 13.2  
186 min as shown in [Fig. 1A](#).

187 Each SFC fraction was then purified by preparative LC. All experimental conditions are  
188 gathered in [Table 1](#) and chromatograms are illustrated in [Fig. 1B](#). The chromatographic  
189 conditions were chosen after a screening step using neutral or acidic medium and two stationary  
190 phases Kromasil C18 and CSH C18. The elution gradient was optimized for each separation.

191 The two steps of purification with polar (SFC) and non-polar (LC) chromatographic  
192 phases led to the purification of 16 compounds. The purity of each preparative LC fraction  
193 (except that containing compounds **15** and **16** which could not be separated) was evaluated from  
194 the UHPLC-UV chromatograms performed in routine analytical conditions and was in the range  
195 88.8-99.3% (mean 96.3%). It can be noticed that 14 products eluted between 2.07 and 2.92 min  
196 ([Table 2](#)), which also justifies the use of two orthogonal chromatographic methods that provided  
197 complementary selectivity to achieve the purification.

### 200 3.3. Characterization of purified compounds

201 The chemical structures of the two major adulterants methisosildenafil (**1**) and sildenafil  
202 (**12**) deduced from the <sup>1</sup>H NMR analysis of the raw dietary supplement (*vide supra*) were  
203 confirmed by their UV ( $\lambda_{\text{max}} \approx 225$  and 295 nm [10]), HRMS ( $[\text{M}+\text{H}]^+$  489.2277 and 475.3126  
204 respectively) and 1D and 2D NMR spectra (Fig. 3, Tables 3-5). Some clues on the structures of  
205 the other isolated compounds could be deduced from their UV and HRMS characteristics.

206 The UV spectrum of compound **2** with the same profile and  $\lambda_{\text{max}}$  than those of  
207 compounds **1** and **12** indicated that its structure also displays the pyrazolo[4,3-d]pyrimidine-7-  
208 one ethoxyphenyl (or propoxyphenyl) sulfonamide moiety [10]. By comparison with the UV  
209 profiles of the 64 selected PDE-5i reported by the USP [10], it can be excluded that the  
210 chromophore of the other compounds is that of pyrazolopyrimidine-7-thione sulfonamide  
211 analogues, pyrazolopyrimidine-7-one or -7-thione with acetyl moieties, or of tadalafil or  
212 vardenafil derivatives. Nevertheless, similar chromophoric skeletons are observed for  
213 compounds **4/5/13**, **6/7** and to a lesser extent **3**, **8/9/14**, and **10/11** (Fig. 3).

#### 215 3.3.1. MS and MS/MS analysis

216 The ESI<sup>+</sup> accurate mass data of parent and fragment ions of all the isolated compounds  
217 are gathered in Table 3. Some characteristic ESI<sup>+</sup> MS/MS spectra together with the structure of  
218 fragment ions are illustrated in Fig. 4A. The MS/MS fragmentations show the presence of a  
219 small (or very small) peak at m/z 166.0973-166.0985 corresponding to the molecular formula  
220 C<sub>8</sub>H<sub>12</sub>N<sub>3</sub>O<sup>+</sup> whose proposed structure (Fig. 4B) is characteristic of the fragmentation of the  
221 pyrazolo[4,3-d]pyrimidine-7-one moiety of the sildenafil derivatives [11] and not of the  
222 isomeric vardenafil skeleton that leads to fragmentation ions at m/z 169 and 151 [12].  
223 Moreover, a peak at m/z 299.1137-299.1151 corresponding to the molecular formula  
224 C<sub>15</sub>H<sub>15</sub>N<sub>4</sub>O<sub>3</sub><sup>+</sup> is found in the fragmentation of all the compounds, except **15** and **16**. Its intensity

225 is markedly lower for compounds **2-7** and **13** but remarkably higher for compounds **8-11** and  
226 **14** than for methisosildenafil (**1**) and sildenafil (**12**). The structure of this ion (Fig. 4B and 4C)  
227 involves the occurrence of a pyrazolopyrimidine-7-one sulfonamide moiety (or that of the  
228 isomeric vardenafil structure which has already been excluded (see above)).

229 In comparison with the fragmentation profile of the dimethylpiperazine moiety of  
230 methisosildenafil **1** which generates major peaks at  $m/z$  113, 99, 84 and 71 (the latter being  
231 characteristic of such a piperazine structure [13,14]), it can be stated that compounds **3, 4, 8** and  
232 **10** bear a dimethylpiperazine entity. Compounds **2, 5, 7, 9** and **11** display the same  
233 fragmentation peaks (except that the ion at  $m/z$  99 is replaced by two ions at  $m/z$  100 and 98)  
234 but also the presence of two intense additional ions at  $m/z$  141 and 127 corresponding  
235 respectively to molecular formulae  $C_8H_{17}N_2^+$  and  $C_7H_{15}N_2^+$  (Fig. 4A and 4D for the proposed  
236 structures of the fragment ions). The increase of 28 and 14  $m/z$  units with respect to the  
237 fragmentation ion of the dimethylpiperazine moiety at  $m/z$  113 (molecular formula  $C_6H_{13}N_2^+$ )  
238 suggests the insertion of an ethyl group on the piperazine ring. Moreover, the presence of a  
239 product ion at  $m/z$  72 (as intense as the ion at  $m/z$  71), characteristic of the fragmentation of a  
240 N-ethylpiperazinyl group [13,14], is a good indication of the presence of a dimethyl-N-ethyl-  
241 piperazine moiety. In comparison with the fragmentation of the N-methylpiperazine  
242 sulfonamide moiety of sildenafil **12** that gives peaks at  $m/z$  163, 100, 99, 70 and 58 [12,13], it  
243 can be concluded that this entity is part of the structure of compounds **13** and **14** (Fig. 4A and  
244 4D). The HRMS and MS/MS spectra of compounds **15** and **16** (not separated) do not show the  
245 presence of any fragmentation ion coming from piperazine or other amine substituent [13].

246 Compounds **2, 3, 4, 6** and **11** with a  $[M+H]^+$  ion at  $m/z$  517.2587-517.2600 suggesting  
247 the molecular formula  $C_{25}H_{37}N_6O_4S^+$  are characterized by a gain of 28 mass units compared to  
248 methisosildenafil **1** at  $m/z$  489.2277 ( $C_{23}H_{33}N_6O_4S^+$ ), thus demonstrating the presence of a  
249 supplementary ethyl group. Compounds **5** and **7** with  $[M+H]^+$  ions at  $m/z$  545.2920 and

250 545.2922 suggesting the molecular formula  $C_{27}H_{41}N_6O_4S^+$  are characterized by a gain of 56  
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2 251 mass units compared to methisosildenafil, thus indicating the presence of two additional ethyl  
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5 252 groups. Because the HRMS/MS spectrum of compound **2** only shows product ions  
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7 253 corresponding to methisosildenafil ( $m/z$  489) and its characteristic fragment ions ( $m/z$  432, 377,  
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10 254 311, 299, 283) as well as those of dimethyl-N-ethylpiperazine (Fig. 4B), it is therefore a  
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12 255 methisosildenafil derivative with an ethyl group on the nitrogen atom of the piperazine ring  
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14 256 (MSildEt<sup>N26</sup>). MS/MS spectra of compounds **3-7** show (i) product ions corresponding to  
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16  
17 257 methisosildenafil and its fragment ions cited above, (ii) those of dimethyl or dimethyl-N-ethyl  
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19 258 piperazine moiety, and (iii) additional peaks at  $m/z$  460, 405, 339 (340 for compound **3**) and  
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22 259 327, i.e. 28 mass units greater than those at  $m/z$  432, 377, 311 and 299, thus demonstrating that  
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24 260 they all have an ethyl group located on the pyrazolopyrimidine part (Fig. 4B). As only tiny  
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27 261 peaks at  $m/z$  141, 127 and 72 are detected, it can be considered that compounds **3** and **4** do not  
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29 262 have the ethyl group on the piperazine ring and therefore have it elsewhere, i.e. on the  
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32 263 pyrazolopyrimidine skeleton. Compounds **5** and **7** have two ethyl groups, one on the piperazine  
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34 264 moiety and the other on the pyrazolopyrimidine skeleton. Although its molecular mass is in  
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36 265 agreement with the presence of only one additional ethyl group compared to methisosildenafil,  
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39 266 the fragmentation of compound **6** is surprising because it shows the characteristic signals of  
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41 267 ethyl groups on the piperazine ring ( $m/z$  141, 127 and 72, although less intense than for **2**, **5**  
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44 268 and **7** for which the presence of the ethyl group on the piperazine ring is well established), and  
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46 269 on the pyrazolopyrimidine skeleton ( $m/z$  460, 405, 339 and 327). A possible explanation of  
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49 270 these observations is proposed below. The MS/MS spectrum of compound **11** shows major  
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51 271 product ions at  $m/z$  327, 311, 299 and 283, and less abundant peaks characteristic of the  
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54 272 dimethyl-N-ethyl piperazine fragmentation at  $m/z$  141, 127, 100, 98, 72 and 71 (Fig. 4C and  
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56 273 4D). The ions at  $m/z$  299 and 283 result from the loss of a  $C_2H_4$  moiety from ions at  $m/z$  327  
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58 274 and 311, respectively. If ions at  $m/z$  311, 299 and 283 are well-known fragments of

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275 methisosildenafil, the ion at  $m/z$  327 is only observed with compounds **3-7** for which it was  
276 unambiguously demonstrated (except for compound **6**) that the pyrazolopyrimidine skeleton  
277 bears an ethyl group. Compound **11** has thus two ethyl groups, one on the piperazine ring and  
278 the second on the pyrazolopyrimidine skeleton, which implies that its benzene ring is not  
279 substituted by an ethoxy group but by a hydroxyl group. Moreover, the absence of the ion at  
280  $m/z$  489 ( $[M+H]^+$  of methisosildenafil) in the MS/MS spectrum, in contrast to what is observed  
281 with compounds **2-7**, confirms that the structure of compound **11** does not derive from that of  
282 methisosildenafil.

283           Compound **10** with the same  $[M+H]^+$  molecular ion than methisosildenafil at  $m/z$   
284 489.2292 has the same fragments ions as compound **11** except that the piperazine product ions  
285 are those of a dimethylpiperazine entity. Therefore, it is most likely a methisosildenafil  
286 derivative with an ethyl group on the pyrazolopyrimidine ring and an OH substituent on the  
287 benzene ring. The  $[M+H]^+$  molecular ion at  $m/z$  461.1973 of compound **8**, corresponding to the  
288 molecular formula  $C_{21}H_{29}N_6O_4S^+$ , is 28 mass units lower than that of methisosildenafil  
289 ( $C_{23}H_{33}N_6O_4S^+$ ). Its MS/MS spectrum shows prominent fragment ions at  $m/z$  347, 299 and 283,  
290 and all the characteristic product ions of the dimethylpiperazine ring. The loss of this piperazine  
291 entity produces the fragment at  $m/z$  347 and the further loss of  $SO_2$  leads to the ion at  $m/z$  283.  
292 As no fragment ions correspond to the loss of an ethyl group, it can be concluded that compound  
293 **8** is a methisosildenafil derivative with an OH instead of an ethoxy group on the benzene ring  
294 (MSildOH). Compound **9** displays the same molecular formula ( $C_{23}H_{33}N_6O_4S^+$ ;  $[M+H]^+$   
295 489.2292) than methisosildenafil, the same fragment ions at  $m/z$  347, 299 and 283 than  
296 compound **8**, and the characteristic product ions of the dimethyl-N-ethylpiperazine moiety. It  
297 can therefore be stated that it is a methisosildenafil derivative with the ethoxy group on the  
298 benzene cycle replaced by an OH, and a dimethyl-N-ethylpiperazine entity (MSildOHEt<sup>N26</sup>).

299 The molecular ions  $[M+H]^+$  of compounds **13** (m/z 503) and **14** (m/z 447) are  
1  
2 300 respectively 28 mass units higher and lower in comparison to that of sildenafil (**12**) at m/z 475.  
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4 301 The MS/MS spectra of the two ions show the presence of N-methylpiperazine product ions (m/z  
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7 302 163, 100, 99, 70 and 58). This is a good indication that compound **13** bears an additional ethyl  
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9 303 group on the sildenafil skeleton and shows that compound **14** is a sildenafil derivative whose  
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11 304 ethoxy group on the benzene ring is replaced by a hydroxyl group (SildOH).

14 305 It should be noted that the ion at m/z 299 is much more intense in the MS/MS spectra  
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16 306 of compounds with the benzene ring substituted by a hydroxyl (**8-11** and **14**) rather than by an  
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18 307 ethoxy group (compare the spectrum from **11** to all other spectra presented in Fig. 4A), this  
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20 308 observation being able to be considered as a good indication of the presence of a hydroxyl group  
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22 309 on the benzene ring. Indeed, the ion at m/z 299 results from a well described fragmentation  
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24 310 mechanism of sulfonamides involving a rearrangement of the SO<sub>2</sub> group resulting in the loss of  
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26 311 SO [15]. But if the fragmentation of the ion at m/z 347 of MSildOH (**8**), MSildOHEt<sup>N26</sup> (**9**) or  
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28 312 SildOH (**14**) leads directly by this way to the ion at m/z 299, that of the ion at m/z 377 of  
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30 313 methisosildenafil (**1**) or sildenafil (**12**) for example, requires a subsequent loss of C<sub>2</sub>H<sub>6</sub> for the  
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32 314 formation of the ion at m/z 299 [16] (Fig. 4B and 4C). The same is true for the ion at m/z 327  
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34 315 (299 + ethyl) which is more intense for compounds **10** and **11** than for compounds **3-7** (compare  
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36 316 the spectrum of **11** with the spectrum of **4** in Fig. 4A).  
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44 317 Only a low amount of the mixture of compounds **15** and **16** was obtained after the  
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46 318 various steps of purification and our attempts to separate them were unfruitful. The MS  
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48 319 spectrum of the mixture shows two  $[M+H]^+$  peaks, one very intense at m/z 313.1663 (compound  
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50 320 **15**) and a second much less intense at m/z 341.1980 (compound **16**), corresponding respectively  
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52 321 to molecular formulae C<sub>17</sub>H<sub>21</sub>N<sub>4</sub>O<sub>2</sub><sup>+</sup> and C<sub>19</sub>H<sub>25</sub>N<sub>4</sub>O<sub>2</sub><sup>+</sup>. The MS/MS spectrum of the parent ion  
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54 322 at m/z 341 generates characteristic peaks of the fragmentation of the pyrazolopyrimidine  
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56 323 skeleton of sildenafil derivatives at m/z 313 and 285 (C<sub>15</sub>H<sub>17</sub>N<sub>4</sub>O<sub>2</sub><sup>+</sup>) involving two successive  
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324 losses of an ethyl group, whereas the MS/MS of the parent ion at  $m/z$  313 produces a fragment  
325 ion at  $m/z$  285 corresponding to the loss of only one ethyl group (Fig. 4B). Moreover, these two  
326 compounds do not have any piperazine or other amine entity (see above). All these findings are  
327 in accordance with a sildenafil-type structure in which the piperazine ring and the sulfonyl  
328 group are removed. Compound **15** is thus desulfosildenafil (DeSild) and compound **16**, DeSild  
329 with an additional ethyl group on the pyrazolopyrimidine skeleton.

330 **Table 4** reports the measured accurate masses of the parent and fragment ions in negative  
331 ESI mode. Two characteristic ESI<sup>-</sup> MS/MS spectra together with the structure of fragment ions  
332 are illustrated in Fig. S1. First, it can be noticed that compounds **3-7** and **13** are not ionized.  
333 The fragmentation of [M-H]<sup>-</sup> molecular ions of methisosildenafil (**1**) and sildenafil (**12**) at  $m/z$   
334 487 and 473 produces ions respectively at 459 and 445 (loss of C<sub>2</sub>H<sub>4</sub>), 310 (loss of the  
335 piperazine-SO<sub>2</sub> moiety) and 282 (successive loss of these two entities). This sequential  
336 fragmentation pathway involves the neutral loss of ethylene from the ethoxyphenyl substituent  
337 and the homolytic cleavage of the C<sub>phenyl</sub>-S bond already reported for sildenafil or vardenafil  
338 and their derivatives [17]. Compound **2** (MSildEt<sup>N26</sup>) follows exactly the same fragmentation  
339 process confirming that the additional ethyl group compared to methisosildenafil is well located  
340 on the piperazine ring (Fig. S1). In contrast, compounds **8**, **9** and **14** only show the loss of a  
341 piperazine-SO<sub>2</sub> entity, which suggests that the ethoxy substituent on the benzene ring is  
342 replaced by an OH group. Thus, from the values of the molecular ion and of the eliminated  
343 piperazine-SO<sub>2</sub> group, it can be concluded that (i) compound **8** which undergoes the loss of a  
344 dimethylpiperazine-SO<sub>2</sub> entity is methisosildenafil with a hydroxyl substituent instead of an  
345 ethoxy on the benzene ring (MSildOH), (ii) compound **9** with the loss of a dimethyl-N-  
346 ethylpiperazine-SO<sub>2</sub> moiety is MSildOH with an additional ethyl group on the piperazine ring  
347 (MSildOHEt<sup>N26</sup>), and (iii) compound **14** with the loss of a methylpiperazine-SO<sub>2</sub> is sildenafil  
348 with an OH instead of an ethoxy group on the benzene cycle (SildOH). MS/MS of the molecular

ions [M-H]<sup>-</sup> of all the compounds, except **15**, generate a small peak at m/z 298 due to the loss of their piperazine-SO moiety (compounds **8-11** and **14**) and an additional loss of C<sub>2</sub>H<sub>4</sub> (compounds **1**, **2** and **12**) (Fig. S1B and S1C) and corresponding to the molecular formula C<sub>15</sub>H<sub>14</sub>N<sub>4</sub>O<sub>3</sub><sup>-</sup> whose structure might be similar to that of the ion at m/z 299 observed in ESI<sup>+</sup> mode (Fig. 4B and 4C). This is a good indication of the replacement of the ethoxy group on the benzene ring (present in compounds **1**, **2** and **12**) by a hydroxyl group in compounds **8-11** and **14**. Moreover, the detection of an ion at m/z 326 (molecular formula C<sub>17</sub>H<sub>18</sub>N<sub>4</sub>O<sub>3</sub><sup>-</sup> with a structure similar to that of the ion at m/z 327 observed in ESI<sup>+</sup> mode (Fig. 4B and 4C)) for compounds **10** and **11**, indicates that they bear an ethyl group located on the pyrazolopyrimidine skeleton. In the MS spectrum of the mixture of compounds **15** and **16**, only the molecular ion [M-H]<sup>-</sup> at m/z 311 of compound **15** is detected and its MS/MS fragmentation confirms the presence of an ethoxy group (loss of C<sub>2</sub>H<sub>4</sub>) and the absence of piperazine or other amine entity as well as of the SO<sub>2</sub> group.

In summary, the careful analysis of the data obtained by HRMS and HRMS/MS allows the unambiguous identification of five compounds (**2** (MSildEt<sup>N26</sup>), **8** (MSildOH), **9** (MSildOHEt<sup>N26</sup>), **14** (SildOH) and **15** (DeSild)). On the other hand, for the other eight compounds (**3-5**, **7**, **10**, **11**, **13** and **16**), the MS data only show that they bear an ethyl group on the pyrazolopyrimidine moiety. Compound **6** cannot be identified because its MS data lead to conflicting conclusions. An NMR study was therefore required to determine the exact structure of all these compounds and to confirm and validate the structures proposed from the MS data.

### 3.3.2. <sup>1</sup>H and <sup>13</sup>C NMR analysis

The <sup>1</sup>H and <sup>13</sup>C NMR data of all the isolated compounds are reported in Table 5. The assignments of the resonances were performed thanks to 1D and 2D (COSY, HSQC, HMBC, NOESY) NMR experiments.

374 <sup>1</sup>H NMR spectra (Fig. 5) of compounds **2-11** all display, like methisosildenafil (**1**), the  
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2 375 characteristic doublet (two for compounds **3**, **4** and **5**) of the methyl groups on the piperazine  
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5 376 ring (CH<sub>3</sub> 29 and 30) between 0.99 and 1.26 ppm, thus allowing to assume they are  
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7 377 methisosildenafil derivatives. In the same way, <sup>1</sup>H NMR spectra of compounds **13** and **14**  
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10 378 exhibit, like sildenafil (**12**), at  $\delta \approx 2.2$  ppm the characteristic singlet of the methyl group (CH<sub>3</sub>  
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12 379 29) linked to the N26 of the piperazine ring, thus indicating that they are sildenafil derivatives.  
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15 380 Compared to <sup>1</sup>H NMR spectra of methisosildenafil or sildenafil, those of compounds **2**, **3**, **4**, **6**  
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17 381 and **13** reveal the presence of additional characteristic resonances of one ethyl group [a triplet  
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19 382 accounting for 3 protons (H32) and a quadruplet (or two quadruplet doublets (qd)) for 2 protons  
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21 383 (H31)], or two ethyl groups for compounds **5** and **7** (H31/32, and H33/34). It is highly likely  
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24 384 that these ethyl groups are positioned on the main nucleophilic sites of methisosildenafil or  
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27 385 sildenafil derivatives, namely the oxygen and nitrogen atoms of the pyrazolo[4,3-d]pyrimidine  
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29 386 moiety and the NH group of the piperazine moiety. The <sup>1</sup>H and <sup>13</sup>C NMR characteristics of the  
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32 387 CH<sub>2</sub> of the ethyl group should thus give a good indication of its location on the methisosildenafil  
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34 388 or sildenafil skeleton. For compound **2**, the H31 quadruplet at 2.84 ppm shows an HMBC  
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36 389 correlation with the C25/C27 (54.9 ppm) of the piperazine ring (Fig. 6A), thus confirming that  
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39 390 C<sub>2</sub>H<sub>5</sub> is positioned on the N26 of the piperazine moiety (MSildEt<sup>N26</sup>). For compound **3**, the two  
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41 391 H31 protons appear as two qd at 4.14 and 3.96 ppm. Although close to the quadruplet of the  
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44 392 CH<sub>2</sub> (H20) of the ethoxy group on the benzene ring (4.23 ppm), the two CH<sub>2</sub> entities can easily  
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46 393 be distinguished by their <sup>13</sup>C resonances at 47.8 (C31) and 68.1 ppm (C20). The HMBC  
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49 394 correlations between the qd and C5 and C9 (158.4 and 130.5 ppm) demonstrate that the N4 of  
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51 395 the pyrazolopyrimidine moiety bears the ethyl group (Fig. 6B) (MSildEt<sup>N4</sup>). For compound **4**,  
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54 396 the CH<sub>2</sub> (H31) resonances show the same pattern than for compound **3** with two qd at 4.12 and  
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56 397 3.62 ppm for the protons and a <sup>13</sup>C signal at 43.5 ppm. The HMBC correlations between CH<sub>2</sub>  
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58 398 (H31) and C5 (153.1 ppm) and C7 (157.1 ppm) are the proof of the presence of the ethyl group  
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399 on the N6 of the pyrazolopyrimidine moiety (Fig. 6C) (MSildEt<sup>N6</sup>). For compound **6**, the strong  
400 <sup>1</sup>H and <sup>13</sup>C deshielding of the CH<sub>2</sub> (H31/C31) signals, respectively at 4.65 and 66.4 ppm, of the  
401 additional ethyl group is characteristic of an O-ethyl entity which can be distinguished from the  
402 CH<sub>2</sub> of the ethoxy group on the benzene ring (H20/C20) thanks to the <sup>1</sup>H NMR H20 δ at 4.21  
403 ppm, the <sup>13</sup>C NMR C20 δ at 67.9 ppm being non discriminant. The HMBC correlation between  
404 the CH<sub>2</sub> (H31) and C7 (159.1 ppm) confirms that the ethyl group is linked to the O7 of the  
405 pyrazolopyrimidine entity (Fig. 6D) (MSildEt<sup>O7</sup>). Hence, a N26-ethyl substitution leads to <sup>1</sup>H  
406 and <sup>13</sup>C CH<sub>2</sub> resonances respectively at ≈2.8 and 42.5 ppm. An N-ethyl location on the  
407 pyrazolopyrimidine entity gives rise to two distinct <sup>1</sup>H qd signals, meaning that the two CH<sub>2</sub>  
408 protons are inequivalent (discussed below). One of the two qd and the <sup>13</sup>C resonance are more  
409 deshielded when the substitution is on N4 compared to N6 (Δδ ≈ +0.33 ppm for <sup>1</sup>H and +4.3  
410 ppm for <sup>13</sup>C). An O7-ethyl substitution leads to <sup>1</sup>H and <sup>13</sup>C CH<sub>2</sub> signals respectively at ≈4.7 pm  
411 and 66.5 ppm. From these considerations on the <sup>1</sup>H and <sup>13</sup>C chemical shifts, it is possible to  
412 conclude that compound **13** is sildenafil substituted by an ethyl group on N6 (SildEt<sup>N6</sup>).  
413 Regarding the two additional ethyl groups in compounds **5** and **7**, one is located on the N26 of  
414 the piperazine cycle and the other is either on the N6 (**5**) (MSildEt<sup>N6</sup>Et<sup>N26</sup>) or on the O7 (**7**) of  
415 the pyrazolopyrimidine skeleton (MSildEt<sup>O7</sup>Et<sup>N26</sup>). All these structures were unambiguously  
416 confirmed by HMBC correlations.

417 Ethyl signals characteristic of the O-CH<sub>2</sub>(20)-CH<sub>3</sub>(21) group are undetected for  
418 compounds **8-11** and **14**, demonstrating the replacement of the ethoxy group on the C19 of the  
419 benzene ring by a hydroxyl group as shown by the C19 δ (166-176 ppm) deshielded with respect  
420 to the δ of the C19 bearing an ethoxy group (163 ppm). This structural modification is also  
421 confirmed by a noticeable change in the δ of the neighboring <sup>1</sup>H and <sup>13</sup>C resonances compared  
422 to those of methisosildenafil (**1**) or sildenafil (**12**) (shielding for H17 and H18, and deshielding  
423 for H15, C5, C18 and C19). Moreover, as no other N- or O-ethyl resonances are detected in

424 compounds **8** and **14**, they can be respectively identified as C19-hydroxymethisosildenafil  
1 (MSildOH) and C19-hydroxysildenafil (SildOH). Compounds **9-11**, all methisosildenafil  
2 derivatives, display respectively one ethyl group characteristic of an O7 substitution, two ethyl  
3 groups characteristic of O7 and N26 substitutions, and one ethyl group characteristic of an N26  
4 substitution. Therefore, they are identified as MeSildOH bearing O7- (**10**) (MSildOHEt<sup>O7</sup>), O7-  
5 and N26- (**11**) (MSildOHEt<sup>O7</sup>Et<sup>N26</sup>), and N26- (**9**) (MSildOHEt<sup>N26</sup>) ethyl groups, all the  
6 structures being confirmed by HMBC correlations. The NMR spectra of the mixture of  
7 compounds **15** and **16**, compared to those of methisosildenafil or sildenafil, show for each  
8 compound the disappearance of the <sup>1</sup>H and <sup>13</sup>C signals of the piperazine ring and the appearance  
9 of an additional <sup>1</sup>H triplet doublet at ≈7.1 ppm, corresponding to the presence of a hydrogen  
10 atom on the C16 of the benzene ring instead of a sulfonamide group. Therefore, the two  
11 compounds are desulfosildenafil derivatives (sildenafil without the piperazine-SO<sub>2</sub> moiety).  
12 Moreover, the minor compound **16** exhibits additional characteristic <sup>1</sup>H and <sup>13</sup>C signals of an  
13 ethyl group located on the N6 of the pyrazolopyrimidine skeleton. Compound **16** is thus  
14 identified as N6-ethyl desulfosildenafil (DeSildEt<sup>N6</sup>) and compound **15**, predominant in the  
15 mixture, as desulfosildenafil (DeSild). The structures of all the compounds purified from the  
16 dietary supplement are illustrated in [Fig. 7](#).

41 The structure of the compounds having been determined, it is possible to make a few  
42 comments on the NMR spectra, in particular on some of the proton inequivalences observed.  
43 Both protons of CH<sub>2</sub> 11 and CH<sub>2</sub> 12 of compound **3** are inequivalent leading respectively to two  
44 very close ( $\Delta\nu = 1.2$  Hz) apparent triplets for CH<sub>2</sub> 11 and apparent sextets for CH<sub>2</sub> 12. This  
45 anisochrony results from the steric hindrance of the close N4-ethyl chain as shown by the nOe  
46 interactions between both CH<sub>2</sub> 11 and CH<sub>2</sub> 12 and the two protons of CH<sub>2</sub> 31, the interaction  
47 with H31 at 4.14 ppm being stronger than with H31 at 3.96 ppm ([Fig. S2A](#)). Confronting NMR  
48 results to calculated privileged conformers confirms that each proton of CH<sub>2</sub> 11 or CH<sub>2</sub> 12 is

449 closer to one of the two protons 31 than to the other (Fig. S3). Moreover, an inequivalence of  
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2 450 the two protons of CH<sub>2</sub> 31 is observed in the <sup>1</sup>H NMR spectra of all compounds that bear an N-  
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4 451 ethyl group on the pyrazolopyrimidine skeleton, i.e. **3**, **4**, **5**, **13** and **16**. The stronger nOe  
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7 452 interaction between one of the two H31 (≈4.15 ppm) and aromatic H15 (≈7.8 ppm for **3**, **4**, **5**,  
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9 453 and **13**, and 7.37 ppm for **16**) explains this finding which is confirmed by the calculated  
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11 454 distances in the privileged conformations (Fig. S2B and S3). The shielding of the H15 signal  
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14 455 that can be noticed for these compounds ( $\Delta\delta \approx -0.44$  ppm relative to methisosildenafil H15  $\delta$   
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17 456 for **3**, **4**, and **5**, and -0.42 and -0.84 ppm compared to sildenafil H15  $\delta$  for **13** and **16**) (Fig. 5)  
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19 457 could also be related to these spatial interactions.  
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### 22 458 23 24 459 *3.3.3. Conclusive remarks*

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27 460 In summary, the NMR study confirms the structures of compounds **2**, **8**, **9**, **14** and **15**  
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29 461 already established from MS data and makes it possible to unambiguously determine the  
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32 462 position of the ethyl group on the N and/or O nucleophilic sites of the pyrazolo[4,3-  
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34 463 d]pyrimidine backbone for compounds **3-7**, **10**, **11**, **13** and **16**, which MS does not allow. In  
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36 464 addition, MS does not make it possible to know whether the additional ethyl group compared  
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39 465 to methisosildenafil in compound **6** is located on the piperazine or pyrazolopyrimidine ring.  
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41 466 Indeed, the fragmentation of its molecular ion [M+H]<sup>+</sup> produces ions characteristic of the two  
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44 467 locations, while NMR clearly shows that it is methisosildenafil substituted by an ethyl group  
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46 468 on the O7. The presence of an ethyl group on the N26 of the piperazine as stated by the MS/MS  
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49 469 fragmentation of the molecular ion [M+H]<sup>+</sup> can be explained by an intramolecular ethyl transfer  
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51 470 from the O7 atom of the pyrazolopyrimidine moiety to the N26 atom of the piperazine. Indeed,  
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54 471 the methyl intramolecular transfer from the piperazine nitrogen atom to the thiocarbonyl sulfur  
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56 472 atom of thiosildenafil derivatives is a dominant fragmentation pathway, while the methyl  
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59 473 migration to the carbonyl oxygen atom of sildenafil may occur but to a very low extent [18].  
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474 Consequently, it can be postulated that the ethyl group on the carbonyl atom of the  
475 pyrazolopyrimidine skeleton of compound **6** can be partially transferred to the N26 of the  
476 piperazine group because when it is thus positioned, it should not migrate significantly on the  
477 carbonyl oxygen atom of methisosildenafil.

478 It should also be noted that identical profiles of UV spectra correspond well to  
479 molecular structures with identical chromophores (Fig. 3). The UV profile of compound **2** is  
480 similar to those of methisosildenafil (**1**) and sildenafil (**12**), indicating that, as expected, an N-  
481 ethyl substitution on the piperazine ring does not alter the chromophore. Compounds **8**, **9** and  
482 **14** with identical UV spectra are methisosildenafil or sildenafil with a hydroxyl group instead  
483 of an ethoxy group on the C19 of the benzene ring. Compared to methisosildenafil or sildenafil  
484 UV spectra, this replacement induces a bathochromic effect of  $\approx 8$  nm for the band at  $\approx 225$  nm,  
485 whereas the band at  $\approx 295$  nm gives rise to a vibrational fine structure between 270 and 350 nm.  
486 Compounds **4**, **5** and **13** with very similar UV spectra are N6-ethyl substituted methisosildenafil  
487 or sildenafil. The identical UV spectra of compounds **6** and **7** in one hand and **10** and **11** on the  
488 other hand are respectively characteristic of O7-ethyl substituted methisosildenafil and O7-  
489 ethyl methisosildenafil with OH instead of  $\text{OC}_2\text{H}_5$  on the benzene ring, this change leading to  
490 bathochromic effects of  $\approx 4$  and 22 nm for the two main bands observed. Finally, the UV  
491 spectrum of compound **3**, a N4-ethyl substituted methisosildenafil, shows the same  $\lambda_{\text{max}}$  than  
492 the O7-ethyl substituted methisosildenafil derivatives (compounds **6** and **7**) but with a different  
493 profile, the most intense band of compound **3** at 205.7 nm appearing as a shoulder for  
494 compounds **6** and **7** at 210 nm.

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496 *3.4. Quantitative analysis of methisosildenafil, sildenafil, and related compounds in the dietary*  
497 *supplement analyzed*

498 The quantities of methisosildenafil (**1**), sildenafil (**12**), and compounds (**8**) (MSildOH)  
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2 499 and (**4**) (MSildEt<sup>N6</sup>) were easily measured from the signal areas of their aromatic protons (Fig.  
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4 500 S4) in the conditions described in reference [1]. They account respectively for 38, 20, 5 and 3  
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7 501 mg per capsule, i.e. 54%, 32%, 7% and 4% of the total contaminants. The amounts of the other  
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9 502 products could only be estimated due to the very low intensity of their <sup>1</sup>H NMR signals (except  
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11 503 for MSildEt<sup>N26</sup> (**2**) which has no distinctive resonances); they range from 0.01 to 0.50 mg per  
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13 504 capsule and represent ≈3% of the total contaminants, i.e. ≈2 mg per capsule. So, each capsule  
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15 505 contains ≈68 mg of methisosildenafil- and sildenafil-related compounds, i.e. between the  
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17 506 recommended (50 mg) and the maximum (100 mg) daily dose of sildenafil in patients.  
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#### 23 24 508 **4. Discussion**

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26 509 This study deals with the purification and characterization of 16 sildenafil derivatives  
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29 510 present in a dietary supplement intended to be commercialized for improving sexual  
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31 511 performance and alleged to be “100% natural”. Two adulterants are found in large quantities:  
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33 512 sildenafil, the lead PDE-5i drug, and methisosildenafil, a sildenafil analogue that has never  
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35 513 received a marketing authorization as a medicine. All other molecules possess the scaffold of  
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37 514 sildenafil, i.e. the 5-phenyl-pyrazolo[4,3-d]pyrimidine moiety, and twelve out of fourteen bear  
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39 515 a sulfonyl group linked to a piperazine ring (Fig. 7). Their structures mainly differ by the  
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41 516 presence or absence of an ethyl group N- or O- linked to either the pyrazolopyrimidine or  
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43 517 piperazine moiety. Besides sildenafil commonly found in adulterated erectile dietary  
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45 518 supplements, five other compounds have already been described in the literature.  
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49 519 Methisosildenafil was first reported in 2007 as an adulterant of an herbal dietary supplement  
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51 520 [19] and MSildEt<sup>N26</sup> (**2**) in 2018 in health foods analyzed by UHPLC coupled with Q-TOF-MS  
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53 521 [20]. DeSild (**15**), also named imidazosagatriazinone, is known as sildenafil lactam impurity  
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56 522 and is commercially available (CAS number 139756-21-1). However, it has never been reported  
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523 in a dietary supplement. SildOH (**14**) is the Sildenafil Impurity C in the European  
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2 524 Pharmacopoeia and is also commercially available (CAS number 139755-91-2). SildEt<sup>N6</sup> (**13**)  
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4 525 was described as an impurity formed during the synthesis of sildenafil but was not thoroughly  
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6 526 characterized [21]. The 10 other chemicals identified in this study have to our knowledge never  
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9 527 been described in the literature.  
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12 528 The presence of numerous sildenafil derivatives in the dietary supplement analyzed is  
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14 529 most probably due to its intentional adulteration with sildenafil and methisosildenafil. It can be  
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16 530 hypothesized that the minor compounds detected are either degradation impurities ensuing from  
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18 531 the effect of humidity and/or light during storage for instance, or by-products of different  
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20 532 reaction steps during the synthesis of these two PDE-5i that have not been properly eliminated  
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22 533 during the purification procedure. The second hypothesis is preferred because neither the  
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24 534 hydrolysis nor the photodegradation of sildenafil and analogues leads to the kind of structures  
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26 535 identified in the present study [19,22]. In order to correlate the chemical structures of the minor  
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28 536 components identified in the dietary supplement with the different synthetic routes to sildenafil  
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30 537 and methisosildenafil, we thus thoroughly analyzed the reaction sequences towards both APIs  
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32 538 from the literature [23,24]. The preparation of sildenafil and methisosildenafil follows the same  
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34 539 overall approach, except for the piperazine used, that is either N-methylated or 2,6-  
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36 540 dimethylated, respectively. Two main types of synthetic routes can be distinguished, the first-  
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38 541 generation ones relying on the late introduction of the sulfonamide moiety and the more recent  
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40 542 ones based on an early sulfonylation step.  
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48 543 According to the chemical structures assigned to the minor compounds detected, one  
49  
50 544 can subdivide the discussion into two main categories, illustrated for sildenafil in Fig. 8. The  
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52 545 first one deals with compounds **15** and **16**, lacking the sulfonamide group. The second one  
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54 546 comprises all the other compounds that display either no ethyl radical (compounds **8** and **14**) or  
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56 547 variable O- or N-ethylation patterns (**2-7**, **9-11**, **13**). It has to be recalled that, in the case of  
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2 548 methisosildenafil, the presence of the free piperazine nitrogen offers an additional opportunity  
3 549 for unwanted ethylation.

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5 550 The presence of unsulfonylated compounds can be explained by at least two principal  
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7 551 reaction sequences. The initial medicinal chemistry route described for sildenafil involves a late  
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9 552 sulfonylation step [25], which is known to be reversible and thus potentially leads to  
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11 553 desulfonylated by-products [26] (Fig. 8, pathway A). As an alternative, a late Friedel-Crafts  
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13 554 process with sulfamoyl chloride in the presence of AlCl<sub>3</sub> was proposed to introduce the  
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15 555 sulfonamide moiety (Fig. 8, pathway B) [23,25]. This procedure was described with only 45%  
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17 556 yield, potentially leaving a large amount of starting reagent in the reaction medium. A neglected  
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19 557 purification step would thus explain the presence of unsulfonylated by-products in both cases.  
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24 558 The occurrence of variable O- or N-ethylation patterns, corresponding to the second  
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26 559 class of by-products, can be correlated with at least three different synthetic pathways. In the  
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28 560 first possible pathway, the late sulfonylation step requires the *para*-chlorosulfonylation of an  
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30 561 ethoxyphenyl intermediate with chlorosulfonic acid (Fig. 8, pathway C). The scaling-up of this  
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32 562 reaction was reported to be troublesome because of the increased quench time of the excess of  
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34 563 chlorosulfonic acid that leads to prolonged exposure to a large amount of hydrochloric acid  
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36 564 [23]. The possible hydrolysis of the O-ethoxy group would explain the presence of a free phenol  
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38 565 in compounds **8**, **10** and **14**, as previously proposed in a study on synthetic impurities of  
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40 566 sildenafil [27]. Such conditions could in turn result in the formation of ethyl chloride susceptible  
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42 567 to alkylate the electron-rich positions of the pyrimidinone core of the molecule. Alternatively,  
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44 568 the highly acidic medium could also potentially induce a direct intra- or intermolecular  
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46 569 nucleophilic transfer of the ethoxy radical. Yet, due to the absence of nucleophilicity of the  
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48 570 protonated methisosildenafil piperazine ring nitrogen, the formation of compounds **2**, **5**, **7**, **9**  
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51 571 and **11** is unlikely under these acidic conditions. The observed random distribution of ethyl  
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53 572 radicals rather suggests side-products arising from neutral or basic reaction media.  
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573 More recent synthetic pathways of sildenafil were reported involving an early  
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2 574 introduction of the sulfonamide moiety so as to circumvent the problems associated with the  
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5 575 late-stage transformation. Two distinct synthetic steps that share the use of basic conditions can  
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7 576 be proposed in this case. The first one relies on a critical intramolecular cyclization reaction,  
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10 577 using for instance *t*-BuOK in *t*-BuOH, to generate the pyrazolopyrimidone core of sildenafil  
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12 578 (Fig. 8, pathway D). The ethoxy moiety present in the benzamide precursor was described to  
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15 579 be doubly activated by the sulfonamide and amide groups, and thus prone to intra- or  
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17 580 intermolecular nucleophilic substitution [23,24]. This transformation would lead to either the  
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19 581 loss of the ethyl radical or its transfer onto the different nucleophilic sites of the molecule.  
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22 582 Another synthetic pathway was then proposed to address this issue, in which the whole  
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24 583 synthesis is run with a free phenol group and the O-alkylation of the phenol is postponed after  
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27 584 the cyclisation reaction [21] (Fig. 8, pathway E). Unless a very specific neutral decarboxylative  
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29 585 alkylation of a carbonate intermediate was used, exposure to standard alkylating agents was  
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32 586 described to lead to pyrimidinone O- or N-ethylation, along with partial phenol alkylation,  
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34 587 eventually giving rise to a product distribution similar to that observed in the present study.

36 588 Overall, the analysis of the reported routes towards sildenafil and methisosildenafil  
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39 589 indicates that all the minor compounds identified in the dietary supplement can be side-products  
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41 590 of relevant synthetic steps. It was not possible to unambiguously decipher the synthetic  
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44 591 sequence used, or even if there was only one. This clearly indicates that the synthesis processes  
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46 592 used by the manufacturers do not meet the standard chemical purification requirements. A  
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49 593 similar problematic of erectile dietary supplement containing synthetic impurities in minor  
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51 594 amounts was previously reported in two articles by Schramek et al. In the first study [28], the  
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54 595 dietary supplement contained dithiodesmethylcarbodenafil and three impurities coming from  
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56 596 its synthetic pathway and in the second study [29], the herbal food supplement contained 14  
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58 597 chemicals (5 never described) bearing the basic structure of sildenafil that might be process-

598 related impurities or by-products of PDE-5i analogues synthesis. So, in addition to the illegal  
599 falsification of the food supplement by PDE-5i, our study shows once again that manufacturers  
600 do not respect good manufacturing practices and do not control the quality of their preparation  
601 before sending it to intermediaries for placing on the market.

602 It must be underlined that there is a real health risk for a consumer who would ingest  
603 the dietary supplement analyzed. First, it contains a non-negligible amount of sildenafil (20 mg  
604 per capsule) which corresponds to about half of the recommended therapeutic daily dose.  
605 Second, it contains methisosildenafil (38 mg per capsule) and other analogues (10 mg per  
606 capsule) whose safety and toxicology profiles are unknown as they have not been subjected to  
607 any clinical trials. Being structurally similar to sildenafil, these analogues may retain the  
608 corresponding pharmacological/toxicological properties or have different properties, thus  
609 leading to unpredictable effects and/or side-effects [3]. For example, a fatal case associated with  
610 the ingestion of a sildenafil analogue (desmethylcarbodenafil) has been reported in 2017 [30].  
611 In the case of the dietary supplement described in this study, beyond the risks posed by each  
612 compound present in the commercial preparation, the possibility of a synergistic effect of the  
613 16 different molecules must also be taken into account.

## 615 5. Conclusion

616 The “all-natural” dietary supplement for enhancing sexual performance investigated in  
617 this study contains a mixture of two major PDE-5i adulterants, sildenafil and methisosildenafil,  
618 intentionally added to increase its effect and fourteen minor compounds. All compounds were  
619 isolated thanks to the orthogonality of SFC and LC separations and their structures elucidated  
620 using UV spectroscopy, high resolution and tandem mass spectrometry and one- and two-  
621 dimensional NMR. The minor contaminants come from poor purification procedures during the

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622 different steps of sildenafil and methisosildenafil synthesis and 10 of them have never been  
623 described in the literature.

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### 625 **Conflicts of interest**

626 The authors declare that there are no conflicts of interest.

627

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633 convention ANSM/UPSn°2012S071).

### 635 **Appendix A. Supplementary data**

636 Supplementary material related to this article can be found, in the online version, at doi:

637 **Figure S1.** (A) HRMS/MS spectra in negative ESI mode of two methisosildenafil-related  
638 compounds (**2**, **11**) purified from the dietary supplement analyzed. The formulae shown  
639 represent the molecules and not the ions. Proposed structures of fragment ions of (B)  
640 compounds with an ethoxy group on the benzene ring, (C) compounds with a hydroxyl group  
641 on the benzene ring.

642 **Figure S2.** Parts of 2D NOESY spectra showing the spatial correlations between CH<sub>2</sub> 11, 12  
643 and H31 for compound **3** and between H15 and CH<sub>2</sub> 31 for compound **4**.

644 **Figure S3.** Representation of a privileged conformation calculated for compound **3** and  
645 interatomic distances between protons of interest.

646 **Figure S4.** Part (6.7-8.9 ppm) of the <sup>1</sup>H NMR spectrum with <sup>13</sup>C GARP broadband decoupling  
647 of the dietary supplement recorded in CD<sub>3</sub>CN/D<sub>2</sub>O (80:20, v/v) with attribution of the signals  
648 of the identified compounds.

649

### 650 Captions for figures

651 **Figure 1.** (A) SFC-UV chromatogram on BetaSil Diol-100 column (the collection windows are  
652 indicated by the vertical dashed lines). (B) Preparative LC-UV chromatograms on C18 reverse  
653 phase columns. All experimental conditions are reported in [Table 1](#). The presence of  
654 compounds annotated 12\* and 1\* in W2 and W3 fractions may be due to the high concentration  
655 of compounds **12** and **1** in the C2 fraction.

656 **Figure 2.** <sup>1</sup>H NMR spectrum with <sup>13</sup>C GARP broadband decoupling of the dietary supplement  
657 recorded in CD<sub>3</sub>CN/D<sub>2</sub>O (80:20, v/v). (A), (B), (C) and (D) correspond to zoomed areas  
658 highlighted by boxes on the full spectrum. The main signals of sildenafil (**12**) and  
659 methisosildenafil (**1**) are shown.

660 **Figure 3.** UV spectra of methisosildenafil (MSild), sildenafil (Sild) and their related  
661 compounds purified from the dietary supplement analyzed. Spectra were obtained from the  
662 UHPLC analysis of the purified fractions.

663 **Figure 4.** (A) HRMS/MS spectra in positive ESI mode of methisosildenafil (MSild, **1**),  
664 sildenafil (Sild, **12**) and related compounds (**2**, **4**, **11** and **13**) purified from the dietary  
665 supplement analyzed. The formulae shown represent the molecules and not the ions. Proposed  
666 structures of fragment ions of (B) compounds with an ethoxy group on the benzene ring, (C)  
667 compounds with a hydroxyl group on the benzene ring, (D) piperazine entities.

668 **Figure 5.** <sup>1</sup>H NMR spectra of methisosildenafil (MSild), sildenafil (Sild), and related  
669 compounds purified from the dietary supplement analyzed. Spectra were recorded in

670 CD<sub>3</sub>CN/D<sub>2</sub>O (80:20, v/v) except for compounds **9** and **14** whose solubilization required the  
671 subsequent addition of 5 μL of 0.5 M NaOD in the NMR tube. Regions 6.7-9.0 ppm (A), 2.5-  
672 5.0 ppm (B), 1.6-2.6 ppm (C) and 0.7-1.6 ppm (D). The intensity scale is different for the  
673 different parts of the spectrum. For compounds **15** and **16** that could not be separated, the prime  
674 symbol (') is used to mark the protons of compound **16**. HOD: residual protons in D<sub>2</sub>O;  
675 CD<sub>2</sub>H<sub>2</sub>CN: residual protons in CD<sub>3</sub>CN; S<sub>CD<sub>2</sub>H<sub>2</sub>CN</sub>: <sup>13</sup>C satellite signals of CD<sub>2</sub>H<sub>2</sub>CN; ax: axial  
676 protons; eq: equatorial protons; FA: fatty acids present in the dietary supplement; MeOH:  
677 methanol; Ac: residual acetate from the chromatographic mobile phase; \*: unknown  
678 contaminants.

679 **Figure 6.** Parts of the <sup>1</sup>H-<sup>13</sup>C HMBC spectra of compounds **2** (A), **3** (B), **4** (C) and **6** (D).  
680 Correlations between protons H31 and specific carbons indicating the position of the ethyl  
681 group in the structure are marked with dashed arrows. See Fig. 7 for chemical structures and  
682 numbering. MeOH: methanol; HOD: residual protons in D<sub>2</sub>O.

683 **Figure 7.** Chemical structures of sildenafil, methisosildenafil and their analogues purified and  
684 characterized in this study. (a) CAS registry number; (b) compound already described in  
685 reference [20]; (c) compound already described in reference [21]; (d) known as Sildenafil  
686 European Pharmacopoeia impurity C; (e) known as imidazosagatriazinone.

687 **Figure 8.** Possible origin of the various impurities found in the food supplement analyzed,  
688 according to the main routes of synthesis of sildenafil and methisosildenafil reported in the  
689 literature [21,23-27]. Pip: N-methylpiperazine or 2,6-dimethylpiperazine.

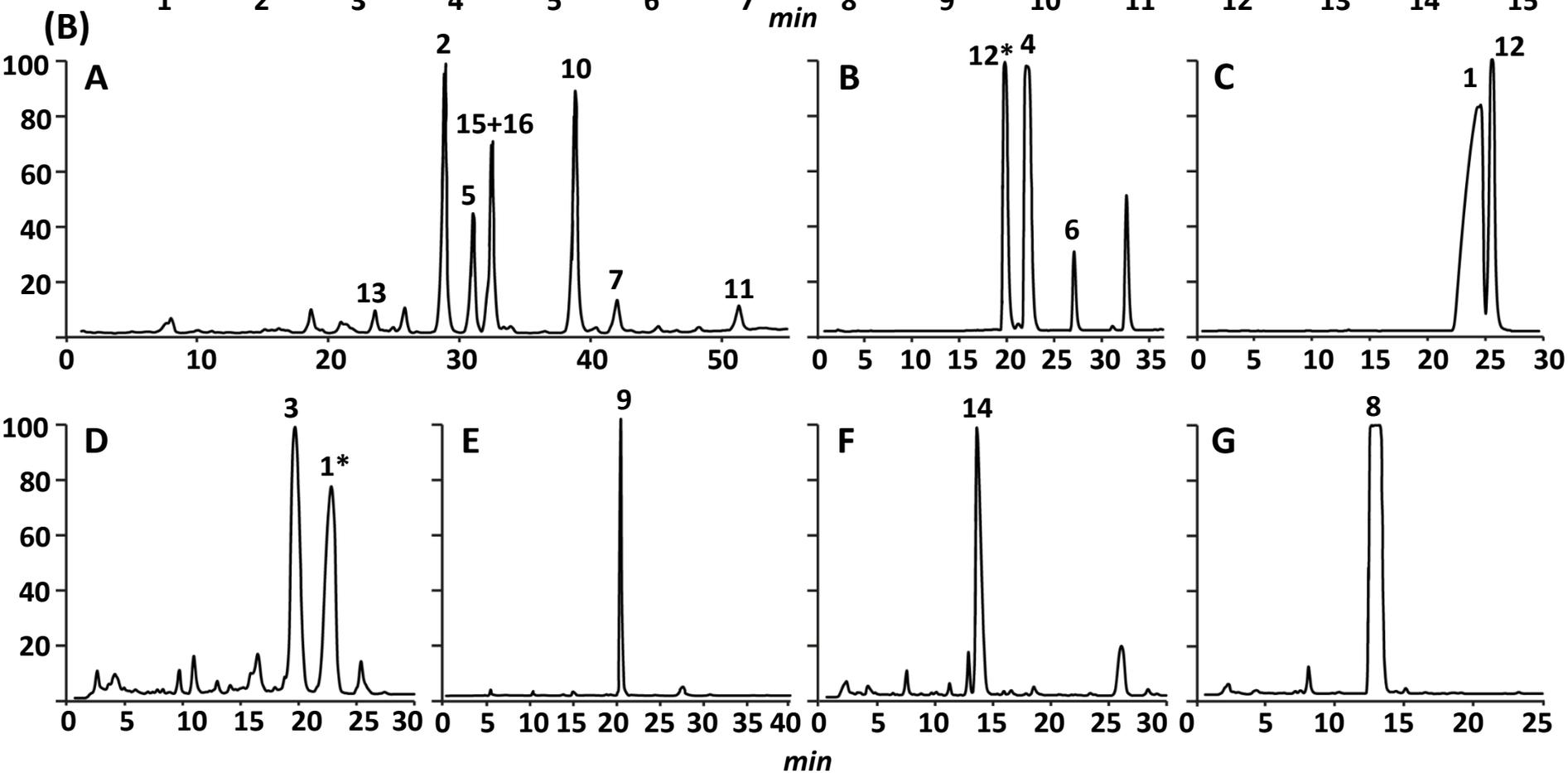
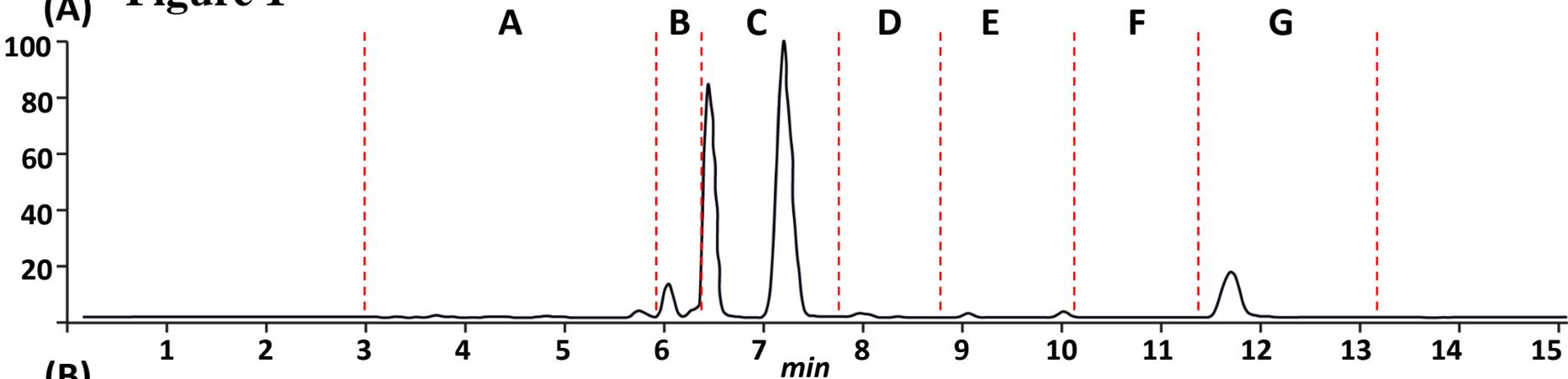
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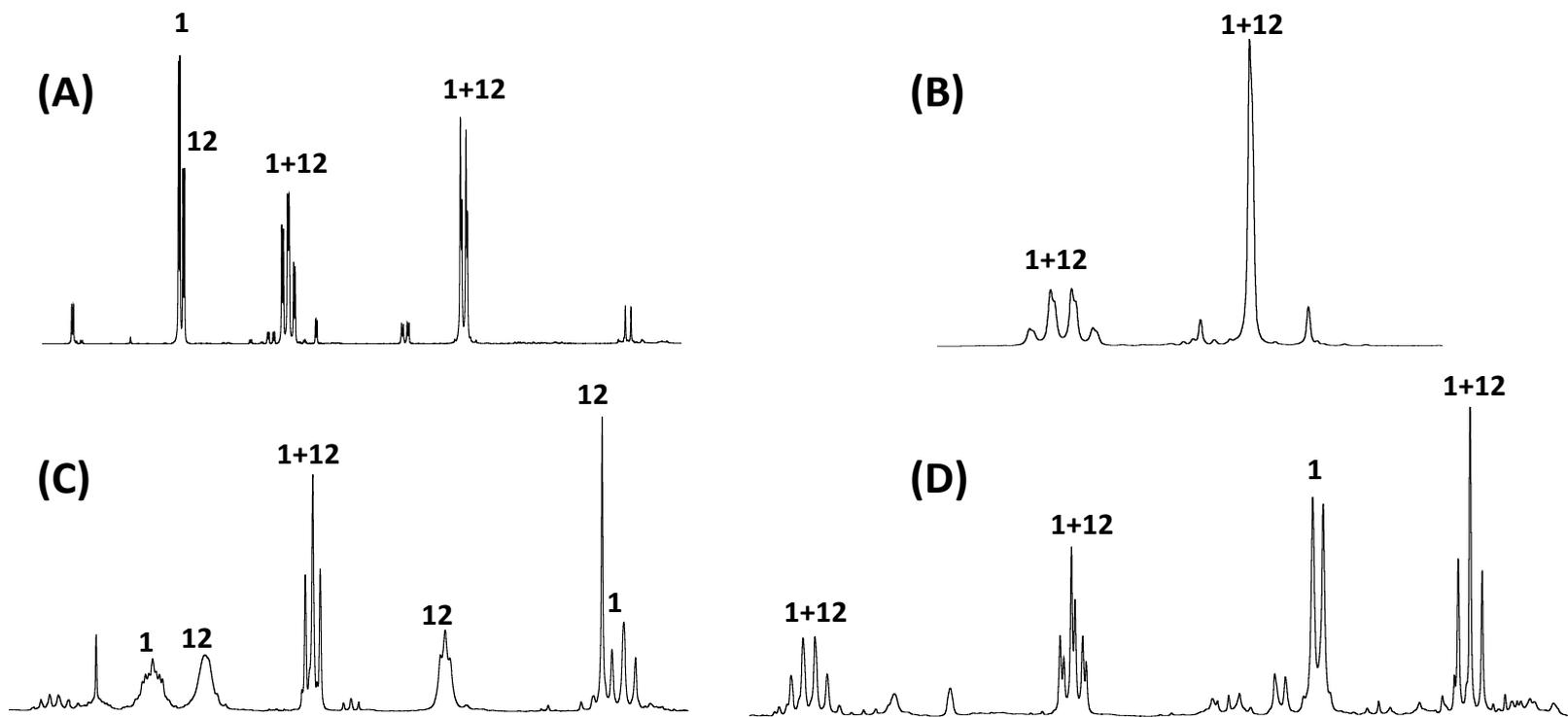
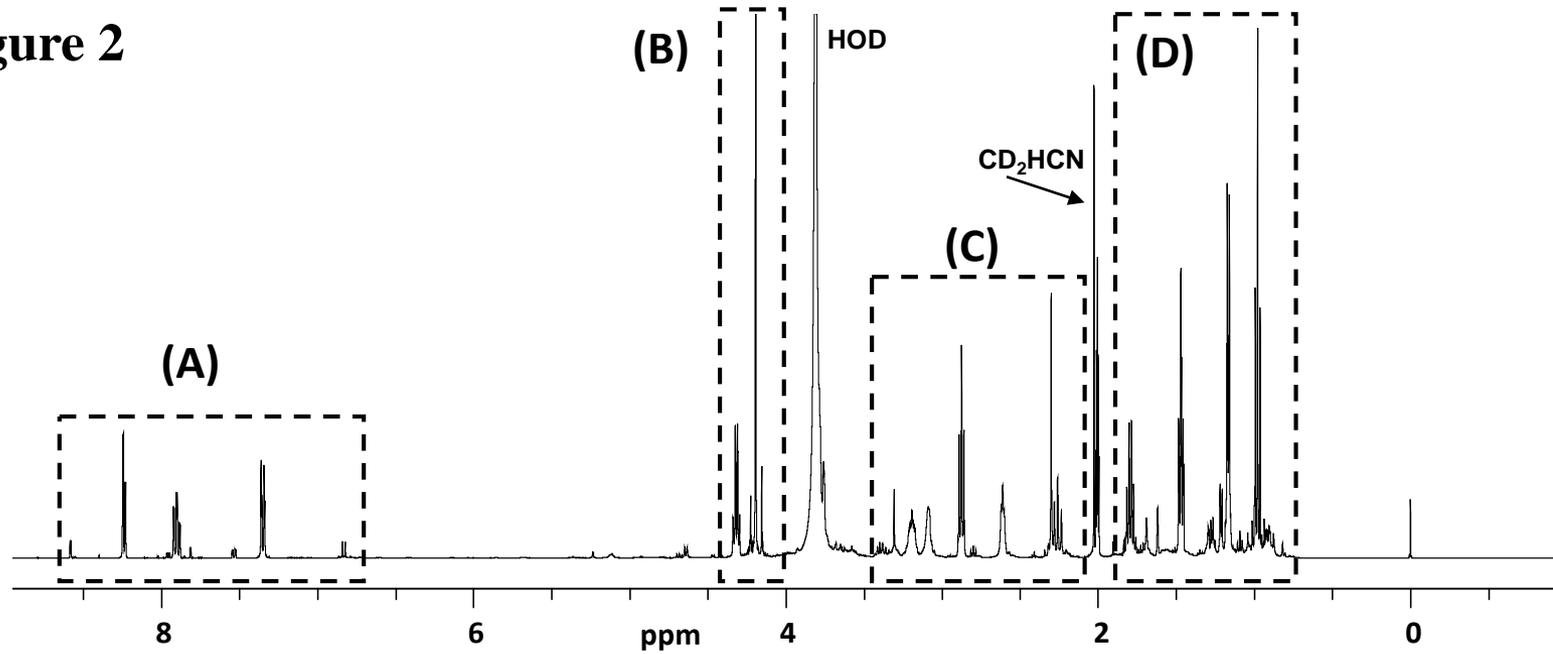
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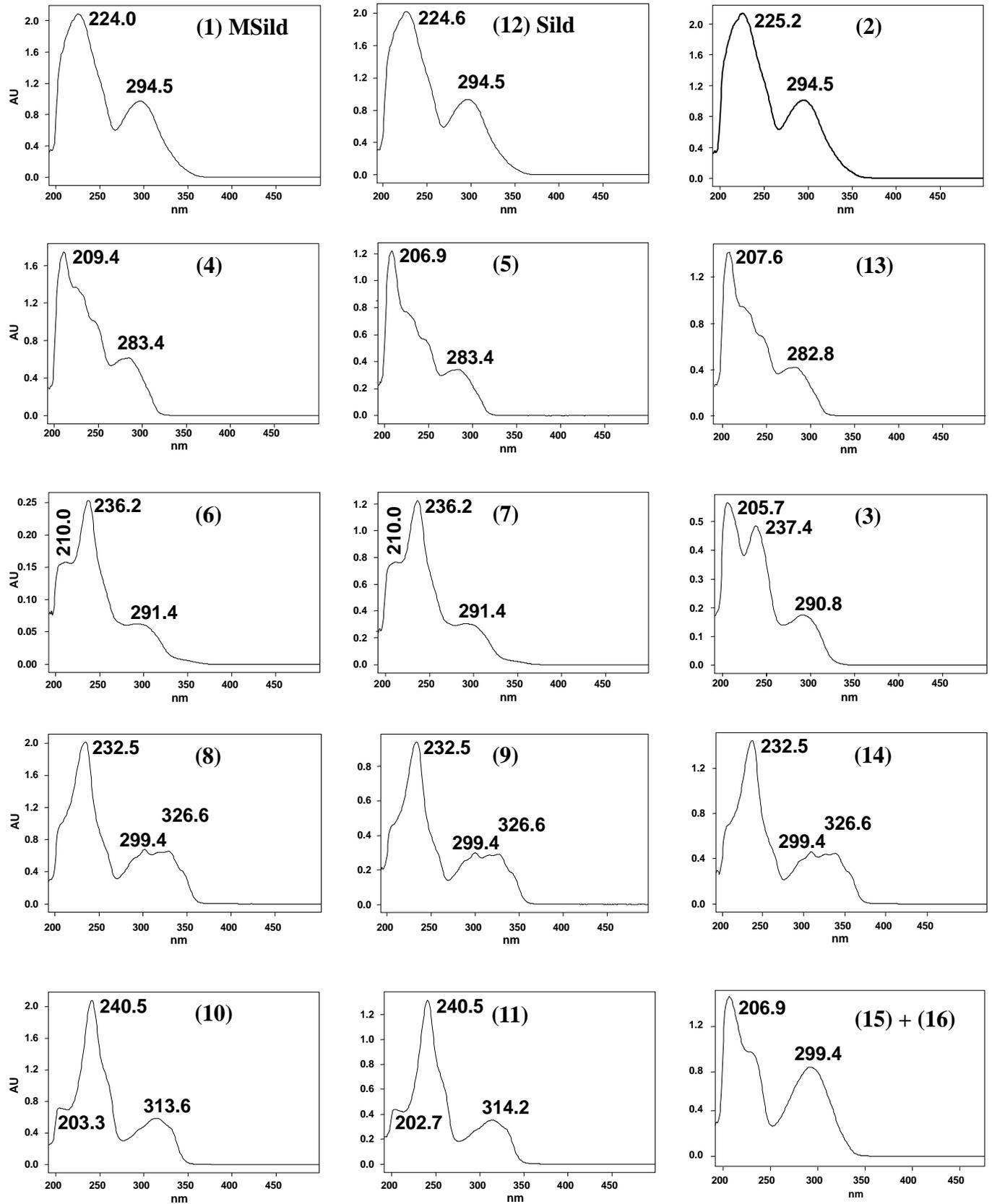
Figures 1-8  
**Figure 1**

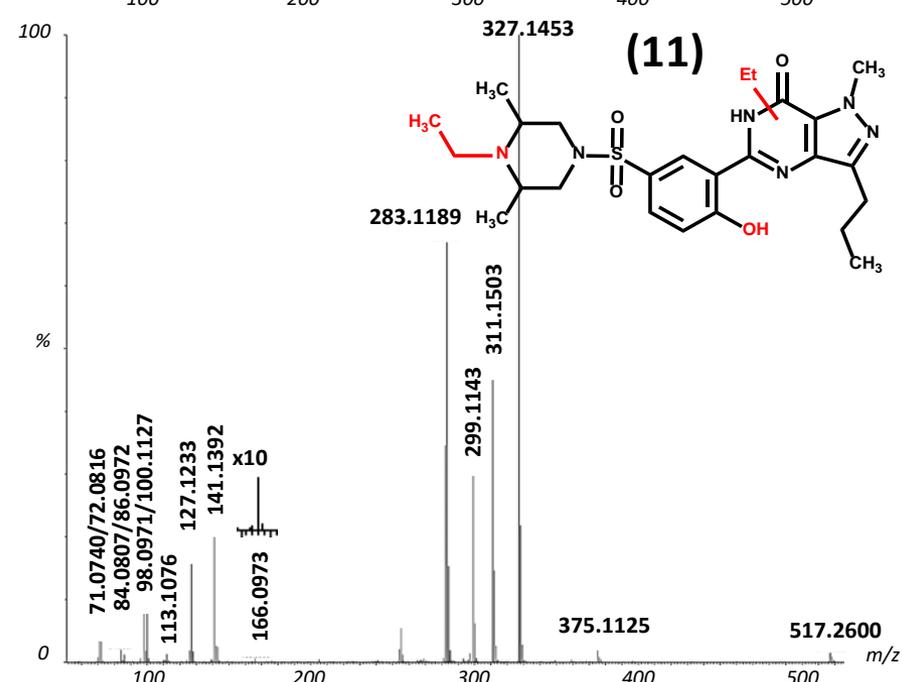
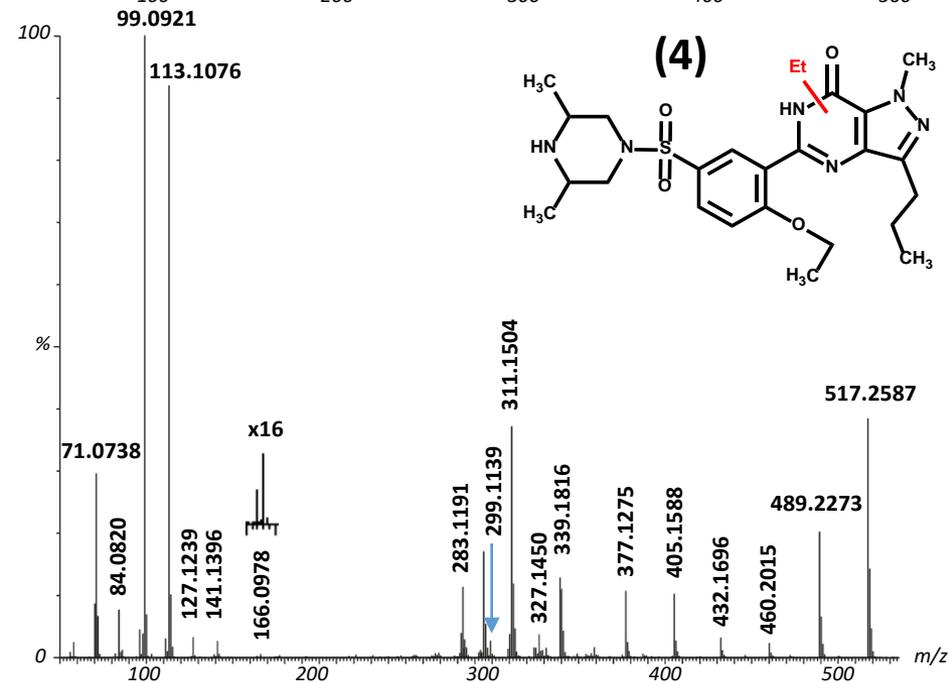
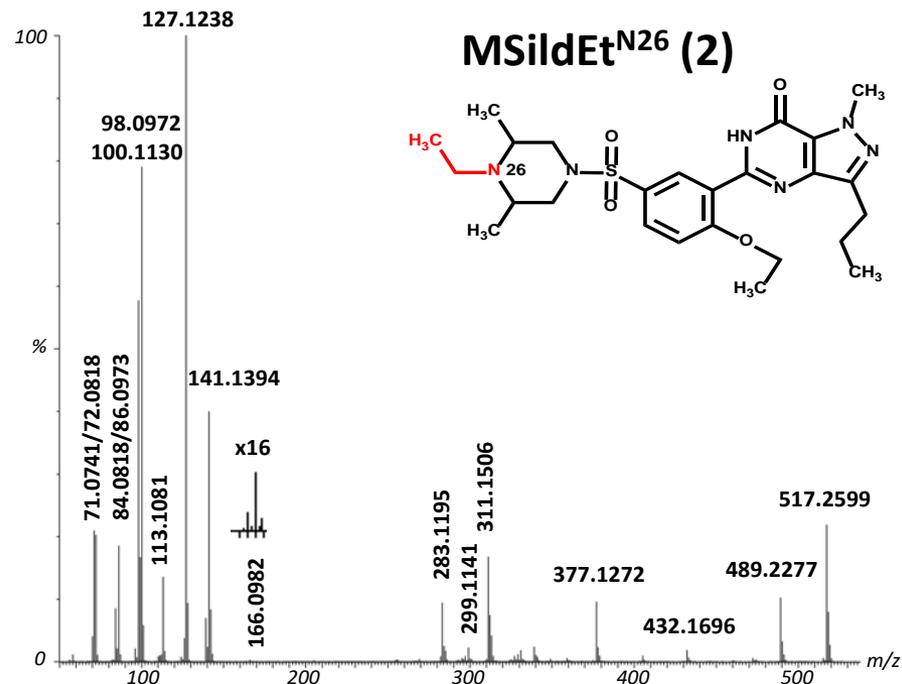
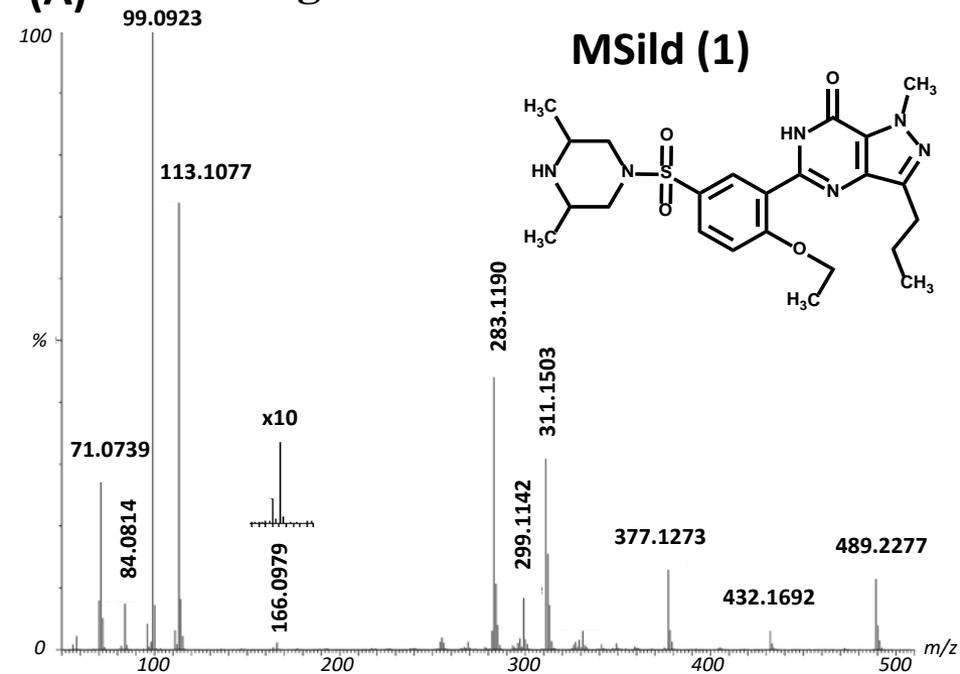


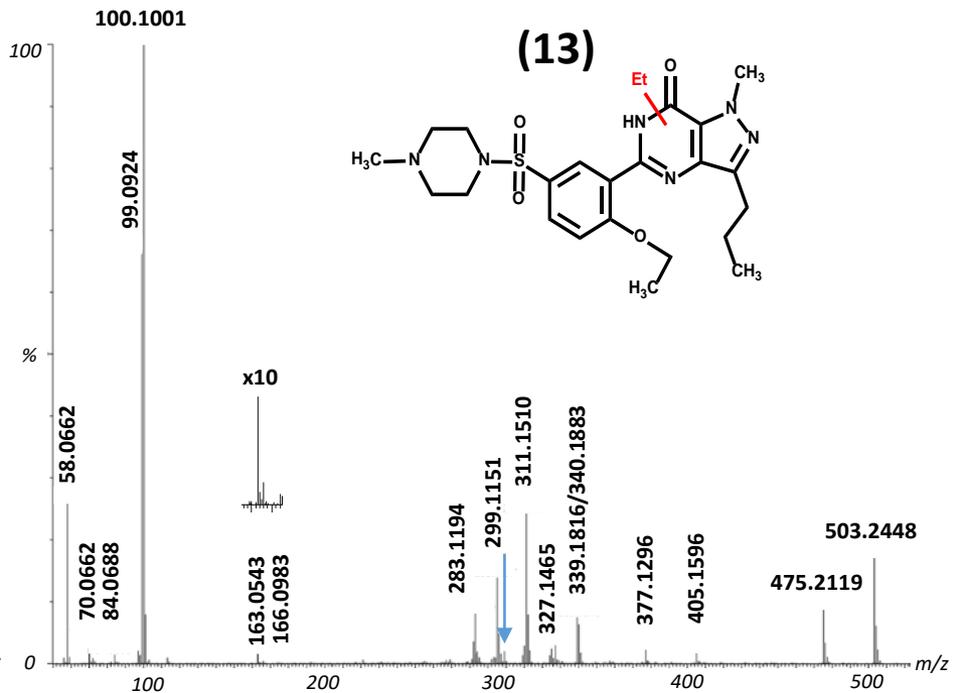
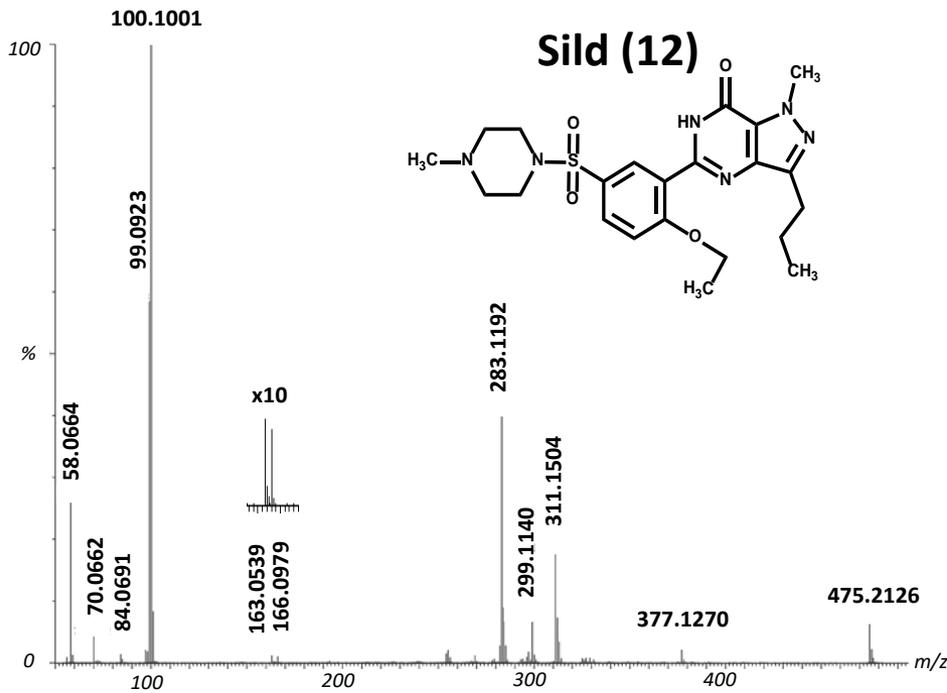
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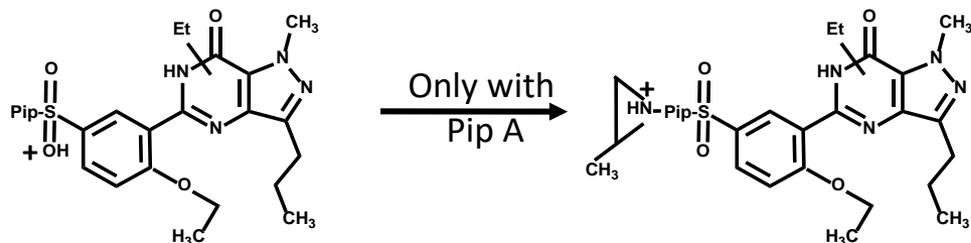


**Figure 3**

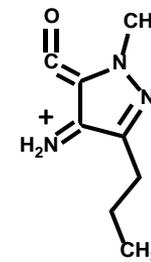


**(A) Figure 4**



**(B)**

m/z  
432 460



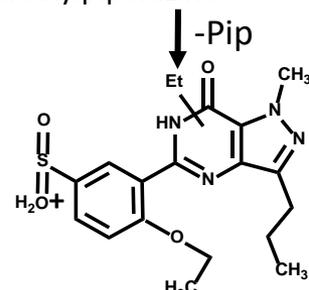
m/z 166  
Common to all  
compounds

m/z without ethyl group on the pyrazolopyrimidine moiety

Pip A: 2,6-dimethylpiperazine	489
Pip B: N1-ethyl-2,6-dimethylpiperazine	517
Pip C: N-methylpiperazine	475

m/z with ethyl group

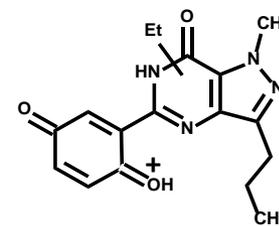
517
545
503



377

405

-SO  
-C<sub>2</sub>H<sub>6</sub>

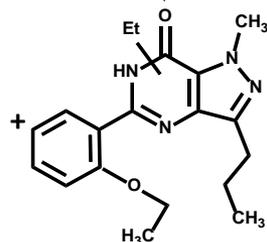


m/z  
299 327

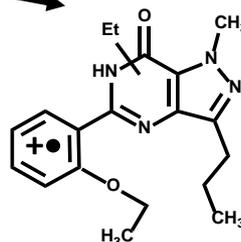
-SO<sub>2</sub>H<sub>2</sub>

-SO<sub>2</sub>H

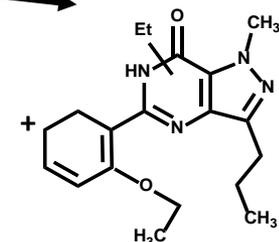
-SO<sub>2</sub>



m/z 311 339



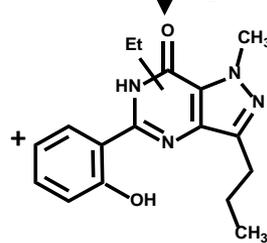
312 340



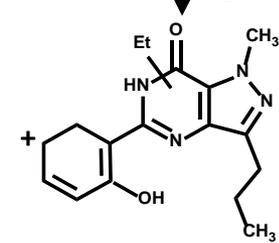
313 341

-C<sub>2</sub>H<sub>4</sub>

-C<sub>2</sub>H<sub>4</sub>

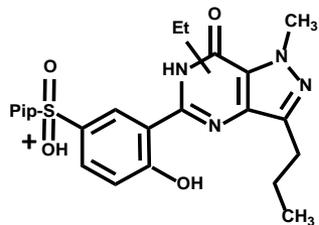


m/z 283 311



285 313

(C)

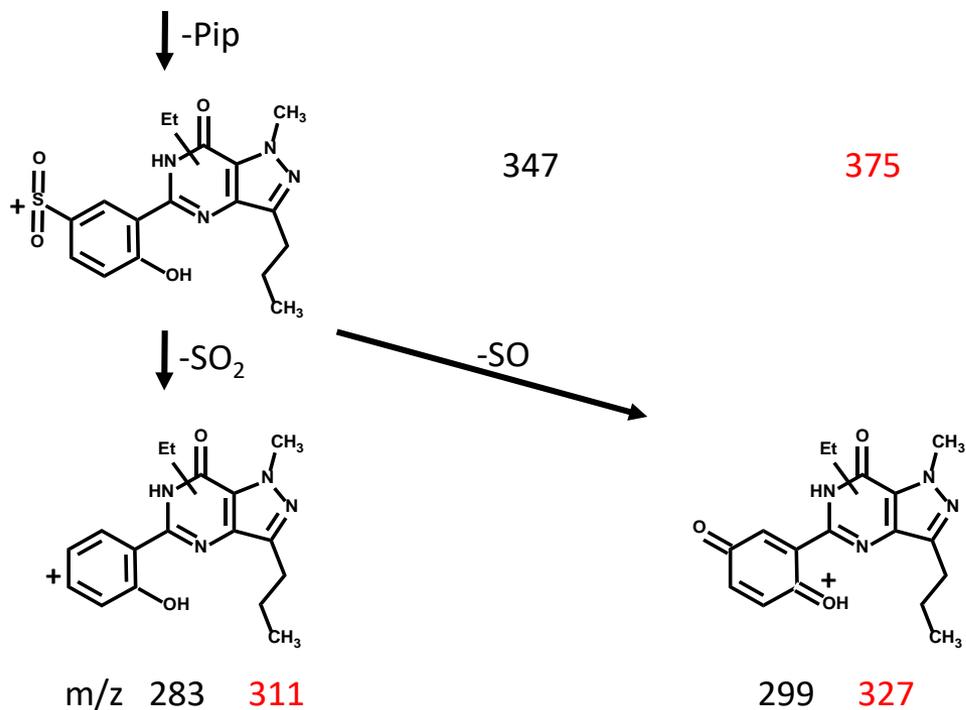


m/z without ethyl group on the pyrazolopyrimidine moiety

Pip A: 2,6-dimethylpiperazine 461  
Pip B: N1-ethyl-2,6-dimethylpiperazine 489  
Pip C: N-methylpiperazine 447

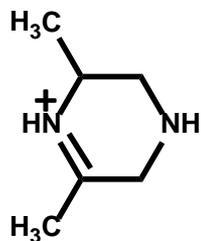
m/z with ethyl group

489  
517

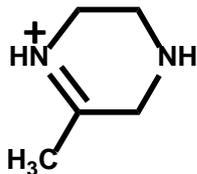


**(D)**

- 2,6-dimethylpiperazine



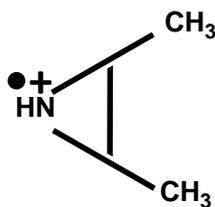
m/z 113  
 $C_6H_{13}N_2^+$



99  
 $C_5H_{11}N_2^+$

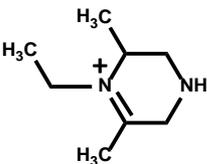


84.0813  
 $C_5H_{10}N^+$

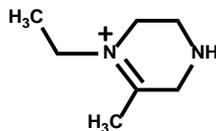


71  
 $C_4H_9N^{\bullet+}$

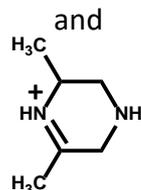
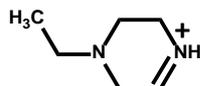
- N1-ethyl-2,6-dimethylpiperazine



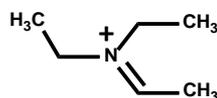
m/z 141  
 $C_8H_{17}N_2^+$



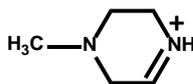
127  
 $C_7H_{15}N_2^+$



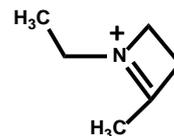
113  
 $C_6H_{13}N_2^+$



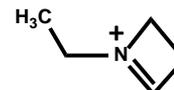
100.1126  
 $C_6H_{14}N^+$



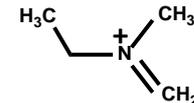
99  
 $C_5H_{11}N_2^+$



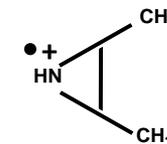
86  
 $C_5H_{12}N^+$



84.0813  
 $C_5H_{10}N^+$

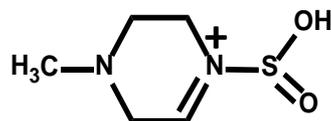


72  
 $C_4H_{10}N^+$

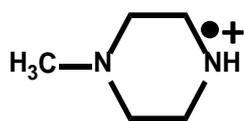


71  
 $C_4H_9N^{\bullet+}$

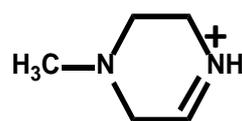
- N1-methylpiperazine



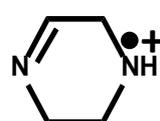
m/z 163  
 $C_5H_{11}N_2O_2S^+$



100.1000  
 $C_5H_{12}N_2^{\bullet+}$



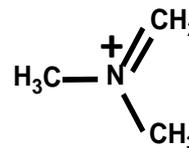
99  
 $C_5H_{11}N_2^+$



84.0688  
 $C_4H_8N_2^{\bullet+}$

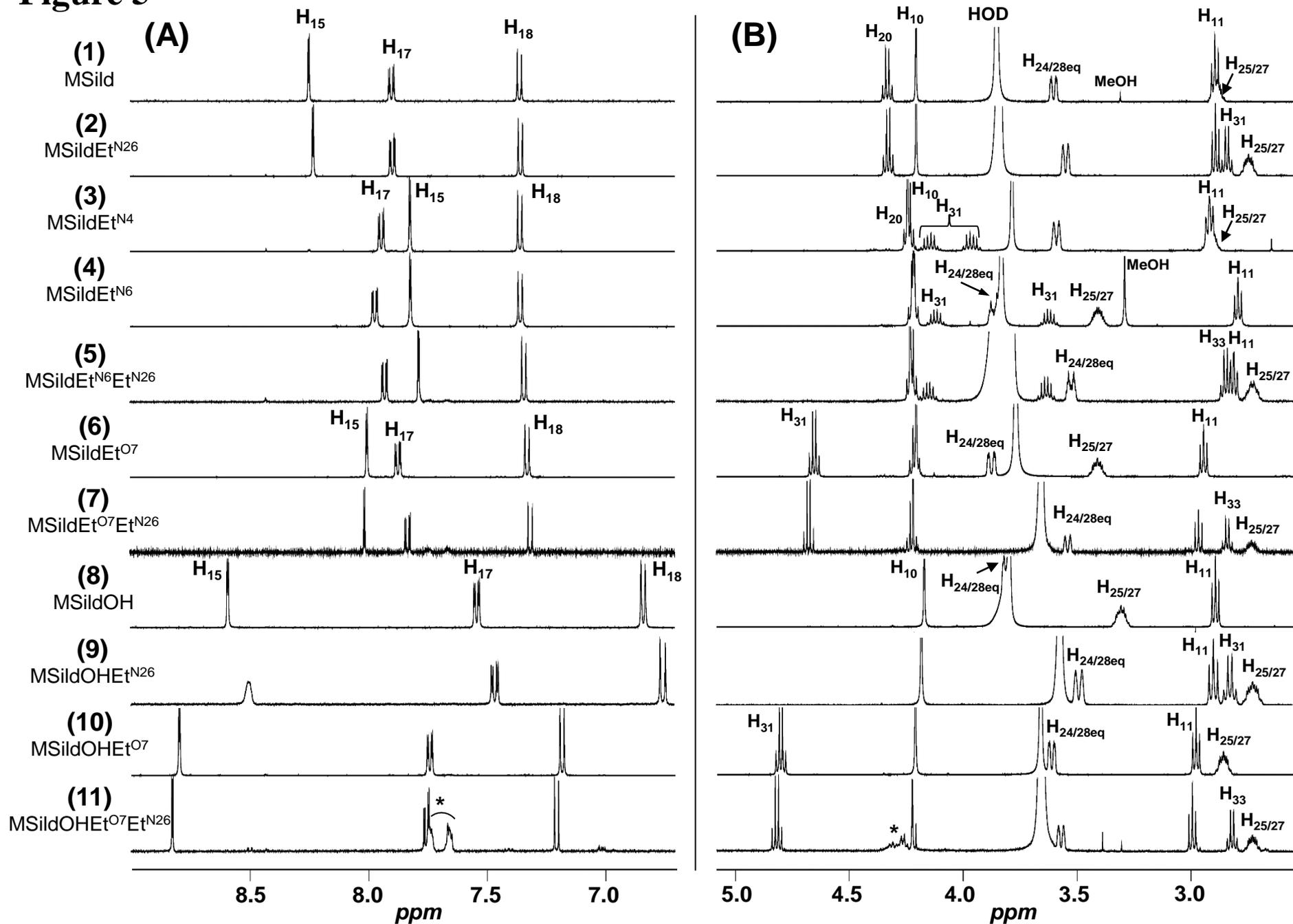


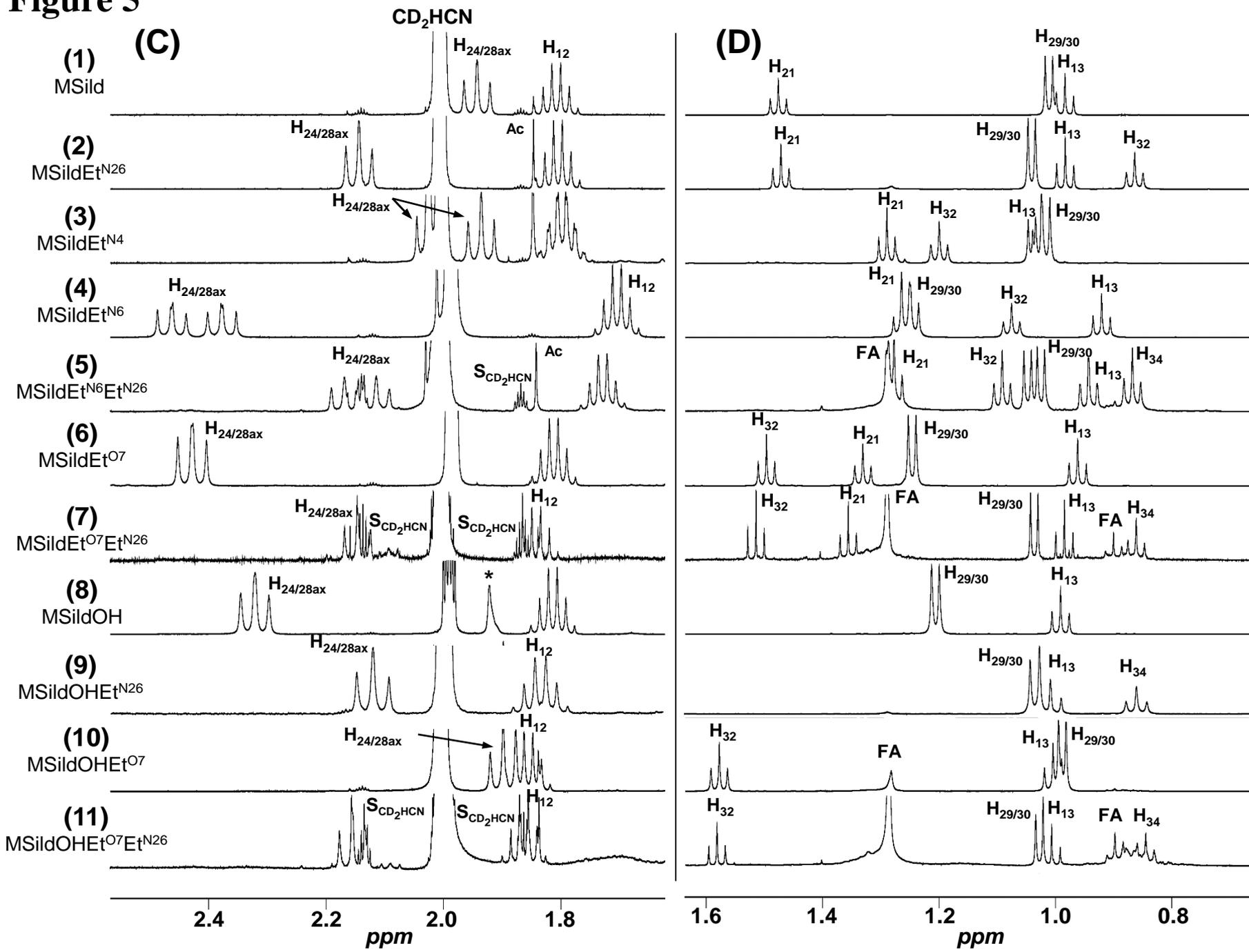
70  
 $C_4H_8N^+$



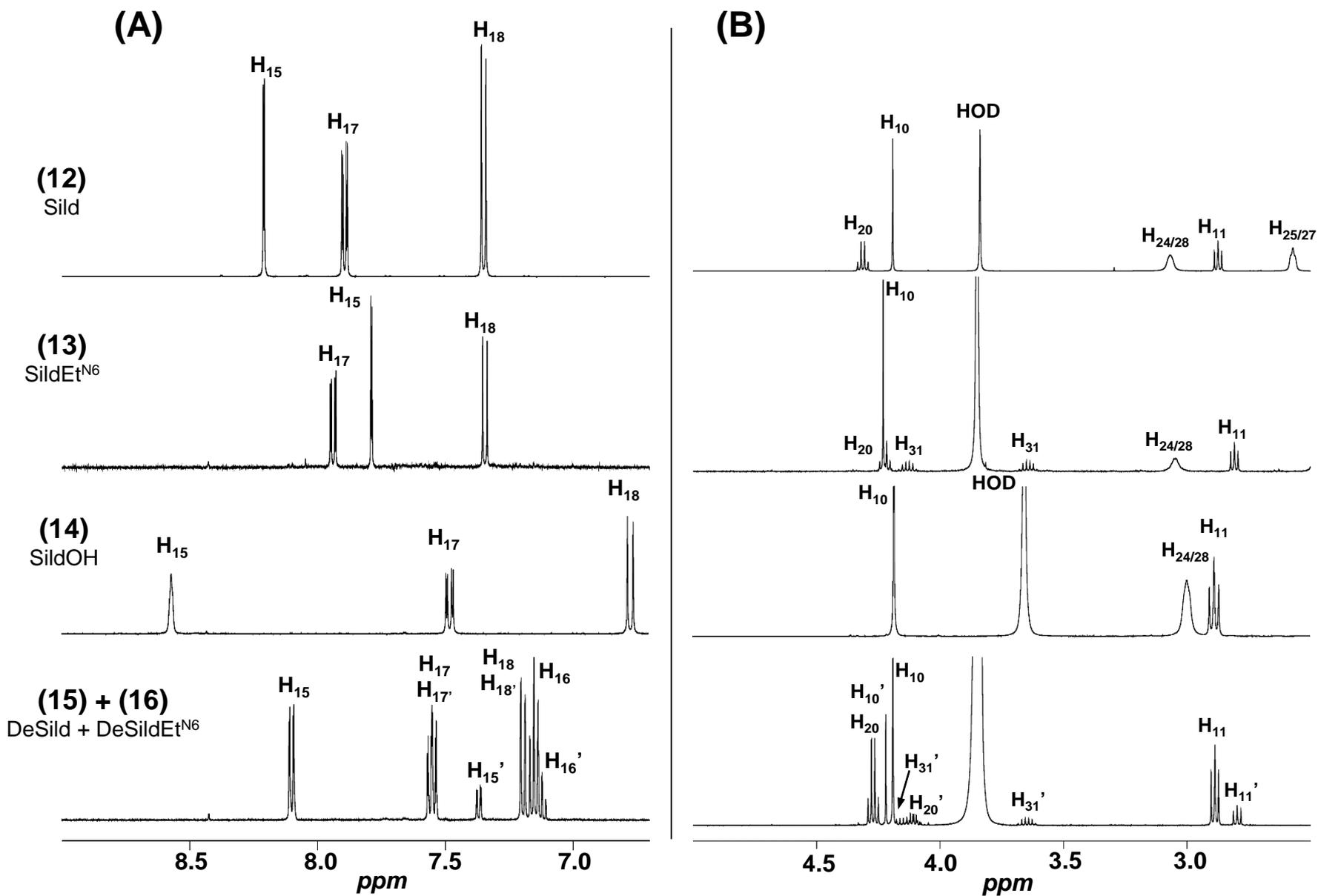
58  
 $C_3H_8N^+$

The two ions at m/z 70 and 58 are also observed with 2,6-dimethylpiperazine and N1-ethyl-2,6-dimethylpiperazine.

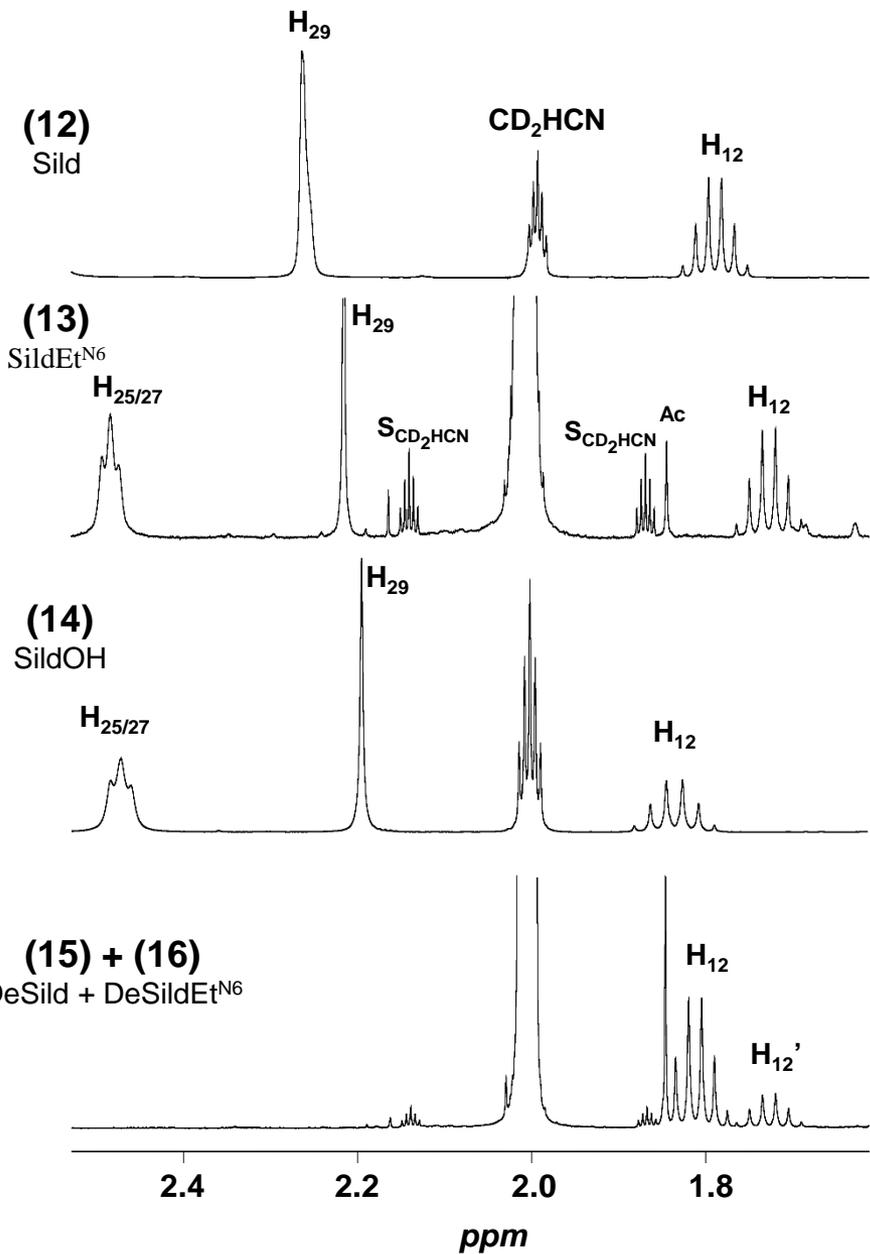
**Figure 5**

**Figure 5**

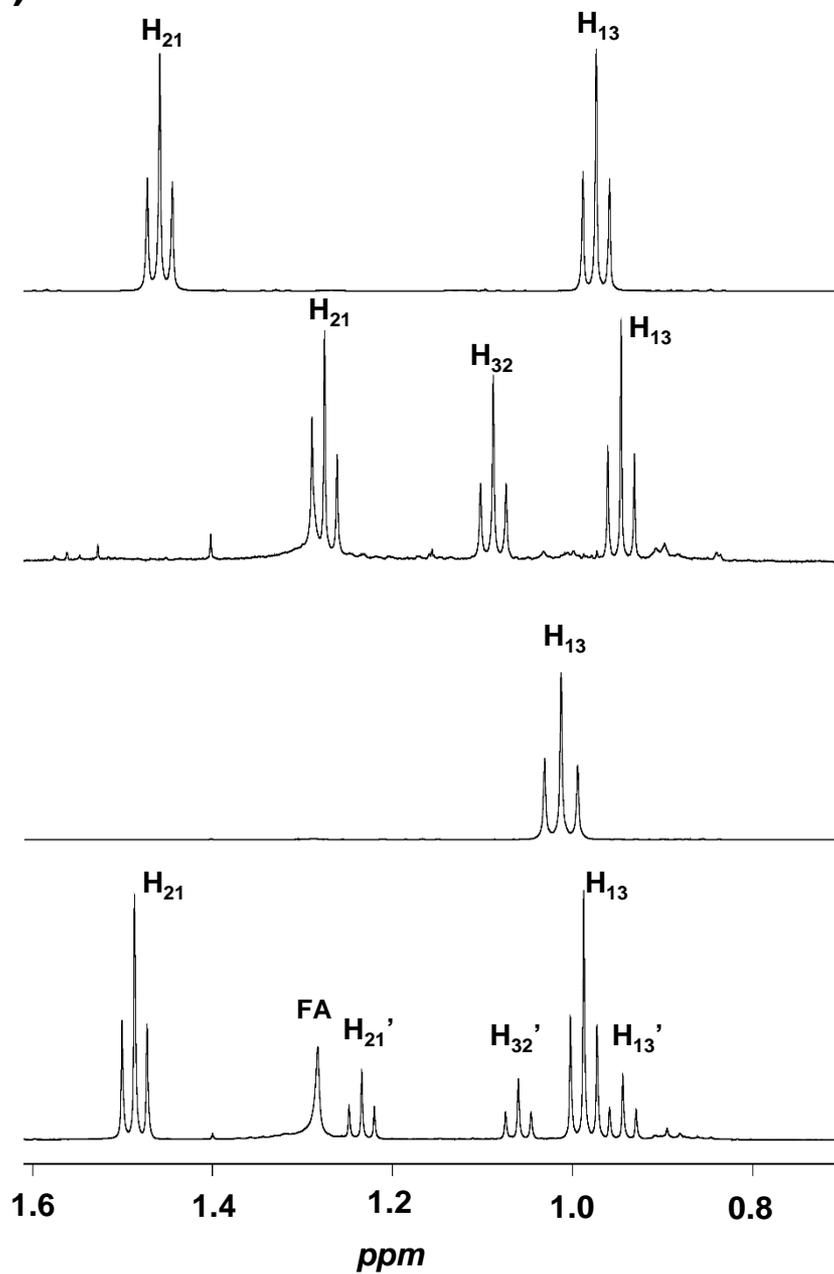
**Figure 5**

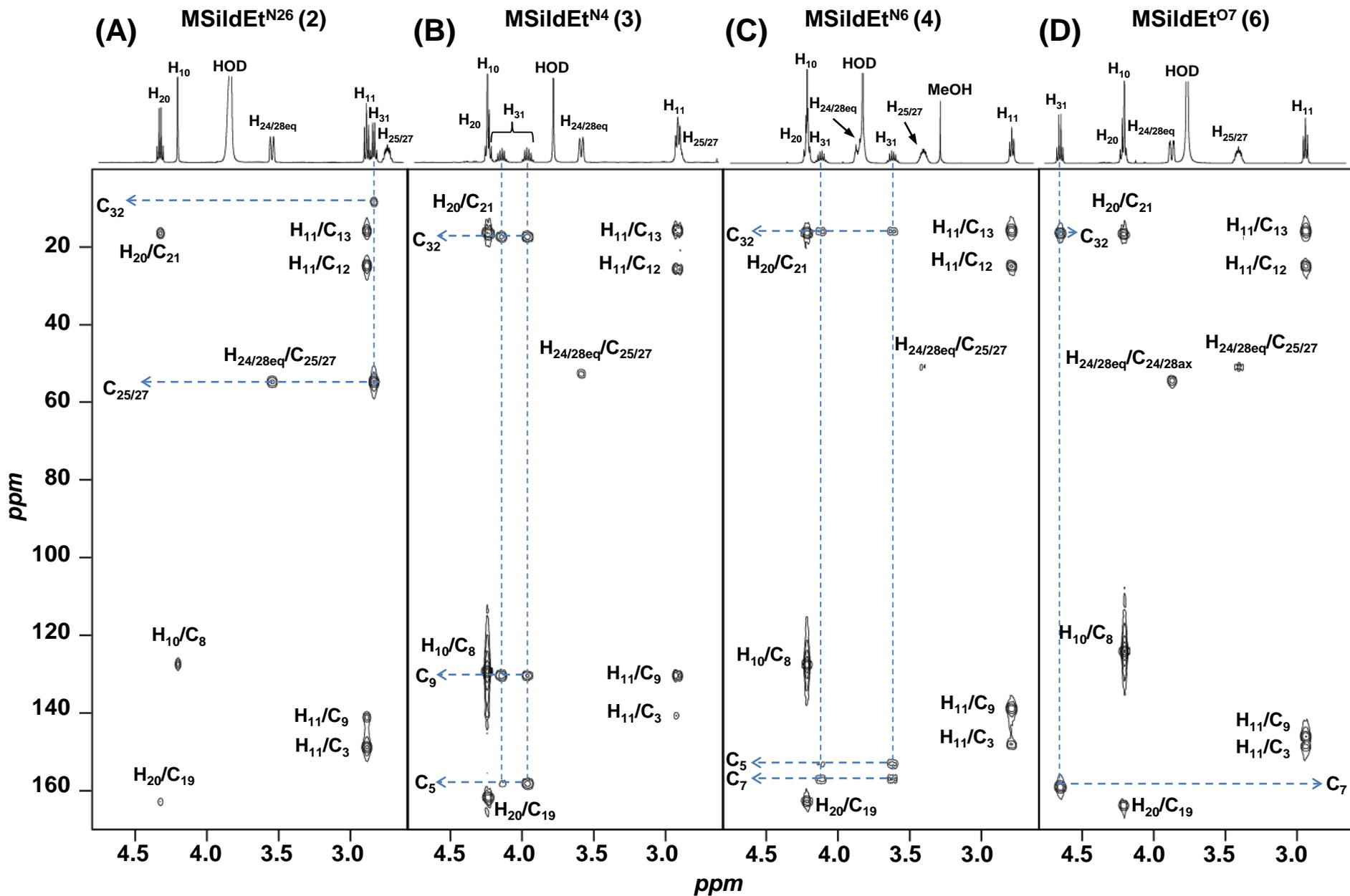


**Figure 5**  
**(C)**

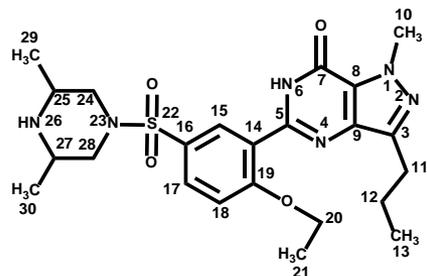


**(D)**

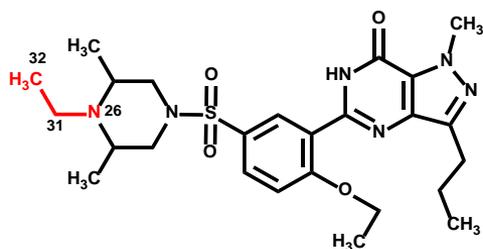


**Figure 6**

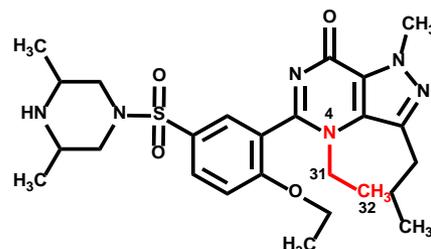
# Figure 7



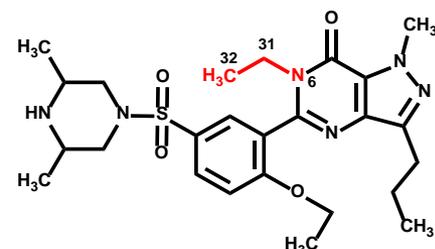
**(1) MSild**  
**Methisosildenafil**  
[496835-35-9]<sup>(a)</sup>



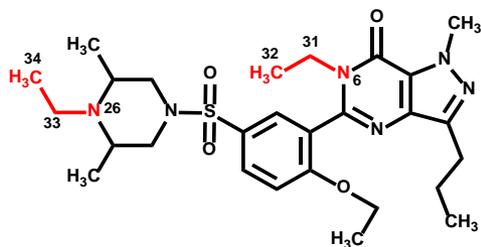
**(2) MSildEt<sup>N26</sup>**  
**N26-ethyl-methisosildenafil** <sup>(b)</sup>



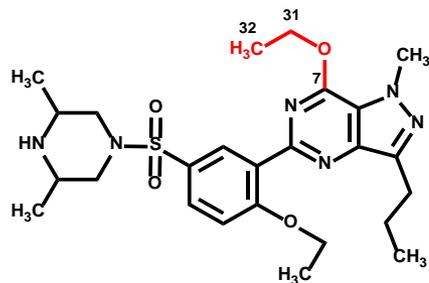
**(3) MSildEt<sup>N4</sup>**  
**N4-ethyl-methisosildenafil**



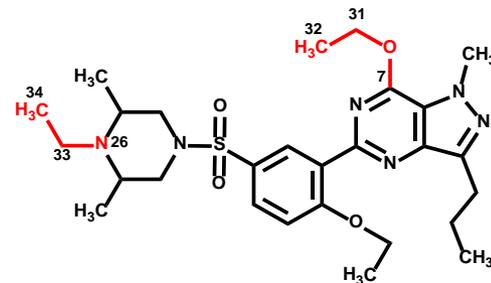
**(4) MSildEt<sup>N6</sup>**  
**N6-ethyl-methisosildenafil**



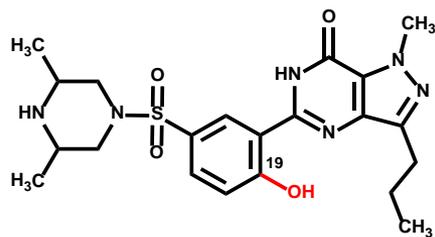
**(5) MSildEt<sup>N6</sup>Et<sup>N26</sup>**  
**N6,N26-diethyl-methisosildenafil**



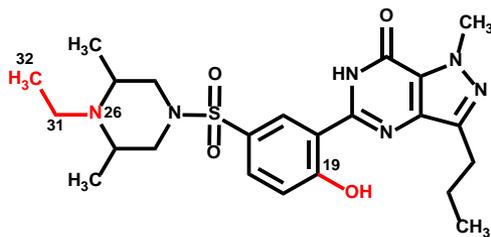
**(6) MSildEt<sup>O7</sup>**  
**O7-ethyl-methisosildenafil**



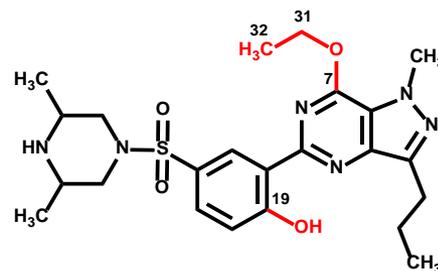
**(7) MSildEt<sup>O7</sup>Et<sup>N26</sup>**  
**O7,N26-diethyl-methisosildenafil**



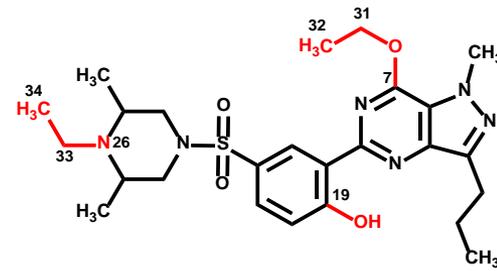
**(8) MSildOH**  
**O-desethyl-methisosildenafil**



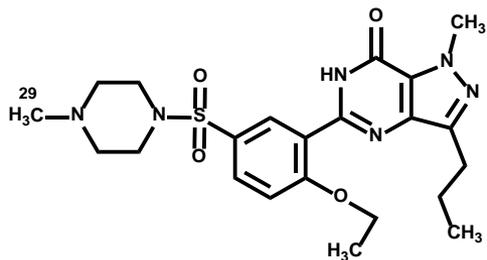
**(9) MSildOHet<sup>N26</sup>**  
**N26-ethyl-O-desethyl-**  
**methisosildenafil**



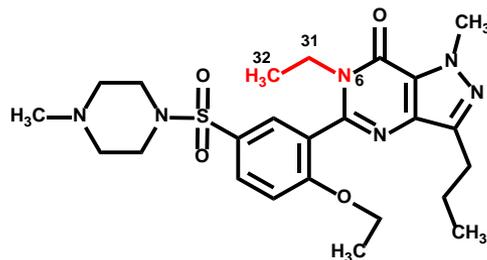
**(10) MSildOHet<sup>O7</sup>**  
**O7-ethyl-O-desethyl-**  
**methisosildenafil**



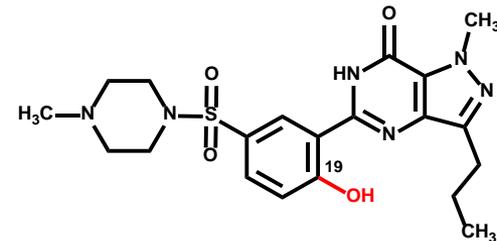
**(11) MSildOHet<sup>O7</sup>Et<sup>N26</sup>**  
**O7,N26-diethyl-O-desethyl-**  
**methisosildenafil**



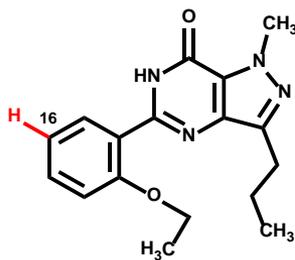
**(12) Sild**  
**Sildenafil**  
[139755-83-2] (a)



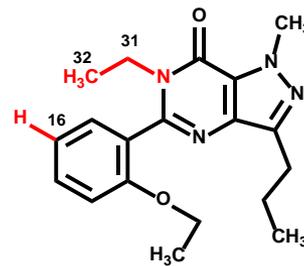
**(13) SildEt<sup>N6</sup>**  
**N6-ethyl-sildenafil** (c)



**(14) SildOH**  
**O-desethyl-sildenafil**  
[139755-91-2] (a,d)



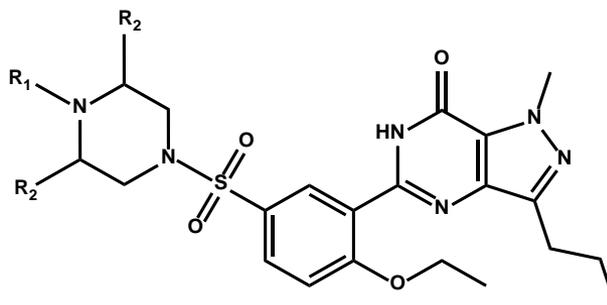
**(15) DeSild**  
**Desulfosildenafil**  
[139756-21-1] (a,e)



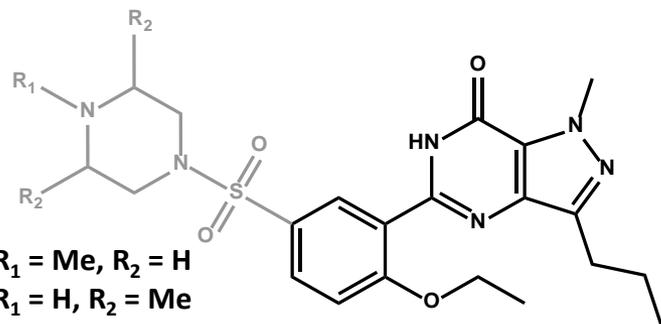
**(16) DeSildEt<sup>N6</sup>**  
**N6-ethyl-desulfosildenafil**

# Figure 8

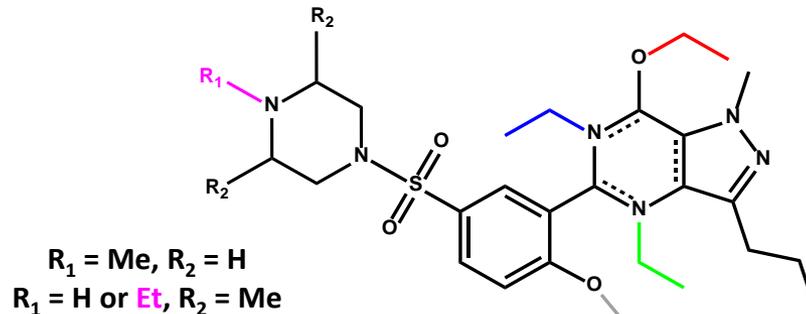
Sildenafil ( $R_1 = \text{Me}$ ,  $R_2 = \text{H}$ )  
Methisosildenafil ( $R_1 = \text{H}$ ,  $R_2 = \text{Me}$ )



Unsulfonylated impurities



Randomly ethylated impurities

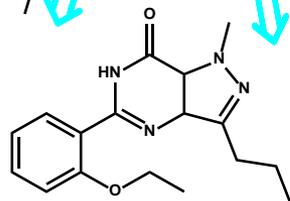


(A)

Chlorosulfonylation

Friedel-Crafts

(B)



Reversible transformation Incomplete conversion

15,16

Late sulfonylation

Early sulfonylation

(C)

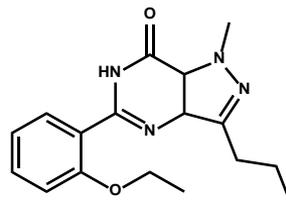
Chlorosulfonylation

Cyclisation

(D)

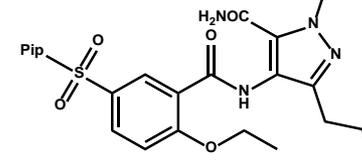
Alkylation

(E)



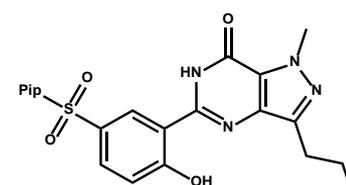
Nucleophilic transfer under acidic conditions

3, 4, 6, 8, 10, 13, 14, 16



Nucleophilic transfer under basic conditions

2-11, 13, 14, 16



Unselective alkylation

### **Author statement**

**Gaëtan Assemat:** Methodology, Validation, Investigation, Writing-original draft preparation, Visualization. **Stéphane Balayssac:** Methodology, Validation, Investigation, Visualization, Supervision. **Véronique Gilard:** Validation, Writing-original draft preparation, Visualization, Supervision, Funding acquisition. **Nathalie-Martins-Froment:** Investigation. **Isabelle Fabing:** Investigation. **Frédéric Rodriguez:** Formal analysis. **Yves Génisson:** Writing-review and editing. **Robert Martino:** Writing-review and editing. **Myriam Malet-Martino:** Conceptualization, Writing-review and editing, Supervision, Funding acquisition.

**Table 1.** Chromatographic conditions for purification of sildenafil and its 15 analogues from the dietary supplement analyzed.

	SFC conditions <sup>a</sup>			Preparative LC conditions <sup>a</sup>								Analytical UHPLC <sup>a</sup>							
Fraction used	Raw			A		B		C		D		E		F		G		All	
Instrument	Berger Multigram II preparative SFC			Waters Prep 150 LC		Waters Prep 150 LC		Waters Prep 150 LC		Waters Prep 150 LC		Waters Prep 150 LC		Waters Prep 150 LC		Waters Prep 150 LC		Waters Acquity UHPLC	
Column	BetaSil Diol-100 <sup>b</sup>			Kromasil C18 <sup>c</sup>		CSH C18 <sup>c</sup>		Kromasil C18 <sup>c</sup>		Kromasil C18 <sup>c</sup>		Kromasil C18 <sup>c</sup>		Kromasil C18 <sup>c</sup>		Kromasil C18 <sup>c</sup>		Acquity UHPLC CSH C18 <sup>d</sup>	
Mobile phase	(A)	CO <sub>2</sub> (100 bar)		10 mM ammonium acetate		0.1% (v/v) TFA <sup>e</sup> in water		10 mM ammonium acetate		0.1% (v/v) TFA <sup>e</sup> in water									
	(B)	0.5% (v/v) isopropylamine in methanol		10% (v/v) of 100 mM ammonium acetate in acetonitrile		10% (v/v) of mobile phase (A) in acetonitrile		10% (v/v) of 100 mM ammonium acetate in acetonitrile		10% (v/v) of 100 mM ammonium acetate in acetonitrile		10% (v/v) of 100 mM ammonium acetate in acetonitrile		10% (v/v) of 100 mM ammonium acetate in acetonitrile		10% (v/v) of 100 mM ammonium acetate in acetonitrile		0.1% (v/v) TFA <sup>e</sup> in acetonitrile	
Gradient elution profile	Step	min	% (B)	min	% (B)	min	% (B)	min	% (B)	min	% (B)	min	% (B)	min	% (B)	min	% (B)	min	% (B)
	1	0 - 0.5	10	0 - 50	30 - 100	0 - 40	10 - 70	0 - 40	30 - 70	0 - 40	25 - 70	0 - 40	25 - 70	0 - 40	25 - 70	0 - 4	5 - 95	0 - 4	5 - 95
	2	0.5 - 8	10 - 28	50 - 55	100	40 - 41	70 - 100	40 - 41	70 - 100	40 - 41	70 - 100	40 - 41	70 - 100	40 - 41	70 - 100	4 - 5.5	95	4 - 5.5	95
	3	8 - 15	28			41 - 50	100	41 - 50	100	41 - 50	100	41 - 50	100	41 - 50	100	5.5 - 5.51	5	5.5 - 5.51	5
	4	15 - 16	28 - 10													5.51 - 7.3	5	5.51 - 7.3	5
5	16 - 20	10																	
Flow rate	42.5 mL/min			120 mL/min		120 mL/min		120 mL/min		120 mL/min		120 mL/min		120 mL/min		120 mL/min		0.6 mL/min	
Temperature	40 °C			RT		RT		RT		RT		RT		RT		RT		30 °C	
Injection volume <sup>f</sup>	0.5 mL			9 mL		7 mL		40 mL		10 mL		10 mL		12 mL		8 mL		1 µL	
Compounds collected	<b>All</b>			<b>13,2,5,15,16,10,7,11</b>		<b>4,6</b>		<b>1,12</b>		<b>3</b>		<b>9</b>		<b>14</b>		<b>8</b>			

<sup>a</sup> UV detection at 235 nm and for analytical UHPLC, cone voltage of QDa mass detector used in positive mode set at 30 V.

<sup>b</sup> 250 mm × 21.2 mm i.d., 5 µm particle size.

<sup>c</sup> 250 mm × 50 mm i.d., 10 µm particle size.

<sup>d</sup> 100 mm × 2.1 mm i.d., 1.7 µm particle size.

<sup>e</sup> TFA: trifluoroacetic acid.

<sup>f</sup> The concentration of powder dissolved in each fraction is different: 50 mg/mL for SFC, 6.3 mg/mL for A, 8.5 mg/mL for B, 4.8 mg/mL for C, 18.6 mg/mL for D, 8.5 mg/mL for E, 6.8 mg/mL for F and 18.5 mg/mL for G.

**Table 2.** Retention times and masses of the parent ions ( $[M+H]^+$ ) of purified compounds in UHPLC.

Compound number	Retention time <sup>a</sup> (min)	UHPLC-MS $[M+H]^+$
<b>1</b>	2.15	489
<b>2</b>	2.21	517
<b>3</b>	2.09	517
<b>4</b>	2.25	517
<b>5</b>	2.31	545
<b>6</b>	2.58	517
<b>7</b>	2.64	545
<b>8</b>	2.22	461
<b>9</b>	2.27	489
<b>10</b>	2.86	489
<b>11</b>	2.92	517
<b>12</b>	2.07	475
<b>13</b>	2.16	503
<b>14</b>	2.15	447
<b>15</b>	3.42	313
<b>16</b>	3.42	341

<sup>a</sup> UHPLC analytical conditions for the control of the chemical purity of preparative LC samples are reported in [Table 1](#).

**Table 3.** Accurate mass data of parent and fragment ions in positive ESI mode for all the compounds (Cpd) isolated from the dietary supplement analyzed. Mass of parent ion is indicated in bold.

**A: methisosildenafil (MSild) and compounds 2-11**

Theoretical mass and formula	Experimental mass of parent and fragment ions (relative mass error in ppm)										
	MSild(1)	Cpd (2)	Cpd (3)	Cpd (4)	Cpd (5)	Cpd (6)	Cpd (7)	Cpd (8)	Cpd (9)	Cpd (10)	Cpd (11)
545.2910					<b>545.2920</b>		<b>545.2922</b>				
C <sub>27</sub> H <sub>41</sub> N <sub>6</sub> O <sub>4</sub> S <sup>+</sup>					(+1.8)		(+2.2)				
517.2597		<b>517.2599</b>	<b>517.2595</b>	<b>517.2587</b>	517.2593	<b>517.2591</b>	517.2587				<b>517.2600</b>
C <sub>25</sub> H <sub>37</sub> N <sub>6</sub> O <sub>4</sub> S <sup>+</sup>		(+0.4)	(-0.4)	(-1.9)	(-0.8)	(-1.2)	(-1.9)				(+0.6)
489.2284	<b>489.2277</b>	489.2277	489.2278	489.2273	489.2272	489.2276	489.2271		<b>489.2292</b>	<b>489.2292</b>	
C <sub>23</sub> H <sub>33</sub> N <sub>6</sub> O <sub>4</sub> S <sup>+</sup>	(-1.4)	(-1.4)	(-1.2)	(-2.2)	(-2.5)	(-1.6)	(-2.7)		(+1.6)	(+1.6)	
461.1971								<b>461.1973</b>			
C <sub>21</sub> H <sub>29</sub> N <sub>6</sub> O <sub>4</sub> S <sup>+</sup>								(+0.4)			
460.2019			460.2007	460.2015	460.2011	460.2008	460.2008				
C <sub>22</sub> H <sub>30</sub> N <sub>5</sub> O <sub>4</sub> S <sup>+</sup>			(-2.6)	(-0.9)	(-1.7)	(-2.4)	(-2.4)				
432.1700	432.1692	432.1696	432.1690	432.1696	432.1694	432.1695	432.1690				
C <sub>20</sub> H <sub>26</sub> N <sub>5</sub> O <sub>4</sub> S <sup>+</sup>	(-1.9)	(-0.9)	(-2.3)	(-0.9)	(-1.4)	(-1.2)	(-2.3)				
405.1597			405.1592	405.1588	405.1591	405.1591	405.1588				
C <sub>19</sub> H <sub>25</sub> N <sub>4</sub> O <sub>4</sub> S <sup>+</sup>			(-1.2)	(-2.2)	(-1.5)	(-1.5)	(-2.2)				
377.1278	377.1273	377.1272	377.1281	377.1275	377.1273	377.1277	377.1274				
C <sub>17</sub> H <sub>21</sub> N <sub>4</sub> O <sub>4</sub> S <sup>+</sup>	(-1.3)	(-1.6)	(+0.8)	(-0.8)	(-1.3)	(-0.3)	(-1.1)				
375.1127										375.1127	375.1125
C <sub>17</sub> H <sub>19</sub> N <sub>4</sub> O <sub>4</sub> S <sup>+</sup>										(0)	(-0.5)
347.0814								347.0812	347.0815		
C <sub>15</sub> H <sub>15</sub> N <sub>4</sub> O <sub>4</sub> S <sup>+</sup>								(-0.6)	(+0.3)		
340.1899			340.1895	340.1897							
C <sub>19</sub> H <sub>24</sub> N <sub>4</sub> O <sub>2</sub> <sup>+</sup>			(-1.2)	(-0.6)							
339.1815				339.1816	339.1815	339.1815	339.1817				
C <sub>19</sub> H <sub>23</sub> N <sub>4</sub> O <sub>2</sub> <sup>+</sup>				(+0.3)	(0)	(0)	(+0.6)				
327.1458			327.1464	327.1450	327.1445	327.1446	327.1472			327.1458	327.1453
C <sub>17</sub> N <sub>19</sub> N <sub>4</sub> O <sub>3</sub> <sup>+</sup>			(+1.8)	(-2.4)	(-4.0)	(-3.7)	(+4.3)			(0)	(-1.5)
312.1580			312.1575	312.1584							
C <sub>17</sub> H <sub>20</sub> N <sub>4</sub> O <sub>2</sub> <sup>+</sup>			(-1.6)	(+1.3)							

311.1502	311.1503	311.1506	311.1508	311.1504	311.1501	311.1505	311.1500			311.1510	311.1503
C <sub>17</sub> H <sub>19</sub> N <sub>4</sub> O <sub>2</sub> <sup>+</sup>	(+0.3)	(+1.3)	(+1.9)	(+0.6)	(-0.3)	(+1.0)	(+0.6)			(+2.6)	(+0.3)
299.1144	299.1142	299.1141	299.1137	299.1139	299.1145	299.1141	299.1138	299.1147	299.1147	299.1146	299.1143
C <sub>15</sub> H <sub>15</sub> N <sub>4</sub> O <sub>3</sub> <sup>+</sup>	(-0.7)	(-1.0)	(-2.3)	(-1.6)	(+0.3)	(-1.0)	(-2.0)	(+1.0)	(+1.0)	(+0.7)	(-0.3)
284.1268			284.1272	284.1269					284.1266		
C <sub>15</sub> H <sub>16</sub> N <sub>4</sub> O <sub>2</sub> <sup>+</sup>			(+1.4)	(+0.4)					(-0.7)		
283.1190	283.1190	283.1195	283.1195	283.1191	283.1188	283.1192	283.1190	283.1198	283.1196	283.1194	283.1189
C <sub>15</sub> H <sub>15</sub> N <sub>4</sub> O <sub>2</sub> <sup>+</sup>	(0)	(+1.8)	(+1.8)	(+0.4)	(-0.7)	(+0.7)	(0)	(+2.8)	(+2.1)	(+1.4)	(-0.4)
166.0980	166.0979	166.0982	166.0985	166.0978	166.0984	166.0981	166.0973	166.0981	166.0980	166.0981	166.0973
C <sub>8</sub> H <sub>12</sub> N <sub>3</sub> O <sup>+</sup>	(-0.6)	(+1.2)	(+3.0)	(-1.2)	(+2.4)	(+0.6)	(-4.2)	(+0.6)	(0)	(+0.6)	(-4.2)
143.1548									143.1551		
C <sub>8</sub> H <sub>19</sub> N <sub>2</sub> <sup>+</sup>									(+2.1)		
141.1392		141.1394	141.1392	141.1396	141.1392	141.1390	141.1391		141.1392		141.1392
C <sub>8</sub> H <sub>17</sub> N <sub>2</sub> <sup>+</sup>		(+1.4)	(0)	(+2.8)	(0)	(-1.4)	(-0.7)		(0)		(0)
127.1235		127.1238	127.1237	127.1239	127.1236	127.1234	127.1236		127.1235		127.1233
C <sub>7</sub> H <sub>15</sub> N <sub>2</sub> <sup>+</sup>		(+2.4)	(+1.6)	(+3.1)	(+0.8)	(-0.8)	(+0.8)		(0)		(-1.6)
115.1230								115.1235			
C <sub>6</sub> H <sub>15</sub> N <sub>2</sub> <sup>+</sup>								(+4.3)			
113.1079	113.1077	113.1081	113.1077	113.1076	113.1079	113.1081	113.1081	113.1080	113.1084	113.1079	113.1076
C <sub>6</sub> H <sub>13</sub> N <sub>2</sub> <sup>+</sup>	(-1.8)	(+1.8)	(-1.8)	(-2.7)	(0)	(+1.8)	(+1.8)	(+0.9)	(+4.4)	(0)	(-2.7)
100.1126		100.1130			100.1127	100.1134	100.1129		100.1127		100.1127
C <sub>6</sub> H <sub>14</sub> N <sup>+</sup>		(+4.0)			(+1.0)	(+8.0)	(+3.0)		(+1.0)		(+1.0)
99.0922	99.0923		99.0928	99.0921		99.0927		99.0924		99.0925	
C <sub>5</sub> H <sub>11</sub> N <sub>2</sub> <sup>+</sup>	(+1.0)		(+6.1)	(-1.0)		(+5.0)		(+2.0)		(+3.0)	
98.0970		98.0972			98.0972	98.0969	98.0972		98.0971		98.0971
C <sub>6</sub> H <sub>12</sub> N <sup>+</sup>		(+2.0)			(+2.0)	(-1.0)	(+2.0)		(+1.0)		(+1.0)
86.0970		86.0973			86.0972	86.0976	86.0973		86.0976		86.0972
C <sub>5</sub> H <sub>12</sub> N <sup>+</sup>		(+3.5)			(+2.3)	(+7.0)	(+3.5)		(+7.0)		(+2.3)
84.0813	84.0814	84.0818	84.0821	84.0820	84.0816	84.0816	84.0813	84.0819	84.0816	84.0816	84.0807
C <sub>5</sub> H <sub>10</sub> N <sup>+</sup>	(+1.2)	(+5.9)	(+9.5)	(+8.3)	(+3.6)	(+3.6)	(0)	(+7.1)	(+3.6)	(+3.6)	(-7.1)
72.0813		72.0818			72.0816	72.0814	72.0812		72.0815		72.0816
C <sub>4</sub> H <sub>10</sub> N <sup>+</sup>		(+6.9)			(+4.2)	(+1.4)	(-1.4)		(+2.8)		(+4.2)
71.0735	71.0739	71.0741	71.0737	71.0738	71.0740	71.0741	71.0741	71.0738	71.0741	71.0741	71.0740
C <sub>4</sub> H <sub>9</sub> N <sup>+</sup>	(+5.6)	(+8.4)	(+2.8)	(+4.2)	(+7.0)	(+8.4)	(+8.4)	(+4.2)	(+8.4)	(+8.4)	(+7.0)

**B: sildenafil (Sild) and compounds 13-16**

Theoretical mass and formula	Experimental mass of parent and fragment ions (relative mass error in ppm)				
	Sild (12)	Cpd (13)	Cpd (14)	Cpd (15)	Cpd (16)
503.2440		<b>503.2448</b>			
C <sub>24</sub> H <sub>35</sub> N <sub>6</sub> O <sub>4</sub> S <sup>+</sup>		(+1.6)			
475.2127	<b>475.2126</b>	475.2119			
C <sub>22</sub> H <sub>31</sub> N <sub>6</sub> O <sub>4</sub> S <sup>+</sup>	(-0.2)	(-1.7)			
447.1815			<b>447.1818</b>		
C <sub>20</sub> H <sub>27</sub> N <sub>6</sub> O <sub>4</sub> S <sup>+</sup>			(+0.7)		
405.1597		405.1596			
C <sub>19</sub> H <sub>25</sub> N <sub>4</sub> O <sub>4</sub> S <sup>+</sup>		(-0.2)			
377.1278	377.1270	377.1296			
C <sub>17</sub> H <sub>21</sub> N <sub>4</sub> O <sub>4</sub> S <sup>+</sup>	(-2.1)	(+4.8)			
347.0814			347.0809		
C <sub>15</sub> H <sub>15</sub> N <sub>4</sub> O <sub>4</sub> S <sup>+</sup>			(+1.4)		
341.1978				<b>341.1980</b>	
C <sub>19</sub> H <sub>25</sub> N <sub>4</sub> O <sub>2</sub> <sup>+</sup>				(+0.6)	
340.1899		340.1883			
C <sub>19</sub> H <sub>24</sub> N <sub>4</sub> O <sub>2</sub> <sup>+</sup>		(-4.7)			
339.1815		339.1816			
C <sub>19</sub> H <sub>23</sub> N <sub>4</sub> O <sub>2</sub> <sup>+</sup>		(+0.3)			
327.1458		327.1465			
C <sub>17</sub> H <sub>19</sub> N <sub>4</sub> O <sub>3</sub> <sup>+</sup>		(+2.1)			
313.1658				<b>313.1663</b>	313.1666
C <sub>17</sub> H <sub>21</sub> N <sub>4</sub> O <sub>2</sub> <sup>+</sup>				(+1.6)	(+2.6)
311.1502	311.1504	311.1510			
C <sub>17</sub> H <sub>19</sub> N <sub>4</sub> O <sub>2</sub> <sup>+</sup>	(+0.6)	(+2.6)			
299.1144	299.1140	299.1151	299.1148		
C <sub>15</sub> H <sub>15</sub> N <sub>4</sub> O <sub>3</sub> <sup>+</sup>	(-1.3)	(+2.3)	(+1.3)		
285.1346				285.1352	285.1356
C <sub>15</sub> H <sub>17</sub> N <sub>4</sub> O <sub>2</sub> <sup>+</sup>				(+2.1)	(+3.5)
283.1190	283.1192	283.1194	283.1198		
C <sub>15</sub> H <sub>15</sub> N <sub>4</sub> O <sub>2</sub> <sup>+</sup>	(+0.7)	(+1.4)	(+2.8)		

166.0980	166.0979	166.0983	166.0981	166.0981	166.0980
C <sub>8</sub> H <sub>12</sub> N <sub>3</sub> O <sup>+</sup>	(-0.6)	(+1.8)	(+0.6)	(+0.6)	(0)
163.0541	163.0539	163.0543	163.0549		
C <sub>5</sub> H <sub>11</sub> N <sub>2</sub> O <sub>2</sub> S <sup>+</sup>	(-1.2)	(+1.2)	(+4.9)		
101.1079			101.1081		
C <sub>5</sub> H <sub>13</sub> N <sub>2</sub> <sup>+</sup>			(+2.0)		
100.1000	100.1001	100.1001	100.1001		
C <sub>5</sub> H <sub>12</sub> N <sub>2</sub> <sup>+•</sup>	(+1.0)	(+1.0)	(+1.0)		
99.0922	99.0923	99.0924	99.0925		
C <sub>5</sub> H <sub>11</sub> N <sub>2</sub> <sup>+</sup>	(+1.0)	(+2.0)	(+3.0)		
84.0688	84.0691	84.0688			
C <sub>4</sub> H <sub>8</sub> N <sub>2</sub> <sup>+•</sup>	(+3.6)	(0)			
70.0657 <sup>a</sup>	70.0662	70.0662	70.0658		
C <sub>4</sub> H <sub>8</sub> N <sup>+</sup>	(+7.1)	(+7.1)	(+1.4)		
58.0661 <sup>a</sup>	58.0664	58.0662	58.0661		
C <sub>3</sub> H <sub>8</sub> N <sup>+</sup>	(+5.2)	(+1.7)	(0)		

<sup>a</sup>The ions at m/z 70 and 58 are also observed in the degradation pathways of methisosildenafil (MSild) and compounds **2-11** although not mentioned in [Table 3A](#).

**Table 4.** Accurate mass data of parent and fragment ions in negative ESI mode for sildenafil (Sild) and some of its analogues found in the dietary supplement analyzed. Mass of parent ion is indicated in bold. Cpd: compound; MSild: methisosildenafil.

Theoretical mass	Formula	Experimental mass (relative mass error in ppm)								
		MSild (1)	Cpd (2)	Cpd (8)	Cpd (9)	Cpd (10)	Cpd (11)	Sild (12)	Cpd (14)	Cpd (15)
515.2440	C <sub>25</sub> H <sub>35</sub> N <sub>6</sub> O <sub>4</sub> S <sup>-</sup>		<b>515.2448</b> (+1.6)					<b>515.2442</b> (+0.4)		
487.2127	C <sub>23</sub> H <sub>31</sub> N <sub>6</sub> O <sub>4</sub> S <sup>-</sup>	<b>487.2133</b> (+1.2)	487.2124 (-0.6)		<b>487.2130</b> (+0.6)	<b>487.2144</b> (+3.5)	487.2124 (-0.6)			
473.1971	C <sub>22</sub> H <sub>29</sub> N <sub>6</sub> O <sub>4</sub> S <sup>-</sup>							<b>473.1979</b> (+1.7)		
459.1814	C <sub>21</sub> H <sub>27</sub> N <sub>6</sub> O <sub>4</sub> S <sup>-</sup>	459.1810 (-0.9)		<b>459.1813</b> (-0.2)		459.1817 (+0.7)				
445.1658	C <sub>20</sub> H <sub>25</sub> N <sub>6</sub> O <sub>4</sub> S <sup>-</sup>							445.1652 (-1.3)	<b>445.1653</b> (-1.1)	
326.1379	C <sub>17</sub> H <sub>18</sub> N <sub>4</sub> O <sub>3</sub> <sup>-</sup>					326.1386 (+2.1)	326.1373 (-1.8)			
311.1508	C <sub>17</sub> H <sub>19</sub> N <sub>4</sub> O <sub>2</sub> <sup>-</sup>									<b>311.1509</b> (+0.3)
310.1430	C <sub>17</sub> H <sub>18</sub> N <sub>4</sub> O <sub>2</sub> <sup>•-</sup>	310.1427 (-1.0)	310.1422 (-2.6)			310.1431 (+0.3)	310.1426 (-1.3)	310.1436 (+1.9)		
298.1066	C <sub>15</sub> H <sub>14</sub> N <sub>4</sub> O <sub>3</sub> <sup>-</sup>	298.1073 (+2.3)	298.1074 (+2.7)	298.1062 (-1.3)	298.1072 (+2.0)	298.1066 (0)	298.1075 (+3.0)	298.1062 (-1.3)	298.1071 (+1.7)	
283.1195	C <sub>15</sub> H <sub>15</sub> N <sub>4</sub> O <sub>2</sub> <sup>-</sup>									283.1192 (-1.1)
282.1117	C <sub>15</sub> H <sub>14</sub> N <sub>4</sub> O <sub>2</sub> <sup>•-</sup>	282.1114 (-1.1)	282.1114 (-1.1)	282.1117 (0)	282.1119 (+0.7)	282.1112 (-1.8)	282.1116 (-0.4)	282.1115 (-0.7)	282.1114 (-1.1)	

**Table 5.**  $^1\text{H}$  and  $^{13}\text{C}$  NMR data of methisosildenafil (MSild), sildenafil (Sild) and their related compounds purified from the dietary supplement analyzed.  $\delta$  were measured in  $\text{CD}_3\text{CN}:\text{D}_2\text{O}$  (80:20).

N <sup>o</sup> (a)	MSild (1)				MSildEt <sup>N4</sup> (3)				MSildEt <sup>N6</sup> (4)				MSildEt <sup>O7</sup> (6)			
	$\delta$ $^1\text{H}$ (ppm)	Multiplicity <sup>(b)</sup> (J, Hz)	$\delta$ $^{13}\text{C}$ (ppm)		$\delta$ $^1\text{H}$ (ppm)	Multiplicity <sup>(b)</sup> (J, Hz)	$\delta$ $^{13}\text{C}$ (ppm)		$\delta$ $^1\text{H}$ (ppm)	Multiplicity <sup>(b)</sup> (J, Hz)	$\delta$ $^{13}\text{C}$ (ppm)		$\delta$ $^1\text{H}$ (ppm)	Multiplicity <sup>(b)</sup> (J, Hz)	$\delta$ $^{13}\text{C}$ (ppm)	
3	-	-	-	148.9	-	-	-	140.7	-	-	-	148.2	-	-	-	148.3
5	-	-	-	150.7	-	-	-	158.4	-	-	-	153.1	-	-	-	158.4
7	-	-	-	157.0	-	-	-	164.7	-	-	-	157.1	-	-	-	159.1
8	-	-	-	127.4	-	-	-	129.4	-	-	-	127.5	-	-	-	124.2
9	-	-	-	141.0	-	-	-	130.5	-	-	-	138.9	-	-	-	146.2
10	4.20	3H	s	40.4	4.24	3H	s	40.5	4.21	3H	s	40.4	4.20	3H	s	41.0
11	2.89	2H	t (7.4)	29.9	2.91 <sub>2</sub> /2.91 <sub>5</sub>	2H	2*app t (7.5)	31.8	2.79	2H	t (7.4)	29.6	2.94	2H	t (7.5)	30.2
12	1.81	2H	sext (7.4)	24.8	1.79 <sub>7</sub> /1.79 <sub>9</sub>	2H	2*app sext (7.5)	25.8	1.70	2H	sext (7.4)	25.0	1.81	2H	sext (7.5)	25.0
13	0.98	3H	t (7.4)	15.9	1.02	3H	t (7.5)	15.9	0.92	3H	t (7.4)	15.8	0.96	3H	t (7.5)	16.0
14	-	-	-	124.7	-	-	-	126.3	-	-	-	128.1	-	-	-	132.1
15	8.24	1H	d (2.4)	133.0	7.82	1H	d (2.4)	132.4	7.81	1H	d (2.4)	132.4	8.00	1H	d (2.5)	133.8
16	-	-	-	130.2	-	-	-	130.0	-	-	-	129.2	-	-	-	128.8
17	7.89	1H	dd (8.9; 2.4)	134.7	7.94	1H	dd (8.9; 2.4)	134.8	7.96	1H	dd (8.9; 2.4)	134.6	7.87	1H	dd (8.8; 2.5)	133.5
18	7.35	1H	d (8.9)	116.3	7.35	1H	d (8.9)	116.0	7.35	1H	d (8.9)	116.0	7.32	1H	d (8.8)	116.4
19	-	-	-	162.8	-	-	-	161.9	-	-	-	162.8	-	-	-	163.9
20	4.33	2H	q (7.0)	68.5	4.23	2H	q (7.0)	68.1	4.21	2H	q (7.0)	68.0	4.21	2H	q (7.0)	67.9
21	1.47	3H	t (7.0)	16.5	1.29	3H	t (7.0)	16.5	1.26	3H	t (7.0)	16.4	1.33	3H	t (7.0)	16.8
24/28 <sup>eq</sup> (c)	3.60	2H	dm (11.3)	54.0	3.58	2H	dm (11.2)	54.0/53.9	3.86	2H	dm (13.0)	50.9 <sub>5</sub> /50.9 <sub>3</sub>	3.87	2H	dm (13.0)	51.0
24/28 <sup>ax</sup> (c)	1.94	2H	app t (11.3)	54.0	2.02/1.93	2H	2*app t (11.2)	54.0/53.9	2.46/2.38	2H	2*dd (13.0; 11.4)	50.9 <sub>5</sub> /50.9 <sub>3</sub>	2.43	2H	dd (13.0; 11.6)	51.0
25/27	2.88	2H	m	52.7	2.90	2H	m	53.0/52.9	3.40	2H	m	54.2/54.3	3.40	2H	m	54.5
29/30	1.01	6H	d (6.4)	20.5	1.04/1.01	6H	2*d (6.5)	20.5	1.26/1.24	6H	2*d (6.8)	17.7	1.24	6H	d (6.6)	17.8
31	-	-	-	-	4.14/3.96	2H	2*qd (15.0; 7.2)	47.8	4.12/3.62	2H	2*qd (14.0; 7.1)	43.5	4.65	2H	q (7.1)	66.4
32	-	-	-	-	1.20	3H	t (7.2)	17.5	1.07	3H	t (7.1)	16.1	1.50	3H	t (7.1)	16.5

N <sup>o</sup> (a)	MSildEt <sup>N26</sup> (2)				MSildEt <sup>N6Et</sup> N <sup>26</sup> (5)				MSildEt <sup>O7Et</sup> N <sup>26</sup> (7)			
	$\delta$ <sup>1</sup> H (ppm)		Multiplicity <sup>(b)</sup> (J, Hz)	$\delta$ <sup>13</sup> C (ppm)	$\delta$ <sup>1</sup> H (ppm)		Multiplicity <sup>(b)</sup> (J, Hz)	$\delta$ <sup>13</sup> C (ppm)	$\delta$ <sup>1</sup> H (ppm)		Multiplicity <sup>(b)</sup> (J, Hz)	$\delta$ <sup>13</sup> C (ppm)
3	-	-	-	148.9	-	-	-	148.3	-	-	-	148.3
5	-	-	-	150.9	-	-	-	153.1	-	-	-	158.3
7	-	-	-	157.1	-	-	-	157.2	-	-	-	159.0
8	-	-	-	127.5	-	-	-	127.5	-	-	-	124.1
9	-	-	-	141.1	-	-	-	139.1	-	-	-	146.4
10	4.20	3H	s	40.5	4.23	3H	s	40.4	4.21	3H	s	40.9
11	2.89	2H	t (7.4)	30.0	2.81	2H	t (7.4)	29.6	2.96	2H	t (7.4)	30.2
12	1.80	2H	sext (7.4)	24.9	1.73	2H	sext (7.4)	25.0	1.84	2H	sext (7.4)	24.9
13	0.98	3H	t (7.4)	16.0	0.94	3H	t (7.4)	15.8	0.98	3H	t (7.4)	16.0
14	-	-	-	124.9	-	-	-	129.6	-	-	-	129.1
15	8.22	1H	d (2.4)	133.2	7.78	1H	d (2.4)	132.5	8.01	1H	d (2.5)	133.9
16	-	-	-	130.1	-	-	-	128.0	-	-	-	131.9
17	7.89	1H	dd (8.8; 2.4)	134.9	7.92	1H	dd (8.9; 2.4)	134.4	7.83	1H	dd (8.8; 2.5)	133.3
18	7.35	1H	d (8.8)	116.4	7.33	1H	d (8.9)	115.8	7.31	1H	d (8.8)	116.1
19	-	-	-	163.0	-	-	-	162.4	-	-	-	163.4
20	4.32	2H	q (7.0)	68.6	4.22	2H	q (7.0)	67.9	4.22	2H	q (7.0)	67.7
21	1.47	3H	t (7.0)	16.6	1.28	3H	t (7.0)	16.4	1.35	3H	t (7.0)	16.8
24/28 <sup>eq</sup> (c)	3.54	2H	dm (11.3)	54.8	3.52	2H	dm (11.5)	54.8/54.9	3.53	2H	dm (11.2)	54.9
24/28 <sup>ax</sup> (c)	2.14	2H	app t (11.3)	54.8	2.17/2.11	2H	2*app t (11.1)	54.8/ 54.9	2.14	2H	app t (11.2)	54.9
25/27	2.74	2H	m	54.9	2.72	2H	m	54.75/54.7	2.73	2H	m	54.8
29/30	1.04	6H	d (6.4)	18.9	1.05/1.02	6H	2*d (6.4)	18.9	1.04	6H	d (6.2)	19.0
31	2.84	2H	q (7.2)	42.5	4.15/3.63	2H	2*qd (14.1; 7.1)	43.4	4.67	2H	q (7.1)	66.2
32	0.86	3H	t (7.2)	8.6	1.09	3H	t (7.1)	16.1	1.51	3H	t (7.1)	16.5
33	-	-	-	-	2.84	2H	q (7.2)	42.4	2.83	2H	q (7.2)	42.5
34	-	-	-	-	0.87	3H	t (7.2)	8.5	0.86	3H	t (7.2)	8.6

N <sup>o</sup> (a)	MSildOH (8)			MSildOEt <sup>N26</sup> (9) (d)			MSildOEt <sup>O7</sup> (10)			MSildOEt <sup>O7Et</sup> <sup>N26</sup> (11)		
	$\delta$ <sup>1</sup> H (ppm)	Multiplicity <sup>(b)</sup> (J, Hz)	$\delta$ <sup>13</sup> C (ppm)	$\delta$ <sup>1</sup> H (ppm)	Multiplicity <sup>(b)</sup> (J, Hz)	$\delta$ <sup>13</sup> C (ppm)	$\delta$ <sup>1</sup> H (ppm)	Multiplicity <sup>(b)</sup> (J, Hz)	$\delta$ <sup>13</sup> C (ppm)	$\delta$ <sup>1</sup> H (ppm)	Multiplicity <sup>(b)</sup> (J, Hz)	$\delta$ <sup>13</sup> C (ppm)
3	-	-	147.8	-	-	147.7	-	-	147.2	-	-	147.4
5	-	-	155.5	-	-	156.7	-	-	158.8	-	-	158.9
7	-	-	158.1	-	-	159.1	-	-	159.3	-	-	159.5
8	-	-	127.3	-	-	127.6	-	-	124.0	-	-	124.8
9	-	-	141.8	-	-	142.2	-	-	143.5	-	-	143.6
10	4.17	3H s	40.3	4.18	3H s	40.4	4.21	3H s	41.0	4.22	3H s	41.1
11	2.89	2H t (7.4)	30.0	2.90	2H t (7.4)	30.2	2.97	2H t (7.4)	30.2	2.99	2H t (7.4)	30.3
12	1.81	2H sext (7.4)	24.7	1.84	2H sext (7.4)	24.9	1.85	2H sext (7.4)	24.6	1.86	2H sext (7.4)	24.8
13	0.99	3H t (7.4)	16.0	1.00	3H t (7.4)	16.2	1.00	3H t (7.4)	16.0	1.00	3H t (7.4)	16.1
14	-	-	119.3	-	-	119.8	-	-	122.3	-	-	122.5
15	8.59	1H d (2.7)	132.0	8.50	1H br	132.3	8.79	1H d (2.4)	132.2	8.82	1H d (2.4)	132.4
16	-	-	120.3	-	-	ND <sup>(e)</sup>	-	-	128.7	-	-	128.7
17	7.54	1H dd (8.9; 2.7)	133.7	7.46	1H dd (8.9; 2.6)	133.7	7.73	1H dd (8.7; 2.4)	133.9	7.75	1H dd (8.7; 2.4)	134.2
18	6.83	1H d (8.9)	125.1	6.75	1H d (8.9)	125.0	7.18	1H d (8.7)	121.1	7.20	1H d (8.7)	121.3
19	-	-	176.0	-	-	176.3	-	-	165.9	-	-	166.1
20	-	-	-	-	-	-	-	-	-	-	-	-
21	-	-	-	-	-	-	-	-	-	-	-	-
24/28 <sup>eq</sup> (c)	3.81	2H dm (11.9)	53.7	3.49	2H dm (11.3)	55.1	3.61	2H dm (11.2)	54.5	3.57	2H dm (11.2)	55.0
24/28 <sup>ax</sup> (c)	2.32	2H app t (11.5)	53.7	2.12	2H app t (11.3)	55.1	1.90	2H app t (11.0)	54.5	2.15	2H app t (11.0)	55.0
25/27	3.30	2H m	53.0	2.73	2H m	54.9	2.85	2H m	52.8	2.73	2H m	54.9
29/30	1.21	6H d (6.5)	20.1	1.03	6H d (6.2)	19.1	0.99	6H d (6.4)	20.8	1.03	6H d (6.3)	19.1
31	-	-	-	2.83	2H q (7.1)	42.6	4.80	2H q (7.1)	67.0	4.82	2H q (7.1)	67.1
32	-	-	-	0.86	3H br t (7.1)	8.7	1.58	3H t (7.1)	16.3	1.58	3H t (7.1)	16.3
33	-	-	-	-	-	-	-	-	-	2.82	2H q (7.2)	42.6
34	-	-	-	-	-	-	-	-	-	0.84	3H t (7.1)	8.8

N <sup>o</sup> (a)	Sild (12)			SildEt <sup>N6</sup> (13)			SildOH (14) (d)		
	$\delta$ <sup>1</sup> H (ppm)	Multiplicity <sup>(b)</sup> (J, Hz)	$\delta$ <sup>13</sup> C (ppm)	$\delta$ <sup>1</sup> H (ppm)	Multiplicity <sup>(b)</sup> (J, Hz)	$\delta$ <sup>13</sup> C (ppm)	$\delta$ <sup>1</sup> H (ppm)	Multiplicity <sup>(b)</sup> (J, Hz)	$\delta$ <sup>13</sup> C (ppm)
3	-	-	149.0	-	-	148.3	-	-	147.8
5	-	-	151.0	-	-	153.1	-	-	156.1
7	-	-	157.2	-	-	157.3	-	-	158.6
8	-	-	127.6	-	-	127.6	-	-	127.5
9	-	-	141.1	-	-	139.1	-	-	142.0
10	4.19	3H s	40.6	4.23	3H s	40.4	4.18	3H s	40.4
11	2.87	2H t (7.4)	30.0	2.81	2H t (7.5)	29.6	2.90	2H t (7.4)	30.1
12	1.79	2H sext (7.4)	25.0	1.73	2H sext (7.5)	25.0	1.83	2H sext (7.4)	24.9
13	0.97	3H t (7.4)	16.1	0.94	3H t (7.5)	15.9	1.01	3H t (7.4)	16.2
14	-	-	125.0	-	-	128.0	-	-	120.1
15	8.21	1H d (2.4)	133.2	7.79	1H d (2.4)	132.4	8.57	1H br	132.3
16	-	-	130.2	-	-	129.7	-	-	ND <sup>(e)</sup>
17	7.89	1H dd (8.8; 2.4)	135.0	7.94	1H dd (8.9; 2.4)	134.5	7.48	1H dd (8.8; 2.4)	133.7
18	7.34	1H d (8.9)	116.5	7.34	1H d (8.9)	115.8	6.77	1H d (8.8)	125.2
19	-	-	163.1	-	-	162.4	-	-	176.3
20	4.31	2H q (7.0)	68.7	4.22	2H q (7.0)	67.9	-	-	-
21	1.46	3H t (7.0)	16.7	1.27	3H t (7.0)	16.5	-	-	-
24/28	3.07	4H br	48.3	3.05	4H br	48.1	3.01	2H br	48.6
25/27	2.56	4H br	56.2	2.48	4H br app t (4.7)	56.2	2.48	2H br app t (4.8)	56.4
29	2.26	3H s	47.3	2.21	3H s	47.3	2.20	3H s	47.5
31	-	-	-	4.13/3.64	2H 2*qd (14.1; 7.2)	43.4	-	-	-
32	-	-	-	1.09	3H t (7.0)	16.1	-	-	-

N <sup>o</sup> (a)	DeSild ( <b>15</b> ) <sup>(f)</sup>				DeSildEt <sup>N6</sup> ( <b>16</b> ) <sup>(f)</sup>			
	$\delta$ <sup>1</sup> H (ppm)	Multiplicity <sup>(b)</sup> (J, Hz)		$\delta$ <sup>13</sup> C (ppm)	$\delta$ <sup>1</sup> H (ppm)	Multiplicity <sup>(b)</sup> (J, Hz)		$\delta$ <sup>13</sup> C (ppm)
3	-	-	-	148.7	-	-	-	148.2
5	-	-	-	152.0	-	-	-	153.5
7	-	-	-	157.0	-	-	-	158.5 or 157.4
8	-	-	-	127.2	-	-	-	127.4
9	-	-	-	141.3	-	-	-	139.2
10	4.19	3H	s	40.4	4.22	3H	s	40.3
11	2.89	2H	t (7.4)	29.9	2.79	2H	t (7.5)	29.7
12	1.81	2H	sext (7.4)	24.8	1.73	2H	sext (7.5)	24.9
13	0.99	3H	t (7.4)	15.9	0.94	3H	t (7.5)	15.9
14	-	-	-	123.2	-	-	-	124.8
15	8.10	1H	dd (7.8; 1.7)	133.3	7.37	1H	dd (7.4; 1.8)	132.6
16	7.15	1H	td (7.5; 1.0)	124.0	7.12	1H	td (7.5; 0.9)	123.4
17	7.55 <sub>2</sub>	1H	ddd (8.5; 7.4; 1.8)	135.5	7.54 <sub>9</sub>	1H	os	134.2
18	7.19	1H	d (8.5)	115.9	7.16	1H	os	115.1
19	-	-	-	159.4	-	-	-	158.5 or 157.4
20	4.27	2H	q (7.0)	67.8	4.11	2H	q (7.0)	66.8
21	1.48	3H	t (7.0)	16.7	1.23	3H	t (7.0)	16.7
31	-	-	-	-	4.15/3.65	2H	2*qd (14.0; 7.0)	43.1
32	-	-	-	-	1.06	3H	t (7.0)	15.9

<sup>a</sup> Position number indicates either hydrogen or carbon.

<sup>b</sup> Multiplicity: s, singlet; d, doublet; dd, doublet of doublet; ddd, doublet of doublet of doublet; t, triplet; td, triplet of doublet; q, quartet; qd: quartet of doublet; sext: sextet; m: multiplet; dm: doublet of multiplet; app: apparent; br: broad; os overlapped signal.

<sup>c</sup> ax: axial; eq: equatorial.

<sup>d</sup> Due to the low solubility of the compound,  $\delta$  were measured in CD<sub>3</sub>CN:D<sub>2</sub>O (80:20) after basification of the medium by 5  $\mu$ L of 0.5 M NaOD added in the NMR tube.

<sup>e</sup> ND: not detected.

<sup>f</sup> Compounds **15** and **16** were not separated.

# Isolation and identification of ten new sildenafil derivatives in an alleged herbal supplement for sexual enhancement

## Supplementary information

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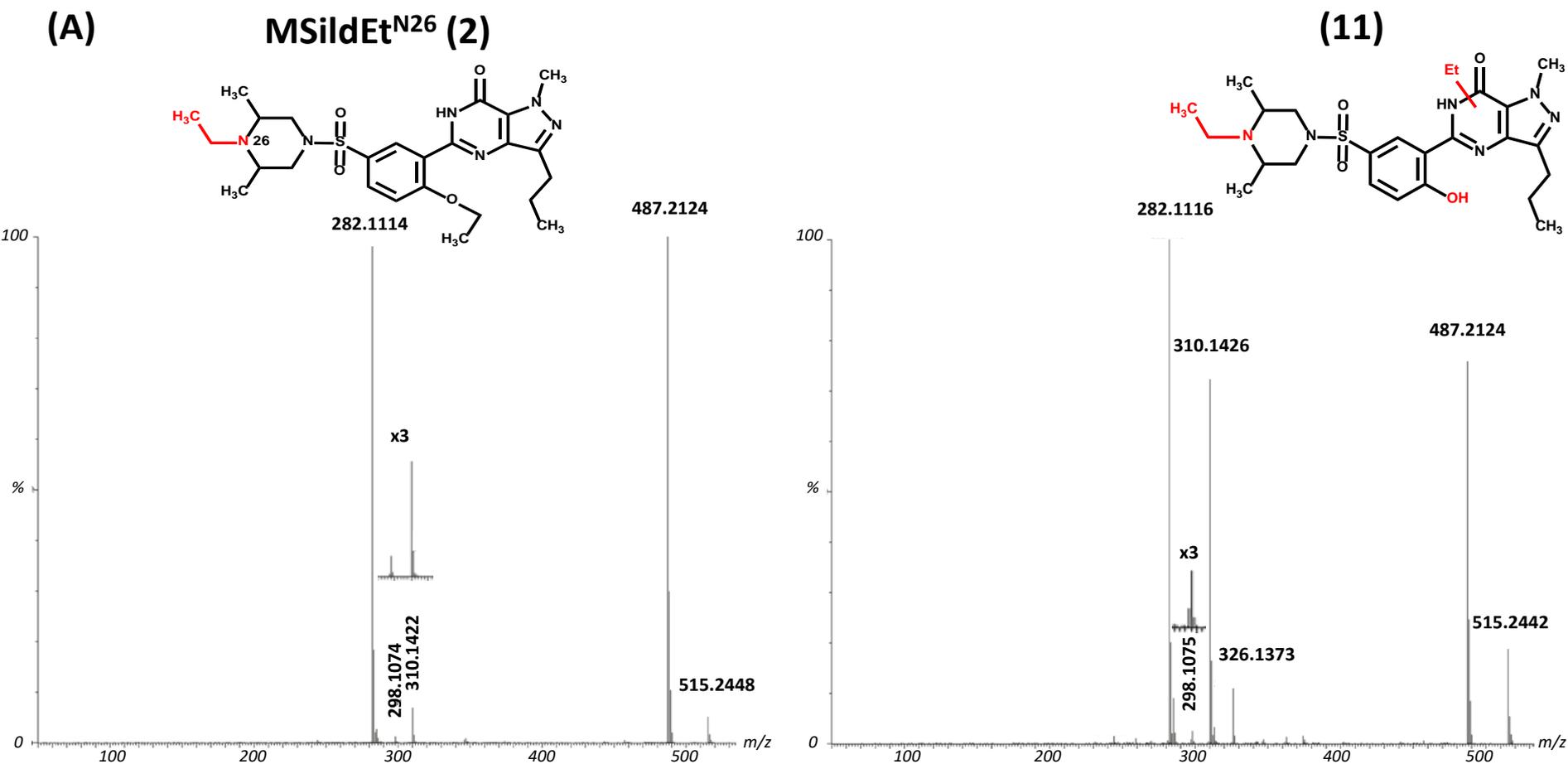
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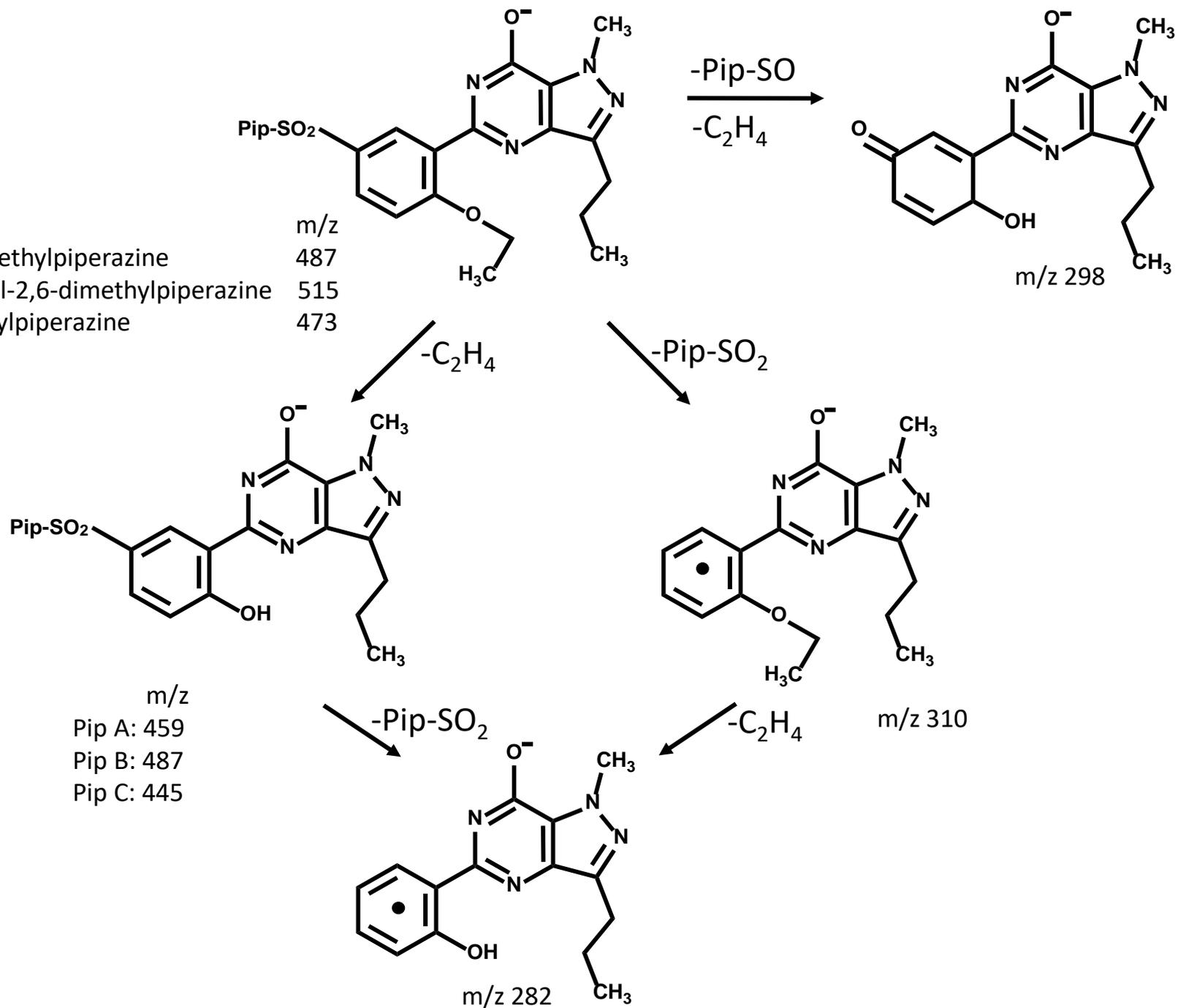
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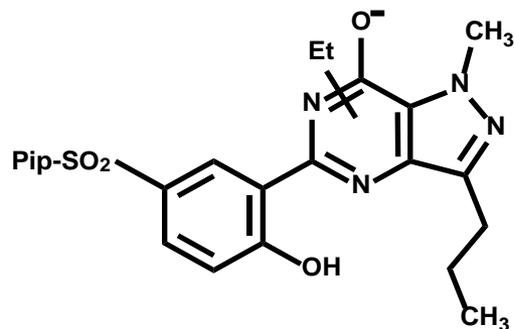
**Figure S1.** (A) HRMS/MS spectra in negative ESI mode of two methisosildenafil-related compounds (**2**, **11**) purified from the dietary supplement analyzed. The formulae shown represent the molecules and not the ions. Proposed structures of fragment ions of (B) compounds with an ethoxy group on the benzene ring, (C) compounds with a hydroxyl group on the benzene ring.



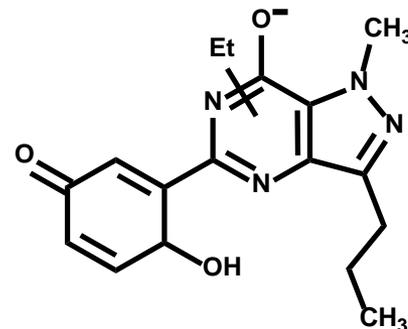
(B)



(C)



-Pip-SO



m/z  
298 326

m/z without ethyl group on the pyrazolopyrimidine moiety

459 Pip A: 2,6-dimethylpiperazine

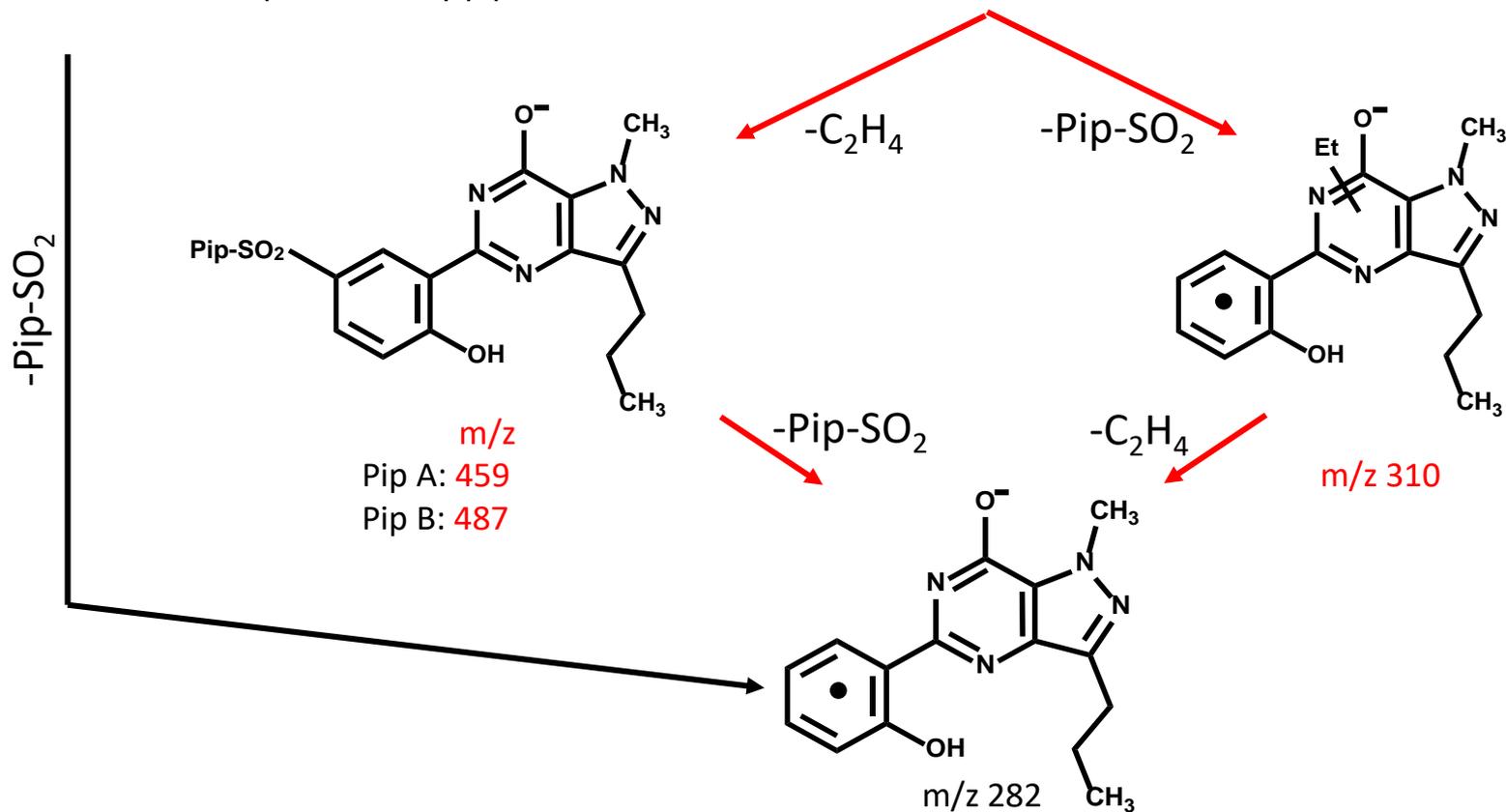
487 Pip B: N1-ethyl-2,6-dimethylpiperazine

445 Pip C: N-methylpiperazine

m/z with ethyl group

487

515



m/z

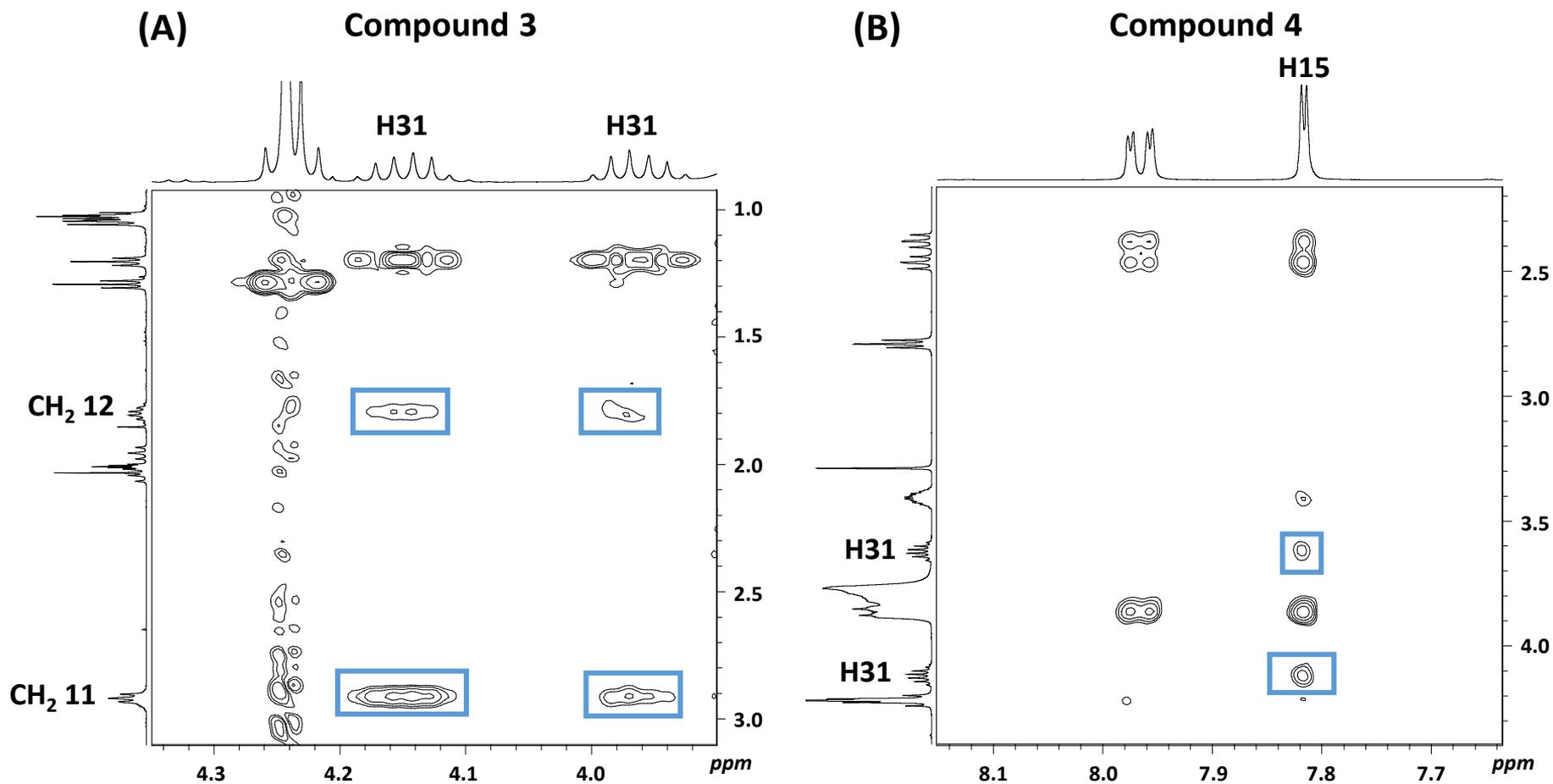
Pip A: 459

Pip B: 487

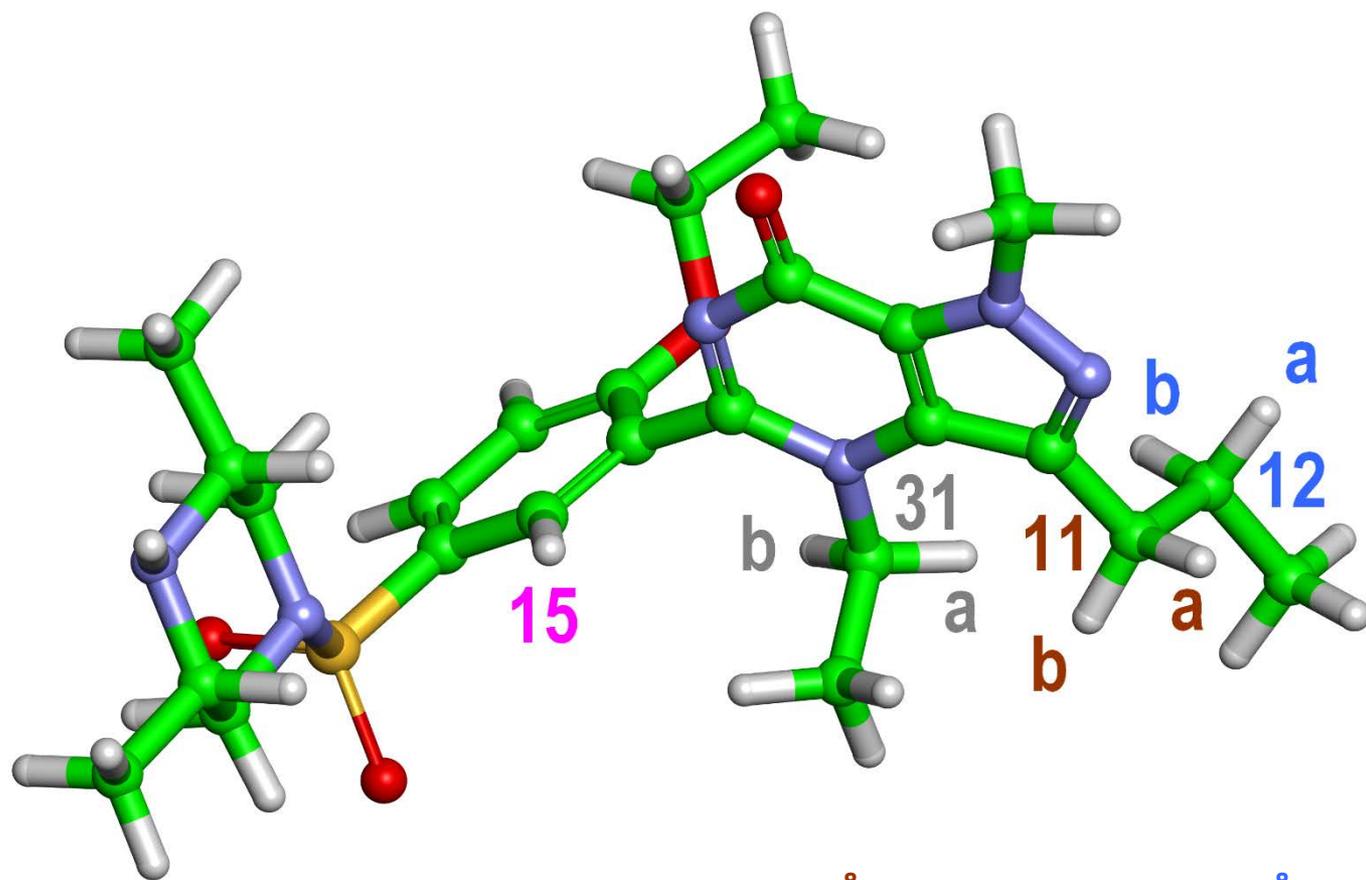
m/z 310

m/z 282

**Figure S2.** Parts of 2D NOESY spectra showing the spatial correlations between CH<sub>2</sub> 11, 12 and H31 for compound **3** (MSildEt<sup>N4</sup>, N4-ethyl-methisosildenafil) and between H15 and CH<sub>2</sub> 31 for compound **4** (MSildEt<sup>N6</sup>, N6-ethyl-methisosildenafil).

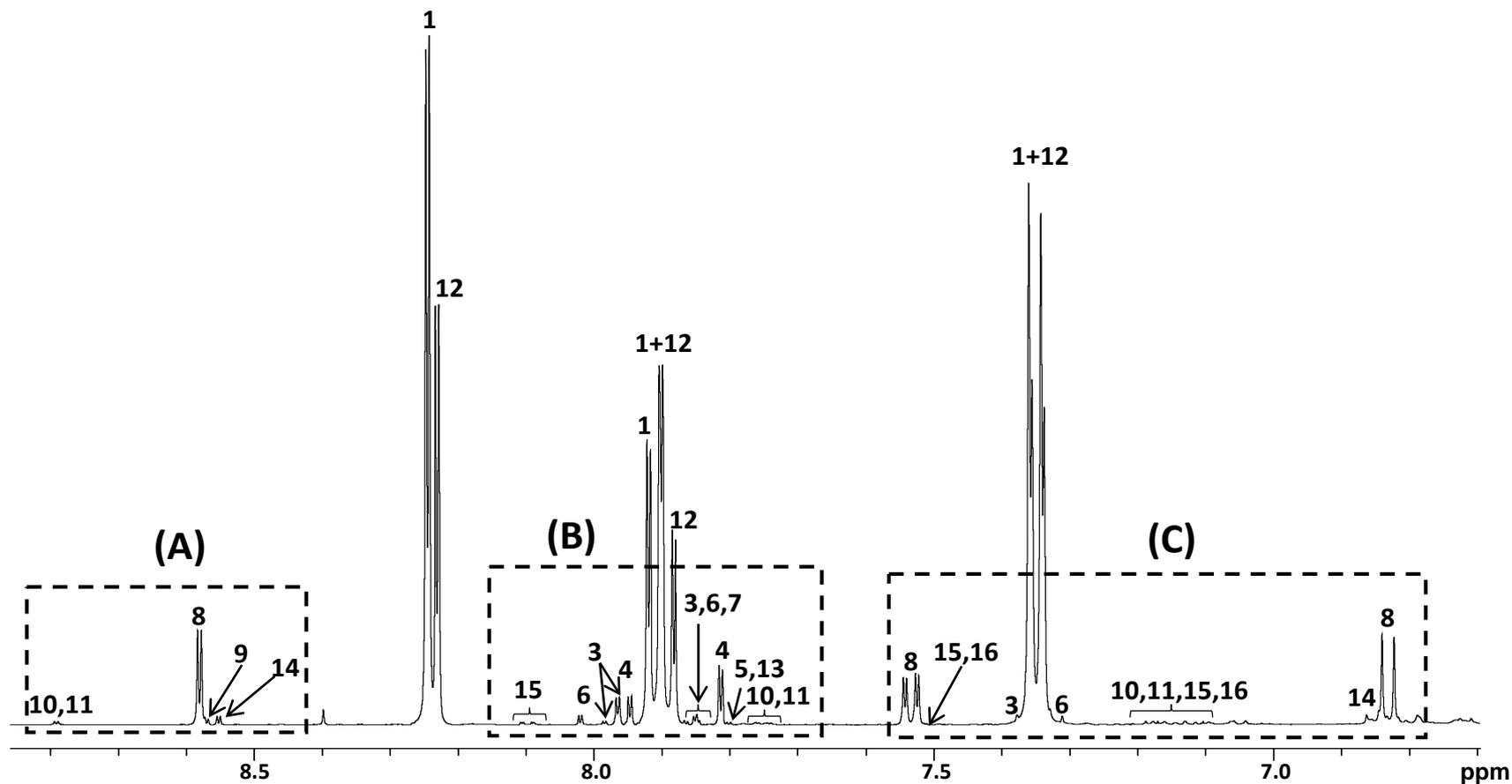


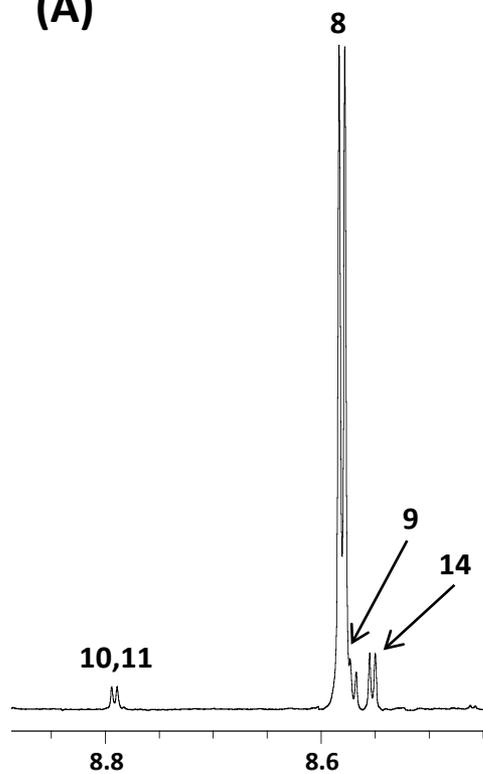
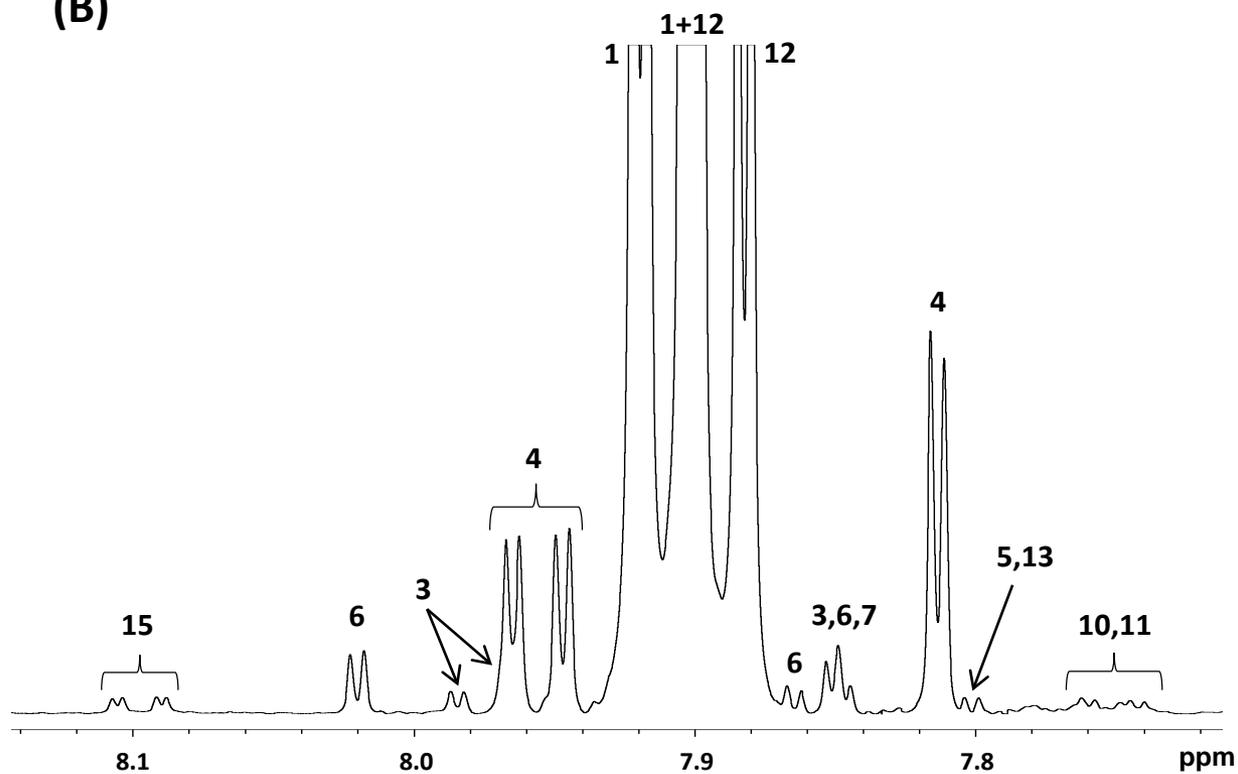
**Figure S3.** Representation of a privileged conformation calculated for compound **3** (MSildEt<sup>N4</sup>, N4-ethyl-methisosildenafil) and interatomic distances between protons of interest.



H31a-H15	4.97 Å	H31a-H11a	3.77 Å	H31a-H12a	3.88 Å
H31b-H15	3.88 Å	H31a-H11b	2.27 Å	H31a-H12b	2.38 Å
		H31b-H11a	5.36 Å	H31b-H12a	5.46 Å
		H31b-H11b	3.82 Å	H31b-H12b	3.87 Å

**Figure S4.** Part (6.7-8.9 ppm) of the  $^1\text{H}$  NMR spectrum with  $^{13}\text{C}$  GARP broadband decoupling of the dietary supplement recorded in  $\text{CD}_3\text{CN}/\text{D}_2\text{O}$  (80:20, v/v) with attribution of the signals of the identified compounds.



**(A)****(B)****(C)**