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Supplementary data

In search for a 5-CT alternative. *In vitro* and *in vivo* evaluation of novel pharmacological tools: 3-(1-alkyl-1*H*-imidazol-5-yl)-1*H*-indole-5-carboxamides, low-basicity 5-HT$_7$ receptor agonists

Gniewomir Latacz, Adam S. Hogendorf, Agata Hogendorf, Annamaria Lubelska, Joanna M. Wierońska, Monika Woźniak, Paulina Cieślik, Katarzyna Kieć-Kononowicz, Jadwiga Handzlik, Andrzej J. Bojarski

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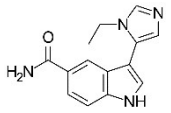
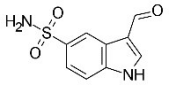
1. Synthesis and characterisation of intermediates and final compounds

3-(1-Ethyl-1*H*-imidazol-5-yl)-1*H*-indole-5-carboxamide (7, AH-494)



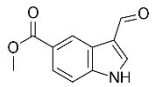
Compound was synthesized from **11** (3-(1-ethyl-1*H*-imidazol-5-yl)-1*H*-indole-5-carbonitrile, 2 mmol) according to general procedure 3. Pale yellow crystalline solid, 40% yield. LC-MS [M+H$^+$]: 255.11, t_R = 0.5 min.

3-Formyl-1*H*-indole-5-sulfonamide (8)



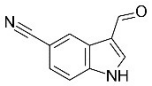
Compound was synthesized from 1*H*-indole-5-sulfonamide (5.1 mmol) according to general procedure 1. Beige solid, 22% yield LC-MS [M+H$^+$]: 224.92, t_R = 0.63 min.

Methyl 3-formyl-1*H*-indole-5-carboxylate (9)



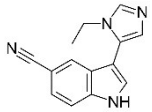
Compound was synthesized from methyl 1*H*-indole-5-carboxylate (11 mmol) according to general procedure 1. Pink solid, 87% yield. LC-MS [M+H$^+$]: 203.93, t_R = 1.99 min.

3-Formyl-1*H*-indole-5-carbonitrile (10)



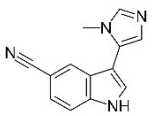
Compound was synthesized from 1*H*-indole-5-carbonitrile (28 mmol) according to general procedure 1. Light brown crystals, 78% yield. LC-MS [M+H$^+$]: 171.09, t_R = 1.66 min.

3-(1-Ethyl-1*H*-imidazol-5-yl)-1*H*-indole-5-carbonitrile (11)



Compound was synthesized from **10** (3-formyl-1*H*-indole-5-carbonitrile, 28.6 mmol) according to general procedure 2. 82% yield. LC-MS [M+H$^+$]: 237.1, t_R = 0.94 min.

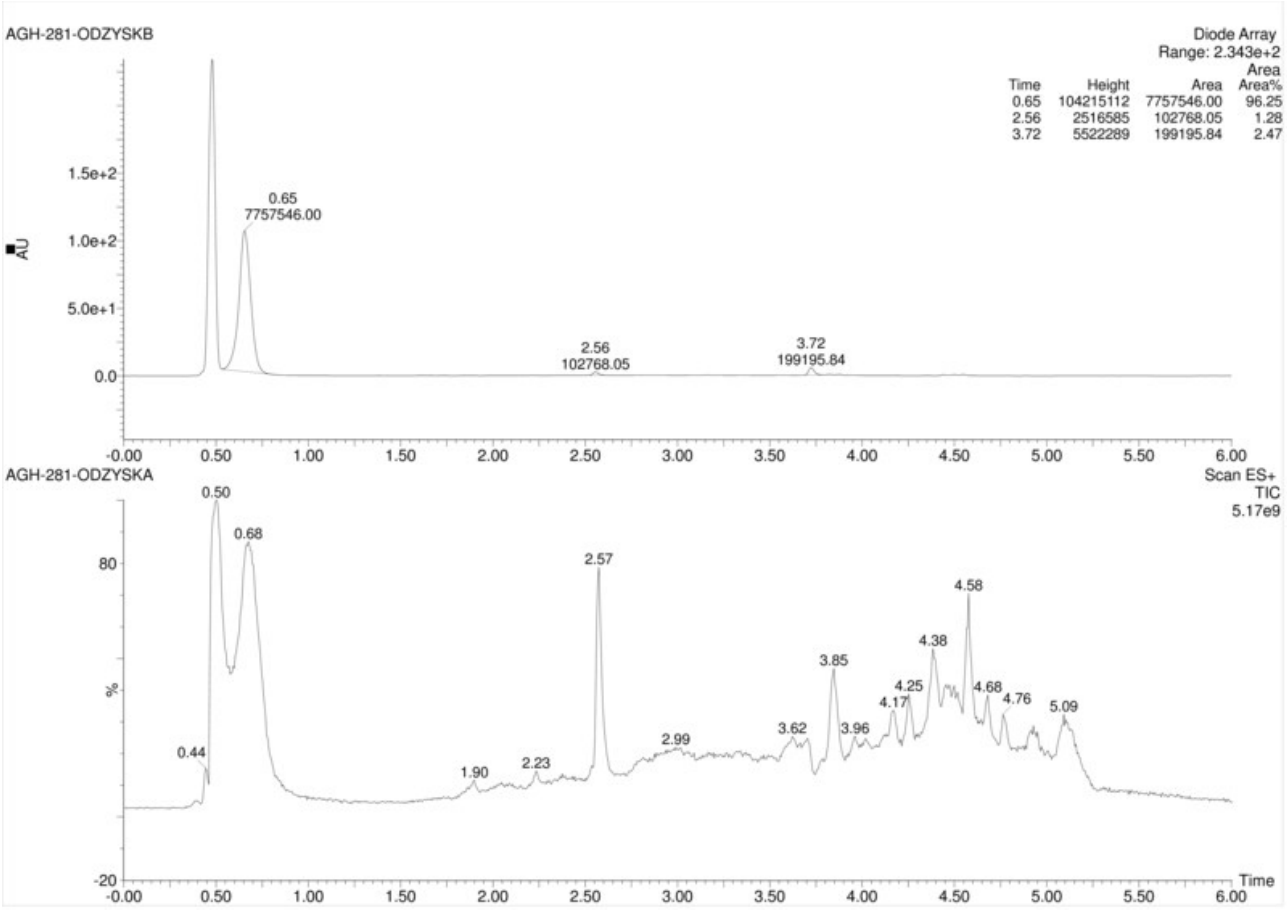
3-(1-Methyl-1*H*-imidazol-5-yl)-1*H*-indole-5-carbonitrile (12)



Compound was synthesized from **10** (3-formyl-1*H*-indole-5-carbonitrile, 11.75mmol) according to general procedure 2. Pale beige solid, 36% yield. LC-MS [$M+H^+$]: 223.05, t_R = 0.61 min.

^{1}H NMR (400 MHz, DMSO-d_6) δ 12.05 (s, 1H), 8.08 (dd, *J* = 1.5, 0.7 Hz, 1H), 7.84 (s, 1H), 7.76 – 7.71 (m, 1H), 7.64 (dd, *J* = 8.4, 0.7 Hz, 1H), 7.53 (dd, *J* = 8.4, 1.6 Hz, 1H), 7.20 (d, *J* = 1.1 Hz, 1H), 3.68 (s, 3H).

^{13}C NMR (101 MHz, DMSO-d_6) δ 139.21, 138.23, 127.73, 126.85, 126.35, 125.87, 125.22, 125.00, 121.00, 113.58, 105.55, 102.30, 32.53.


AGH-281-ODZYSKB
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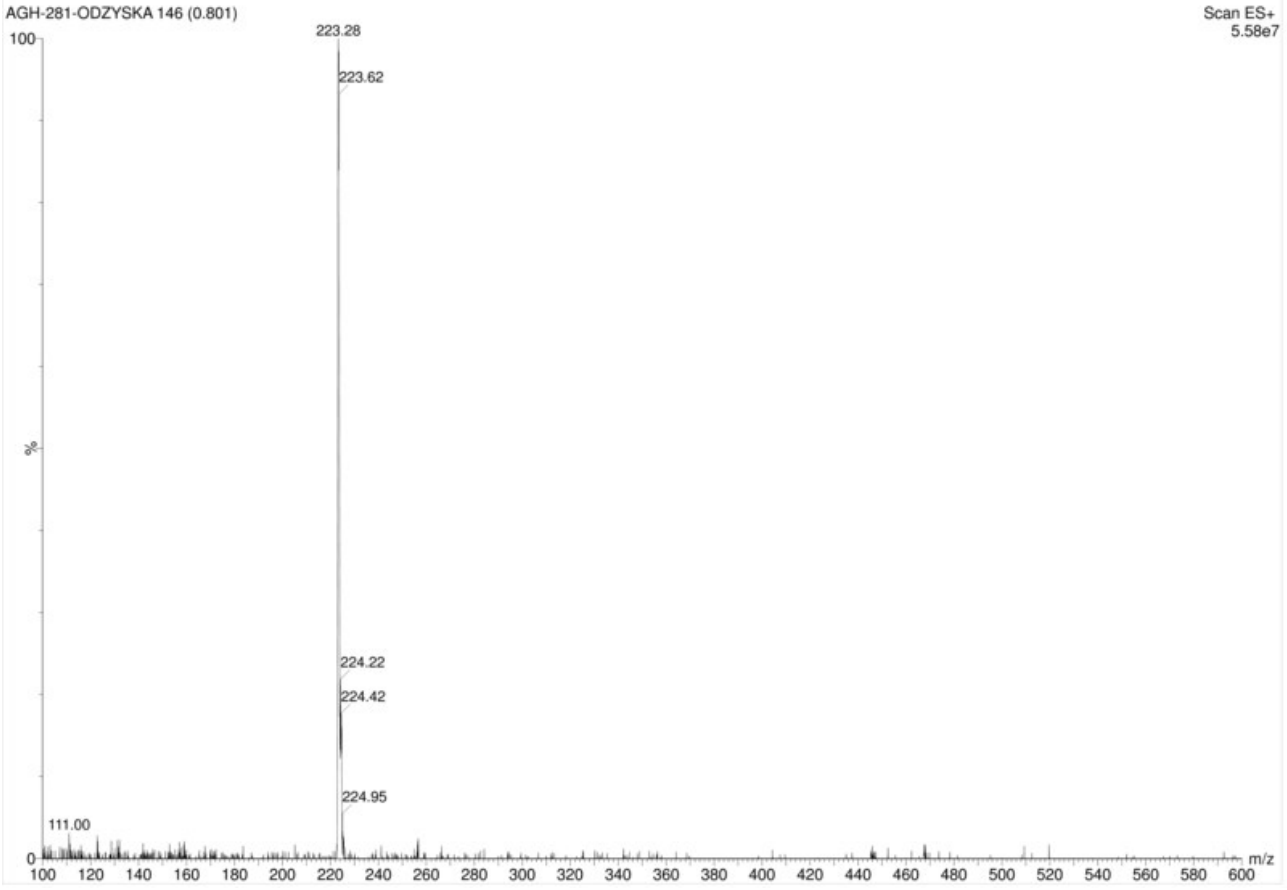
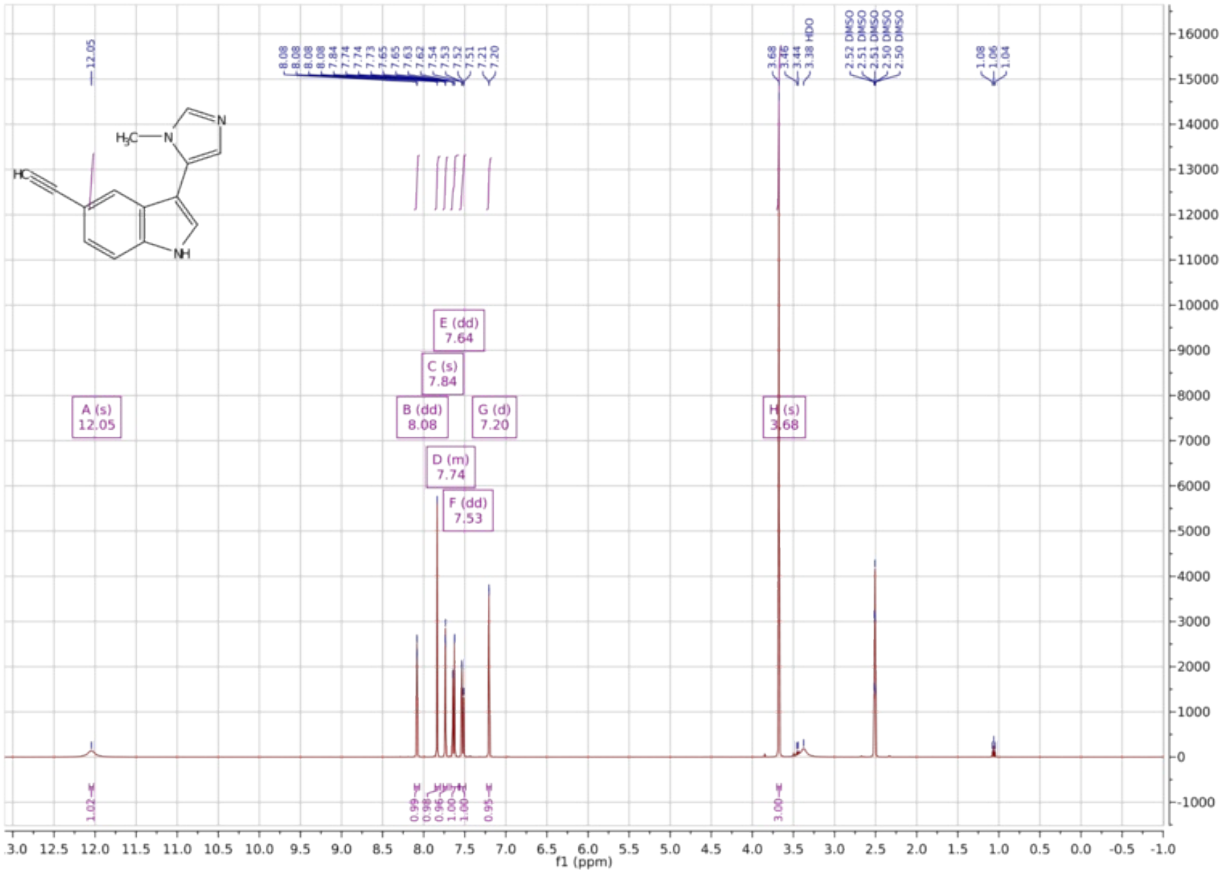
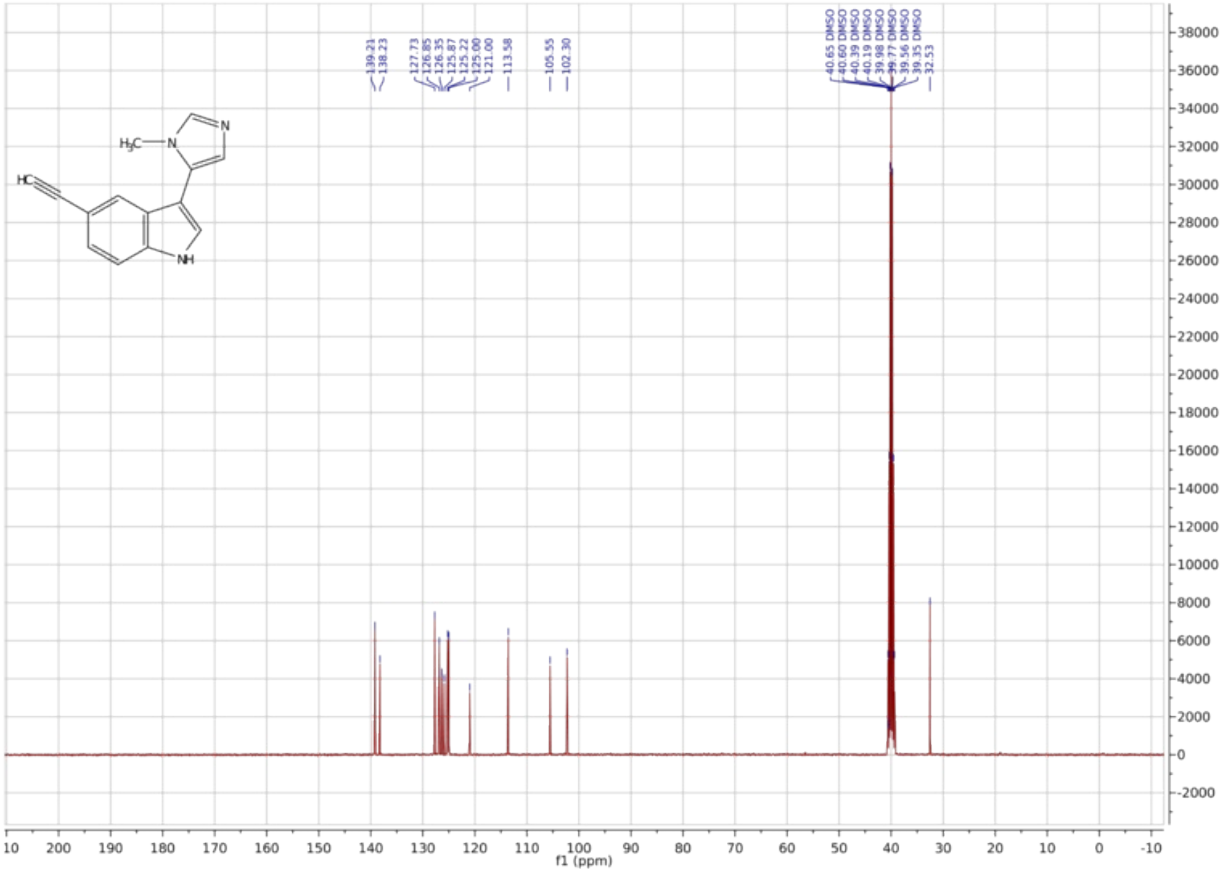
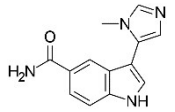
Range: 2.343e+2
Area
Time Height Area Area%
0.65 104215112 7757546.00 96.25
2.56 2516585 102768.05 1.28
3.72 5522289 199195.84 2.47
AU
0.65
7757546.00
2.56
102768.05
3.72
199195.84
AGH-281-ODZYSKA
Scan ES+
TIC
5.17e9
%
Time
0.44
0.50
0.68
1.90
2.23
2.57
2.99
3.62
3.85
3.96
4.17
4.25
4.38
4.58
4.68
4.76
5.09


AGH-281-ODZYSKA 146 (0.801)
Scan ES+
5.58e7
%
m/z
223.28
223.62
224.22
224.42
224.95
111.00


A (s)
12.05
B (dd)
8.08
C (s)
7.84
D (m)
7.74
E (dd)
7.64
F (dd)
7.53
G (d)
7.20
H (s)
3.68
f1 (ppm)


f1 (ppm)

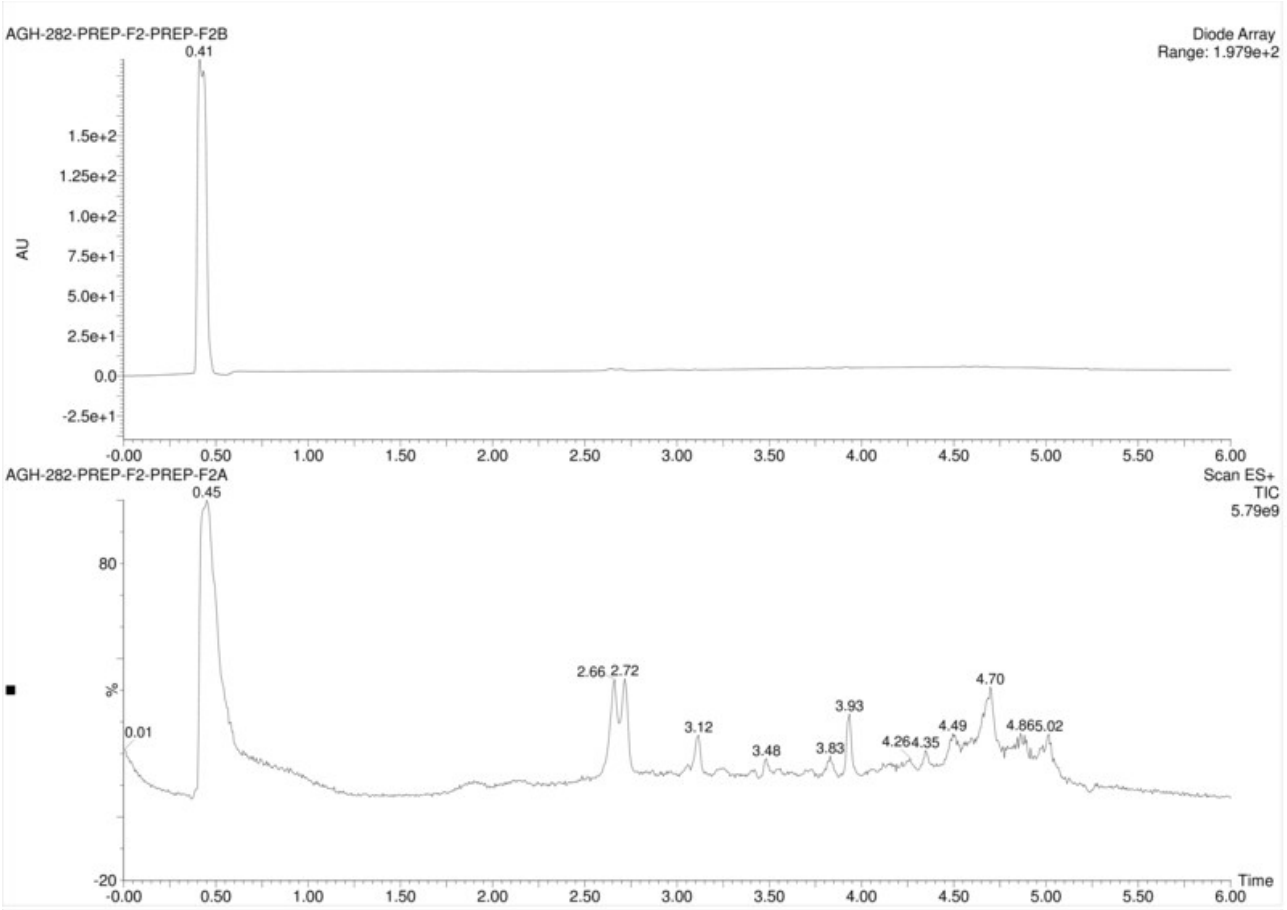
3-(1-Methyl-1*H*-imidazol-5-yl)-1*H*-indole-5-carboxamide (13)

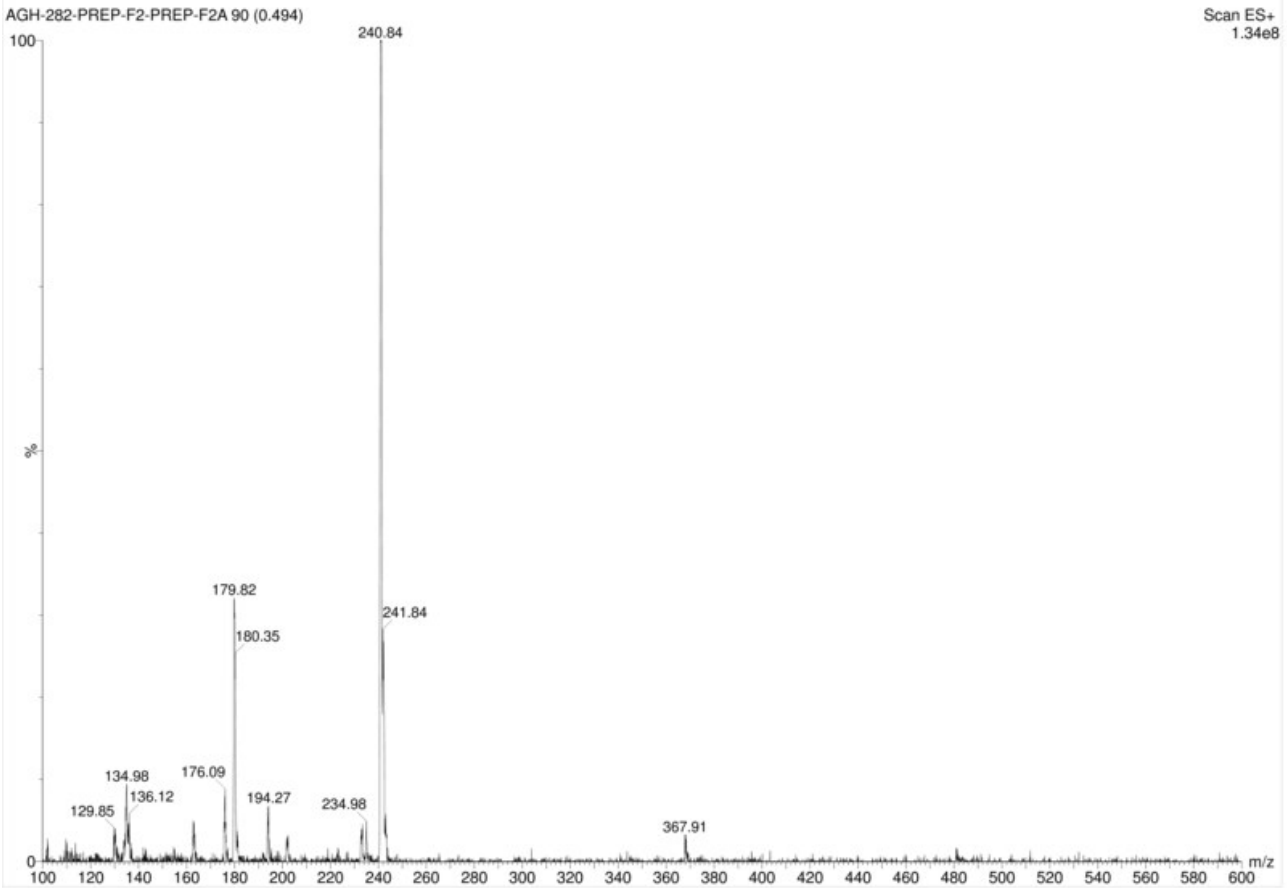


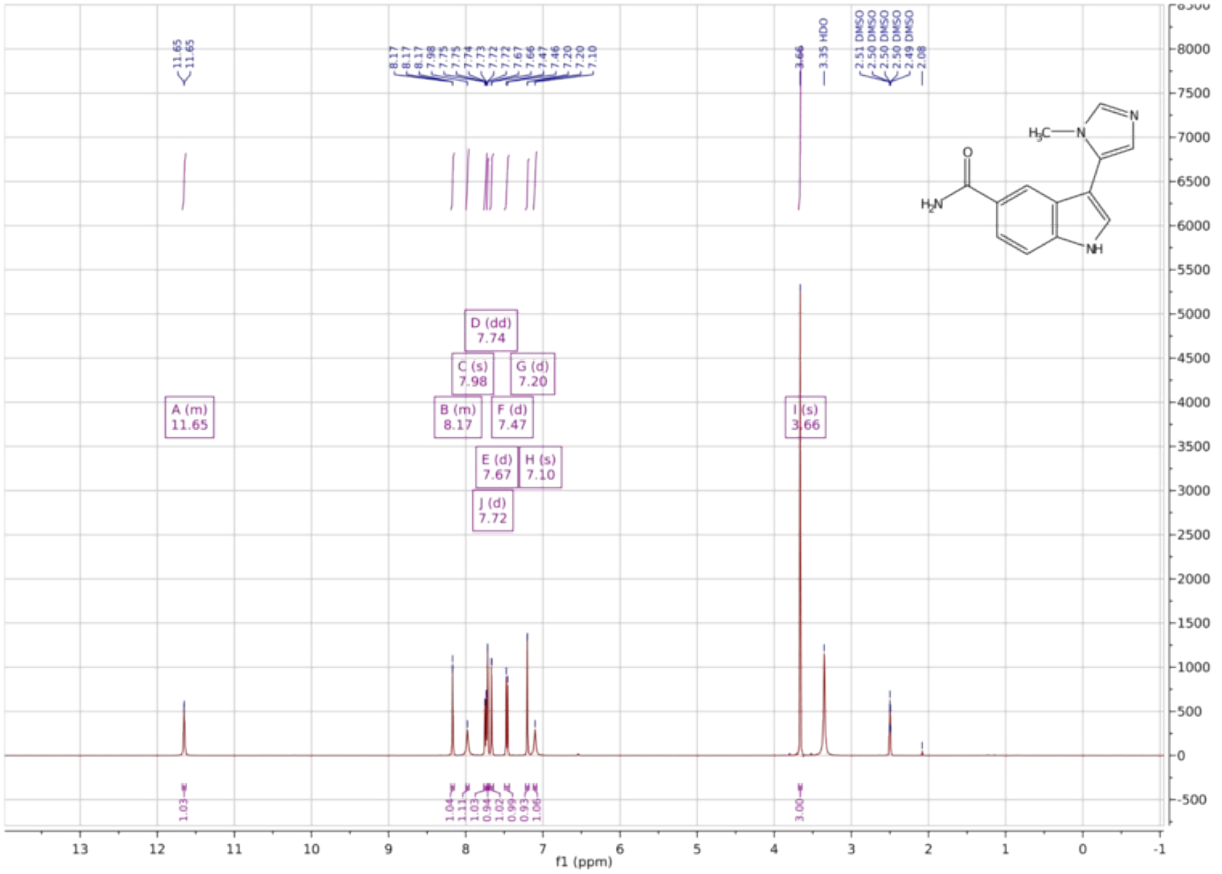
Compound was synthesized from **12** (3-(1-methyl-1*H*-imidazol-5-yl)-1*H*-indole-5-carbonitrile, 2 mmol) according to general procedure 3. Compound was further purified via preparative HPLC on a 250x20 mm C-18 column eluted with Off-white solid, 29% yield. LC-MS [$M+H^+$]: 241.1, t_R = 0.41 min.

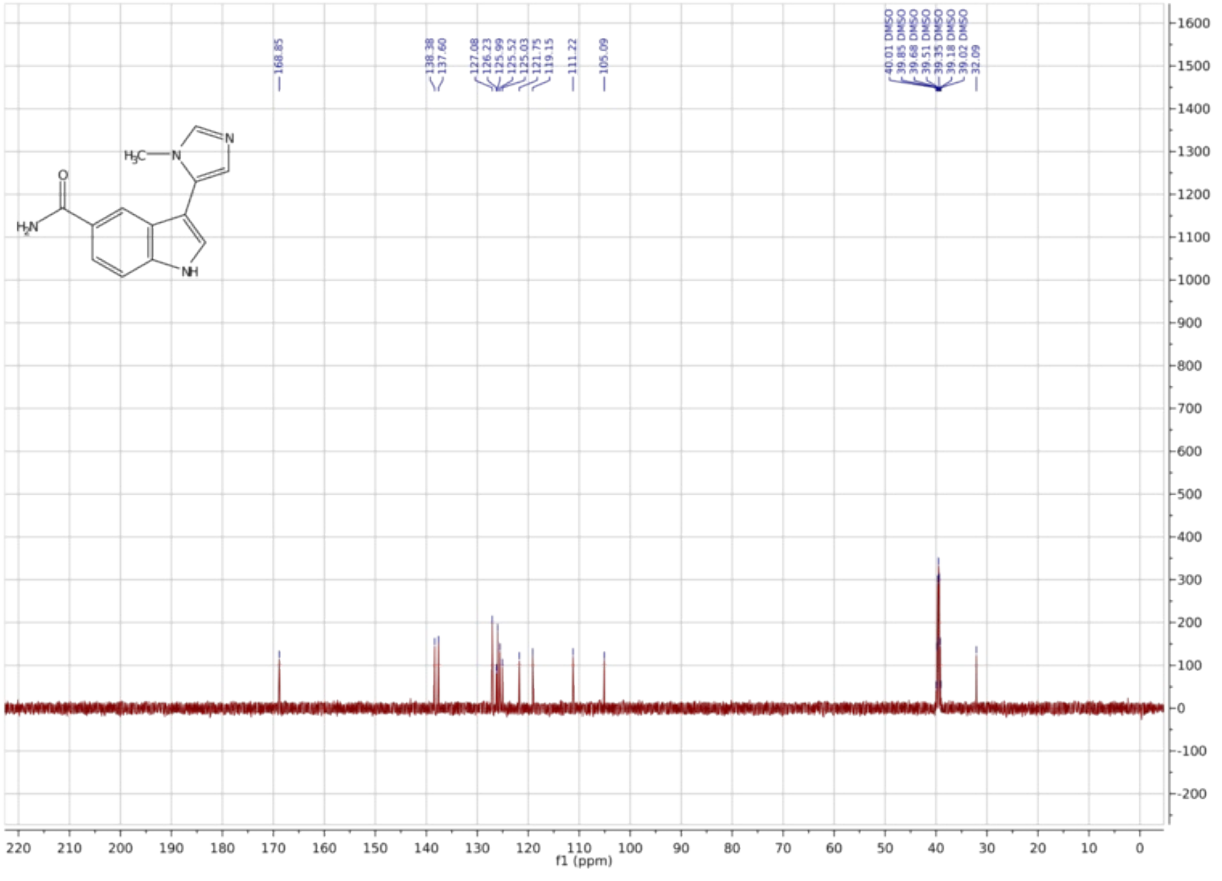
^{1}H NMR (500 MHz, DMSO-d_6) δ 11.67 – 11.63 (m, 1H), 8.19 – 8.15 (m, 1H), 7.98 (s, 1H), 7.74 (dd, *J* = 8.5, 1.7 Hz, 1H), 7.72 (d, *J* = 1.2 Hz, 1H), 7.67 (d, *J* = 2.6 Hz, 1H), 7.47 (d, *J* = 8.5 Hz, 1H), 7.20 (d, *J* = 1.2 Hz, 1H), 7.10 (s, 1H), 3.66 (s, 3H).

^{13}C NMR (126 MHz, DMSO-d_6) δ 168.85, 138.38, 137.60, 127.08, 126.23, 125.99, 125.52, 125.03, 121.75, 119.15, 111.22, 105.09, 32.09.

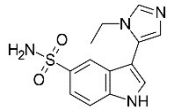

AGH-282-PREP-F2-PREP-F2B
Diode Array
Range: 1.979e2
0.41
AU
AGH-282-PREP-F2-PREP-F2A
Scan ES+
TIC
5.79e9
0.45
0.01
2.66
2.72
3.12
3.48
3.83
3.93
4.26
4.35
4.49
4.70
4.86
5.02
%
Time


AGH-282-PREP-F2-PREP-F2A 90 (0.494)
Scan ES+
1.34e8
240.84
179.82
180.35
241.84
134.98
136.12
129.85
176.09
194.27
234.98
367.91
%
m/z


11.65
11.65
8.17
8.17
8.17
7.98
7.75
7.74
7.73
7.72
7.72
7.67
7.67
7.47
7.20
7.10
3.66
3.35 HDO
2.51 DMSO
2.50 DMSO
2.50 DMSO
2.50 DMSO
2.49 DMSO
2.08
A (m) 11.65
B (m) 8.17
C (s) 7.98
D (dd) 7.74
E (d) 7.67
F (d) 7.47
G (d) 7.20
H (s) 7.10
I (s) 3.66
J (d) 7.72
1.03
1.04
1.13
1.03
0.94
1.02
0.99
0.93
1.06
3.00
f1 (ppm)

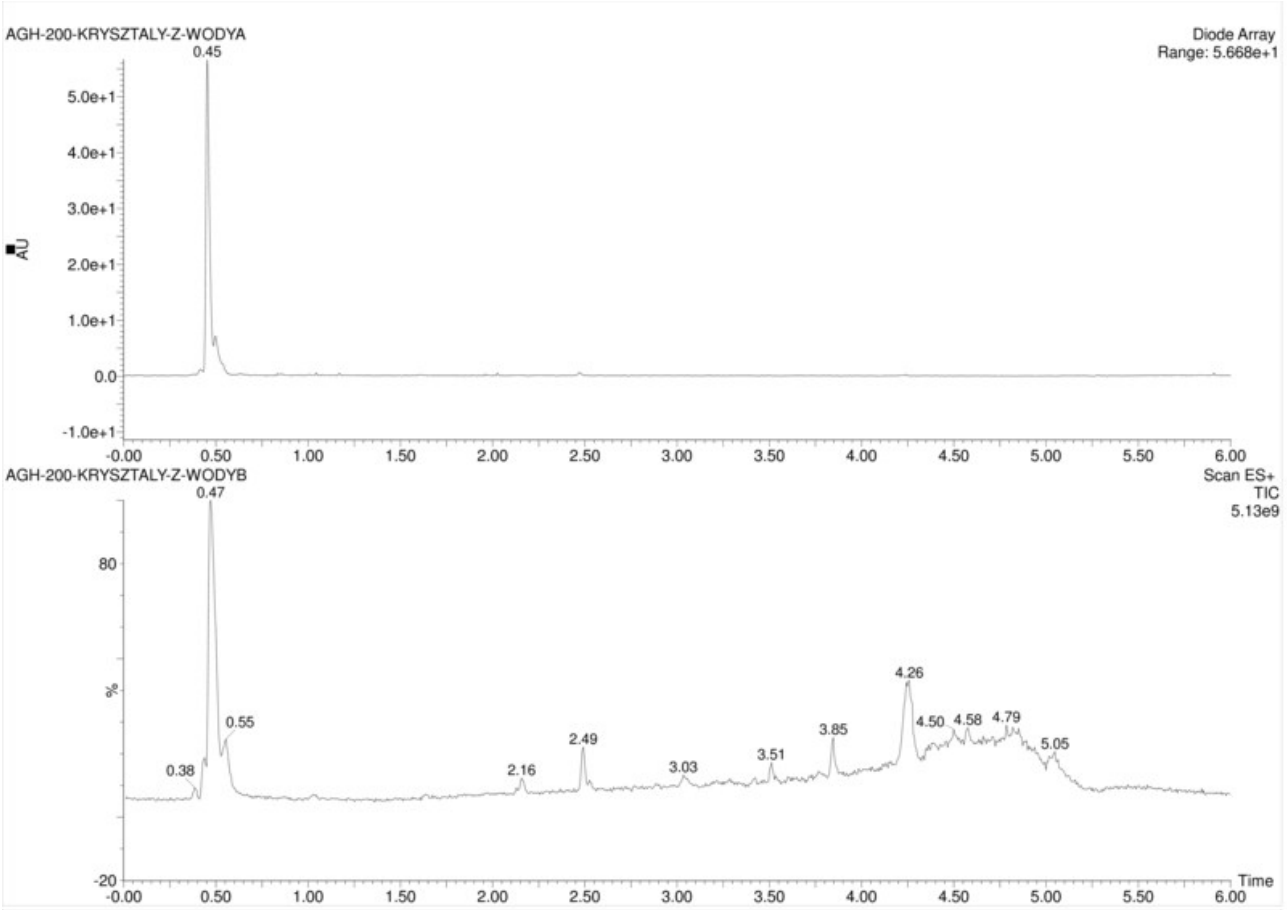

168.85
138.38
137.60
127.08
126.23
125.99
125.52
125.03
121.75
119.15
111.22
105.09
40.01 DMSO
39.85 DMSO
39.68 DMSO
39.51 DMSO
39.35 DMSO
39.18 DMSO
39.02 DMSO
32.09
f1 (ppm)

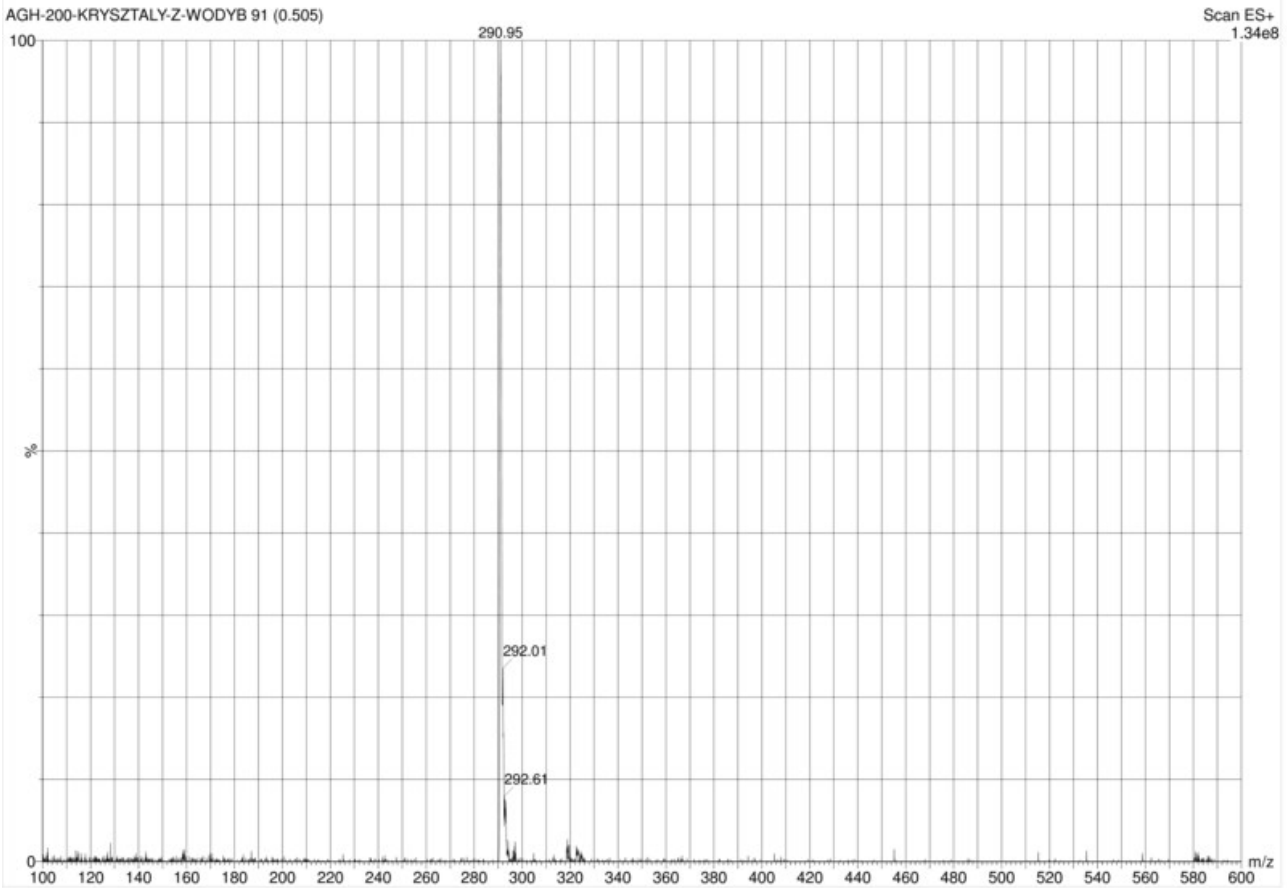
3-(1-Ethyl-1*H*-imidazol-5-yl)-1*H*-indole-5-sulfonamide (14)

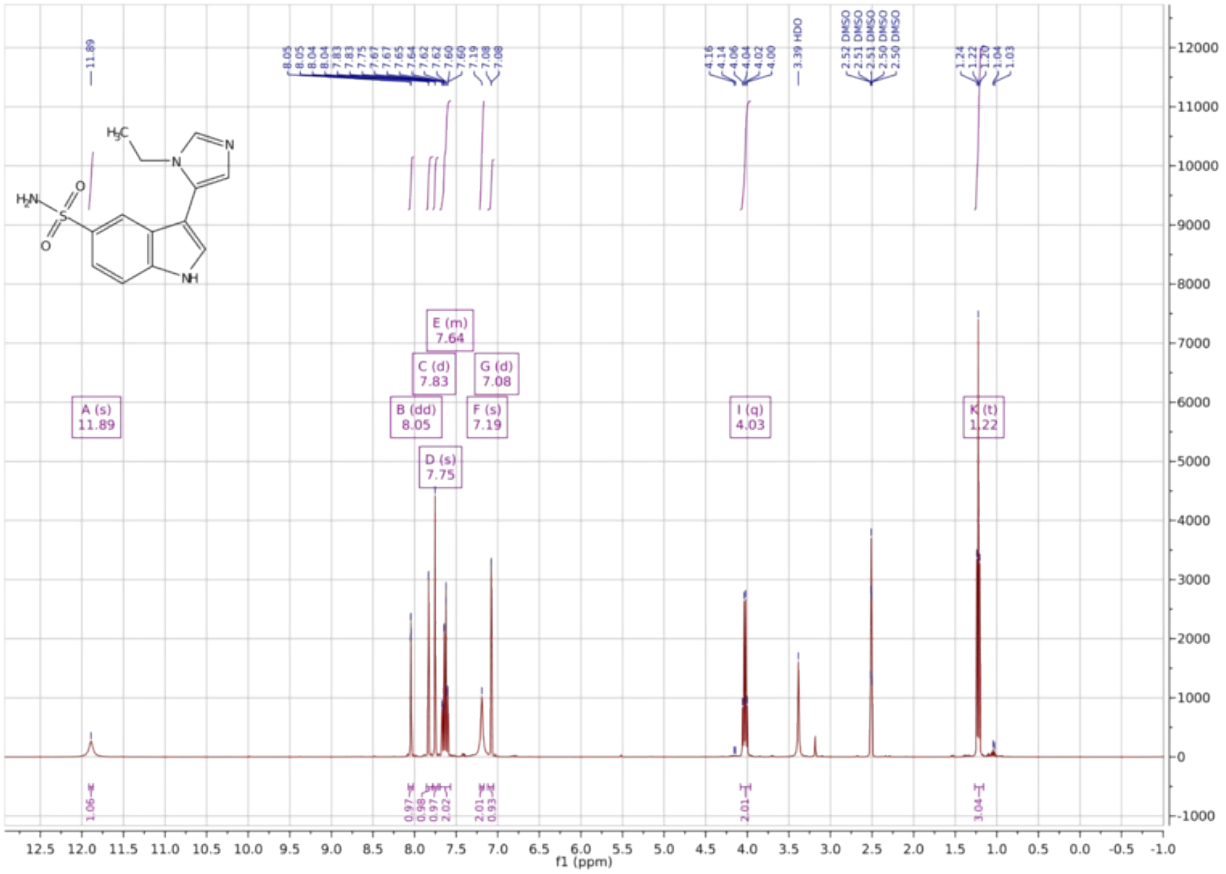


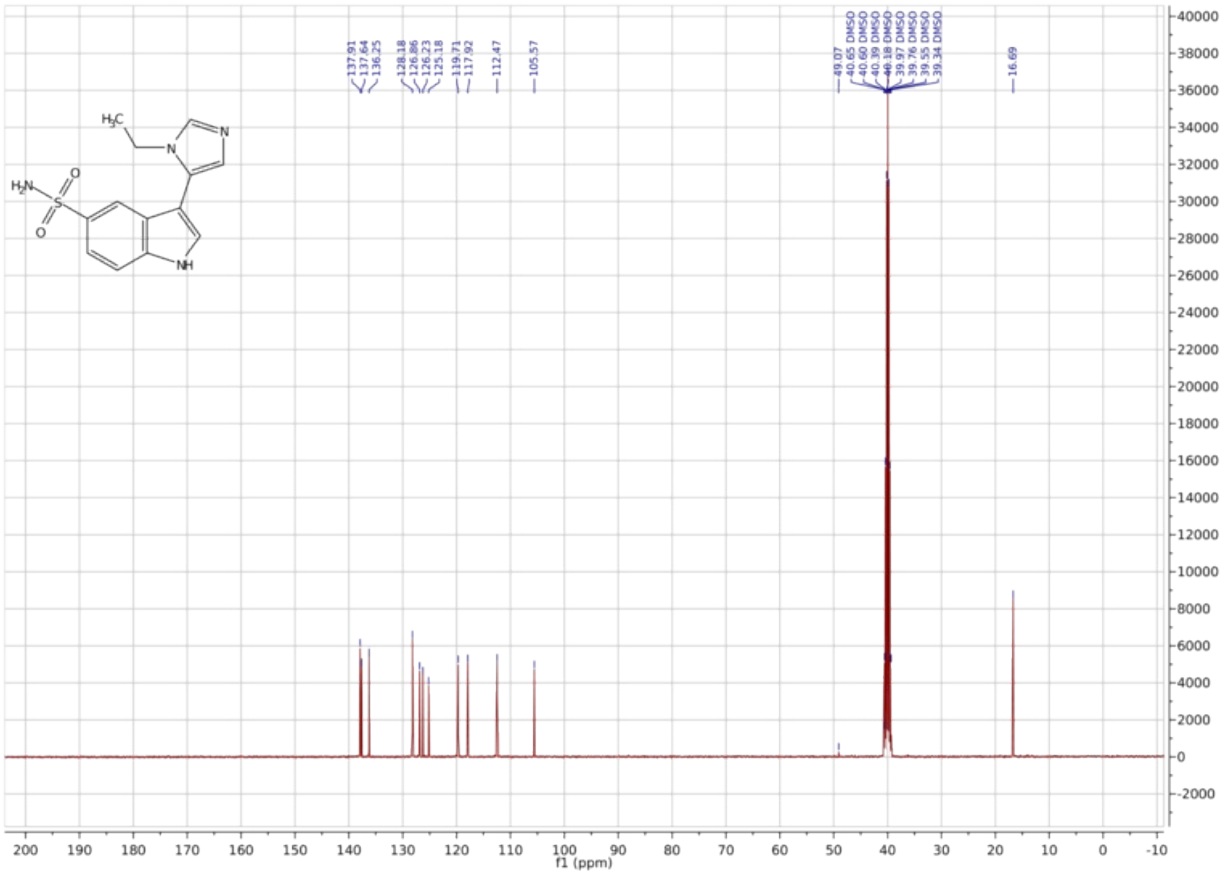
Compound was synthesized from **8** (3-formyl-1*H*-indole-5-sulfonamide, 1.1 mmol) according to general procedure 2. Solid. Pale yellow needles, 57% yield. LC-MS [M+H$^+$]: 290.88, t_R = 0.47 min. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.89 (s, 1H), 8.05 (dd, *J* = 1.8, 0.7 Hz, 1H), 7.83 (d, *J* = 1.1 Hz, 1H), 7.75 (s, 1H), 7.69 – 7.57 (m, 2H), 7.19 (s, 2H), 7.08 (d, *J* = 1.1 Hz, 1H), 4.03 (q, *J* = 7.2 Hz, 2H), 1.22 (t, *J* = 7.2 Hz, 3H).

^{13}C NMR (101 MHz, DMSO-d_6) δ 137.91, 137.64, 136.25, 128.18, 126.86, 126.23, 125.18, 119.71, 117.92, 112.47, 105.57, 49.07, 16.69.

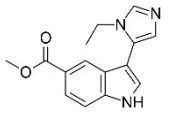

AGH-200-KRYSZTALY-Z-WODYA
Diode Array
Range: 5.668e+1
0.45
AU
AGH-200-KRYSZTALY-Z-WODYB
Scan ES+
TIC
5.13e9
0.47
0.38
0.55
2.16
2.49
3.03
3.51
3.85
4.26
4.50
4.58
4.79
5.05
%
Time


AGH-200-KRYSZTALY-Z-WODYB 91 (0.505)
Scan ES+
1.34e8
290.95
292.01
292.61
%
m/z

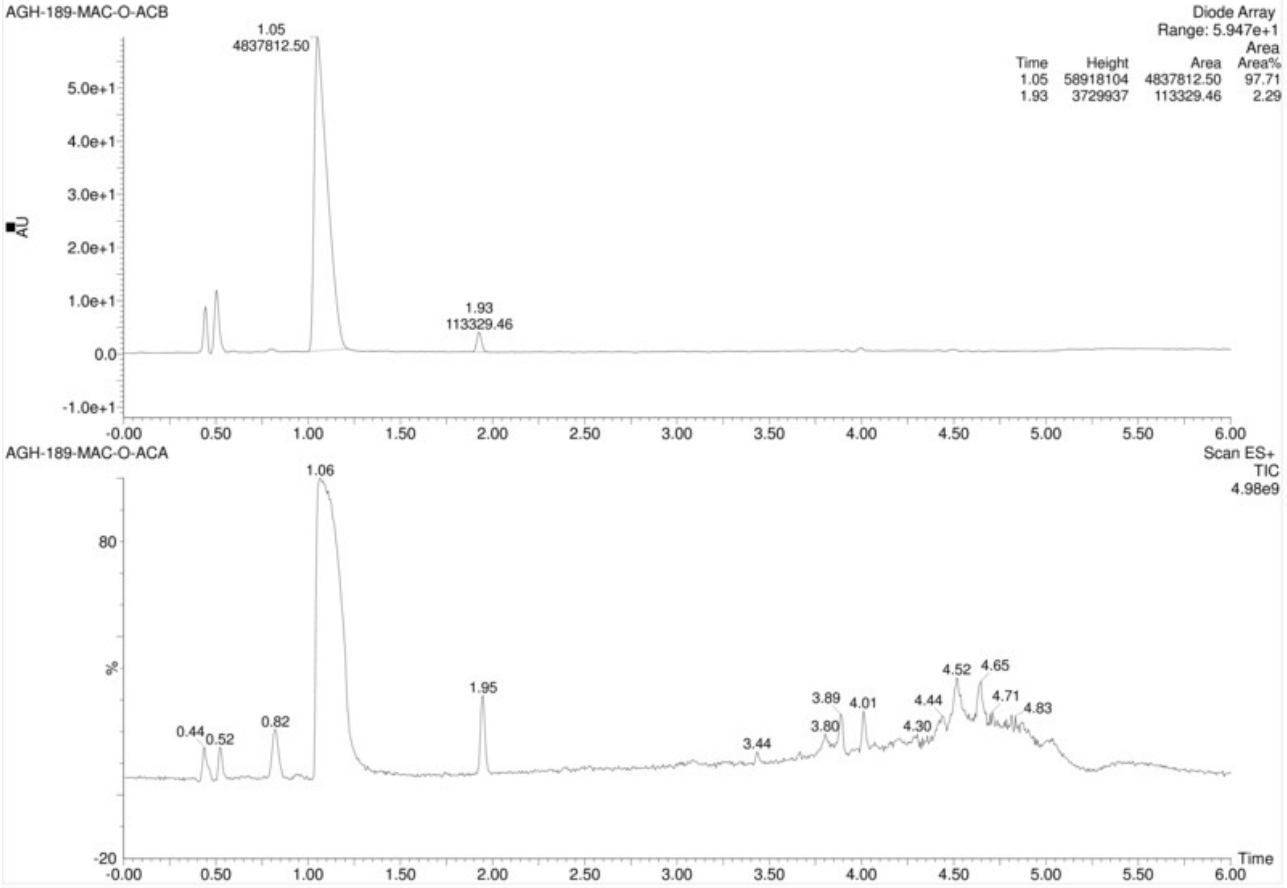

11.89
8.05
8.05
8.04
8.04
7.83
7.83
7.75
7.67
7.67
7.65
7.64
7.62
7.62
7.60
7.60
7.19
7.08
7.08
4.16
4.14
4.06
4.04
4.02
4.00
3.39 HDO
2.52 DMSO
2.51 DMSO
2.51 DMSO
2.50 DMSO
2.50 DMSO
1.24
1.22
1.20
1.04
1.03
A (s) 11.89
B (dd) 8.05
C (d) 7.83
D (s) 7.75
E (m) 7.64
F (s) 7.19
G (d) 7.08
I (q) 4.03
K (t) 1.22
1.06
0.97
0.98
0.97
2.02
2.01
0.93
2.01
3.04
f1 (ppm)

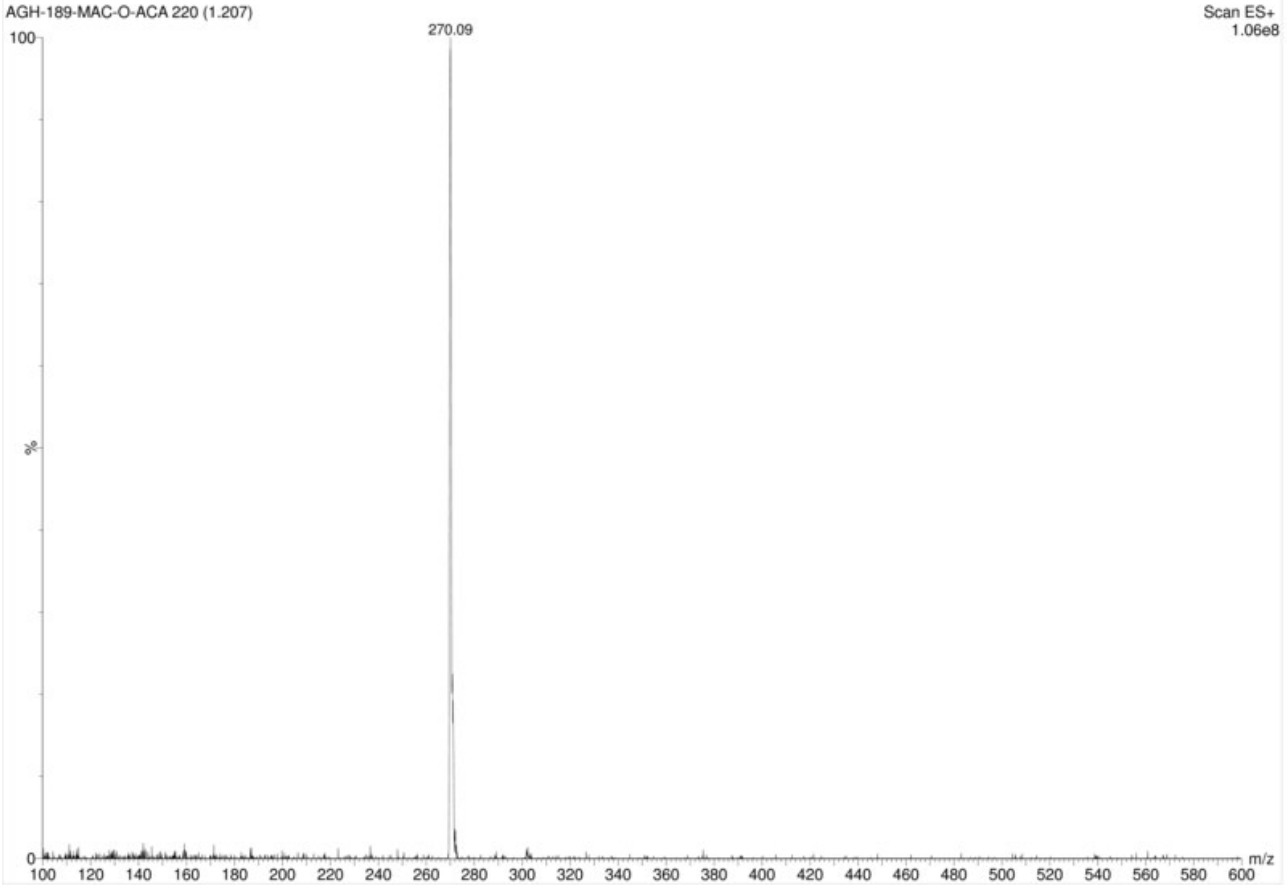

137.91
137.64
136.25
128.18
126.86
126.23
125.18
119.71
117.92
112.47
105.57
49.07
40.65 DMSO
40.60 DMSO
40.39 DMSO
40.18 DMSO
39.97 DMSO
39.76 DMSO
39.55 DMSO
39.34 DMSO
16.69
f1 (ppm)

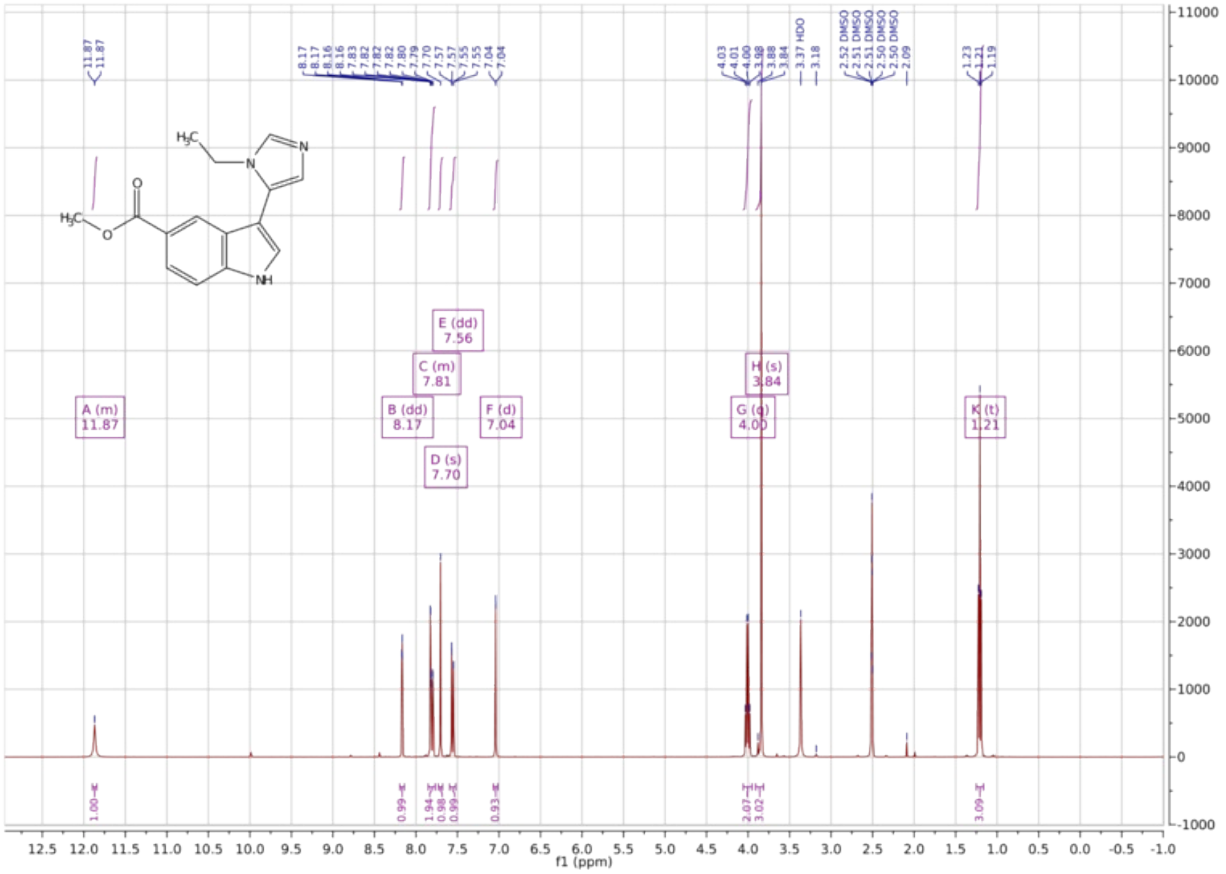
Methyl 3-(1-ethyl-1*H*-imidazol-5-yl)-1*H*-indole-5-carboxylate (15)

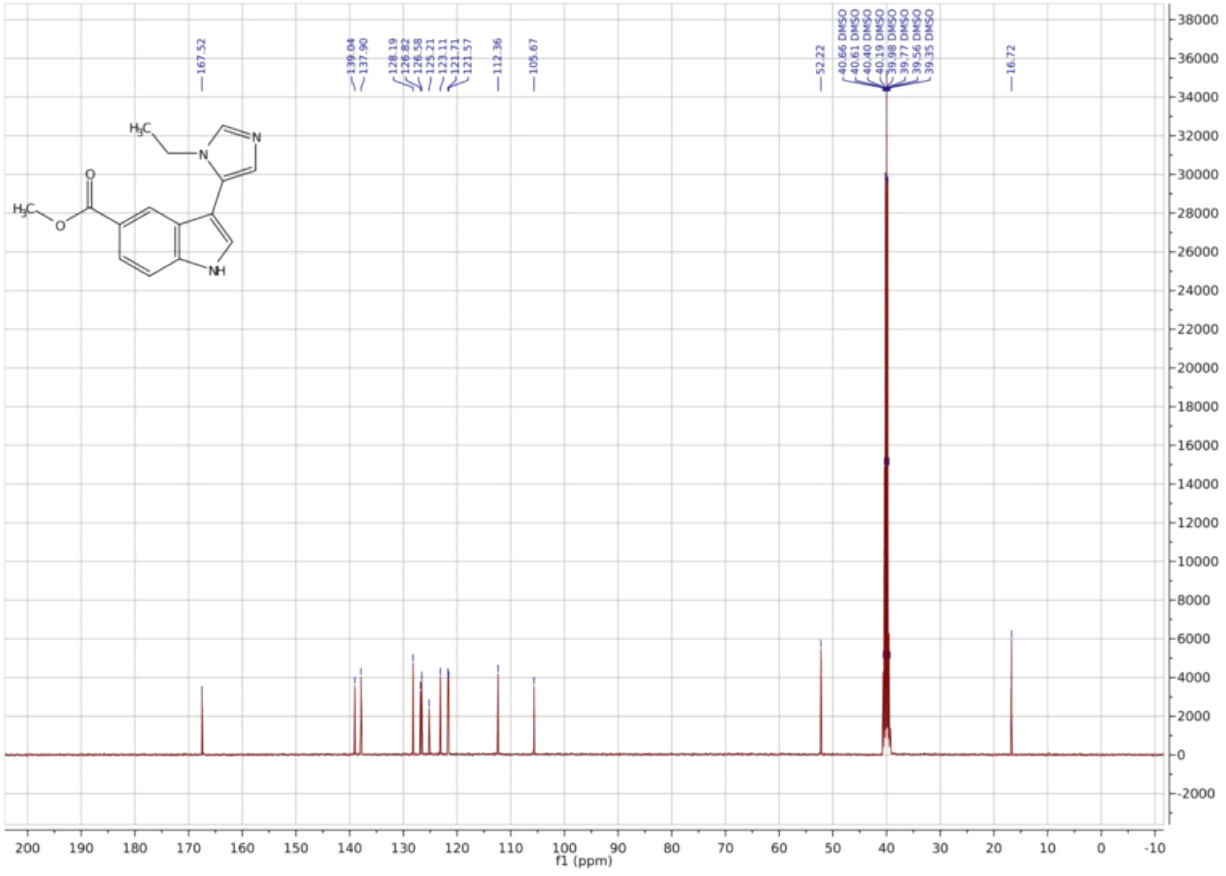


Compound was synthesized from **9** (methyl 3-formyl-1*H*-indole-5-carboxylate, 2 mmol) according to general procedure 2. Orange crystaline solid, 42% yield. LC-MS [M+H$^+$]: 270.09, t_R = 1.05 min.
^{1}H NMR (400 MHz, DMSO-d_6) δ 11.90 – 11.84 (m, 1H), 8.17 (dd, *J* = 1.7, 0.7 Hz, 1H), 7.85 – 7.77 (m, 2H), 7.70 (s, 1H), 7.56 (dd, *J* = 8.5, 0.7 Hz, 1H), 7.04 (d, *J* = 1.1 Hz, 1H), 4.00 (q, *J* = 7.2 Hz, 2H), 3.84 (s, 3H), 1.21 (t, *J* = 7.2 Hz, 3H).
^{13}C NMR (101 MHz, DMSO-d_6) δ 167.52, 139.04, 137.90, 128.19, 126.82, 126.58, 125.21, 123.11, 121.71, 121.57, 112.36, 105.67, 52.22, 16.72.


AGH-189-MAC-O-ACB
Diode Array
Range: 5.947e+1
Area
Time Height Area Area%
1.05 58918104 4837812.50 97.71
1.93 3729937 113329.46 2.29
1.05
4837812.50
1.93
113329.46
AU
AGH-189-MAC-O-ACA
Scan ES+
TIC
4.98e9
1.06
1.95
0.44
0.52
0.82
3.44
3.80
3.89
4.01
4.30
4.44
4.52
4.65
4.71
4.83
%
Time


AGH-189-MAC-O-ACA 220 (1.207)
Scan ES+
1.06e8
270.09
%
m/z


A (m) 11.87
B (dd) 8.17
C (m) 7.81
D (s) 7.70
E (dd) 7.56
F (d) 7.04
G (q) 4.00
H (s) 3.84
K (t) 1.21
f1 (ppm)


167.52
139.04
137.90
128.19
126.58
125.21
123.11
121.71
121.57
112.36
105.67
52.22
16.72
f1 (ppm)

2. 5-HT_{1A}/5-HT_{2A}/5-HT_6/5-HT_7/D_2 receptor radioligand binding assays

The membrane preparation and general assay procedures for the cloned receptors were adjusted to a 96-microwell format, as described in our previous papers.[1,2] The cell pellets were thawed and homogenized in 10 volumes of assay buffer using an Ultra Turrax tissue homogenizer, and were centrifuged twice at 35,000 g for 15 min at 4°C and were incubated for 15 min at 37°C between centrifugation rounds. The composition of the assay buffers was as follows: for 5-HT_{1A}R: 50 mM Tris–HCl, 0.1 mM EDTA, 4 mM $MgCl_2$, 10 μM pargyline and 0.1% ascorbate; for 5-HT_{2A}R: 50 mM Tris–HCl, 0.1 mM EDTA, 4 mM $MgCl_2$ and 0.1% ascorbate; for 5-HT_6R: 50 mM Tris–HCl, 0.5 mM EDTA and 4 mM $MgCl_2$, and for 5-HT_{7b}R: 50 mM Tris–HCl, 4 mM $MgCl_2$, 10 μM pargyline and 0.1% ascorbate; for dopamine D_{2L}R: 50 mM Tris–HCl, 1 mM EDTA, 4 mM $MgCl_2$, 120 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$ and 0.1% ascorbate.

All assays were incubated in a total volume of 200 μL in 96-well microtiter plates for 1 h at 37°C, except for 5-HT_{1A}R and 5-HT_{2A}R, which were incubated at room temperature and 27°C, respectively. The equilibration process was terminated by rapid filtration through Unifilter plates with a 96-well cell harvester and the radioactivity that was retained on the filters was quantified using a Microbeta plate reader (PerkinElmer, USA).

For the displacement studies, the assay samples contained the following as radioligands (PerkinElmer, USA): 1.5 nM [^{3}H]-8-OH-DPAT (135.2 Ci/mmol) for 5-HT_{1A}R; 2 nM [^{3}H]-ketanserin (53.4 Ci/mmol) for 5-HT_{2A}R; 2 nM [^{3}H]-LSD (83.6 Ci/mmol) for 5-HT_6R, 0.6 nM [^{3}H]-5-CT (39.2 Ci/mmol) for 5-HT_7R or [^{3}H]-Raclopride (74.4 Ci/mmol). Non-specific binding was defined using 10 μM of 5-HT in 5-HT_{1A}R and 5-HT_7R binding experiments, whereas 20 μM of mianserin, 10 μM of methiothepine or 1 μM of (+)-butaclamol was used in the 5-HT_{2A}R, 5-HT_6R and D_{2L}R assays, respectively. Each compound was tested in triplicate at 7–8 concentrations (10^{-11}–10^{-4} M). The inhibition constants (K_i) were calculated using the Cheng-Prusoff equation[3] and the results were expressed as the means of at least two independent experiments.

3. Metabolic stability and metabolic site investigation.

3.1 The in silico determination of 5, 7 and 13 metabolic stability

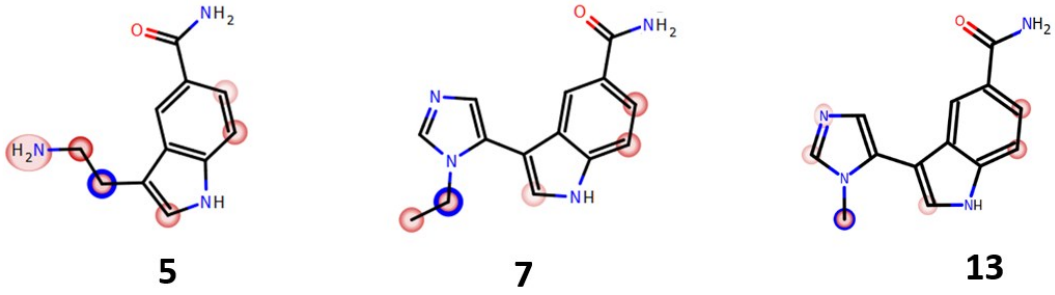


Figure S1 The *in silico* prediction of the most probably sites of compounds' **5, 7** and **13** metabolism using MetaSite 5.1.1.[4] The darker red color of the marked functional group indicates its higher probability to be involved in the metabolism pathway. The blue circle marked the site of compound involved in metabolism with the highest probability (100%).

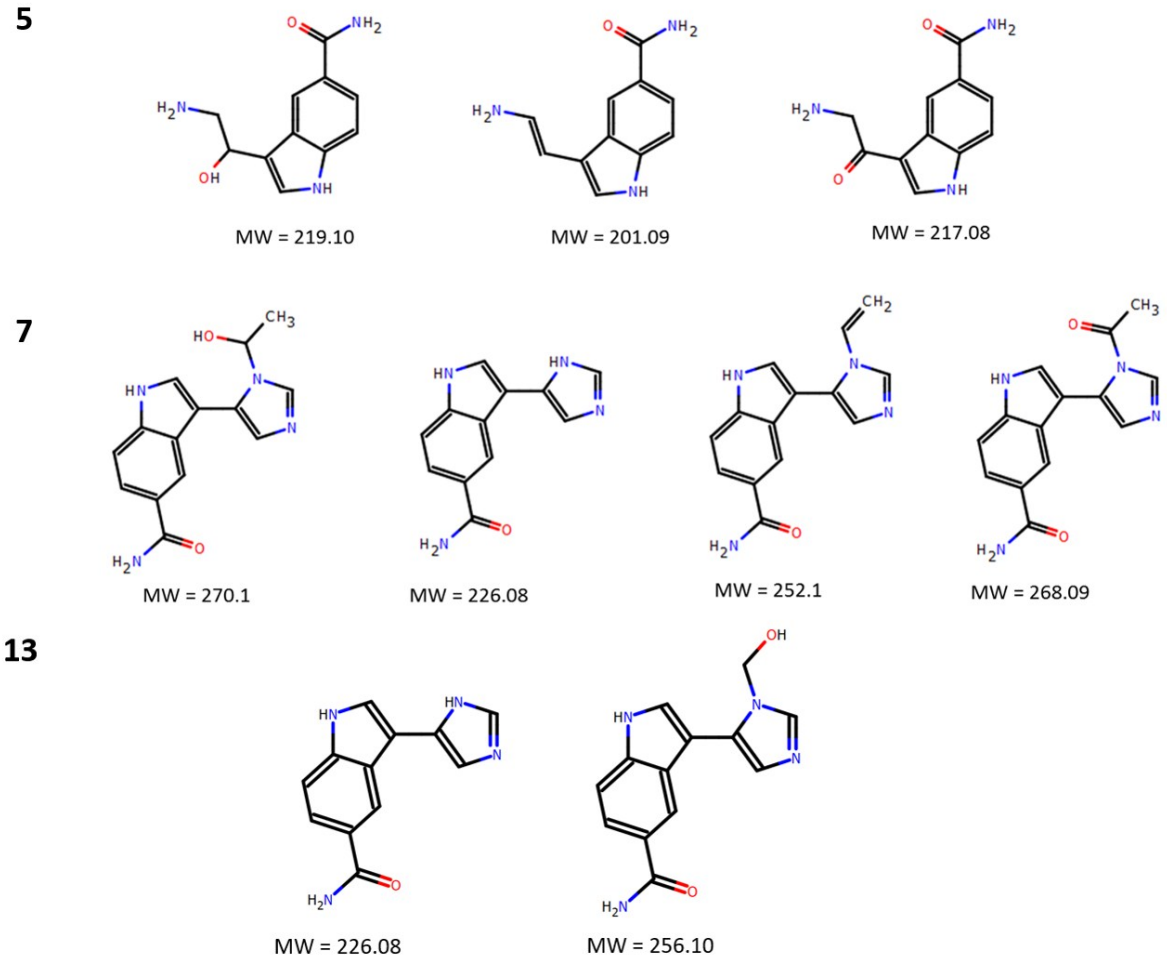


Figure S2 The molecular masses and structures of the most probably compounds' **5**,**7** and **13** metabolites, generated by MetaSite 5.1.1 software.

3.2 The in vitro determination of 5, 7 and 13 metabolic stability by using human liver microsomes (HLMs)

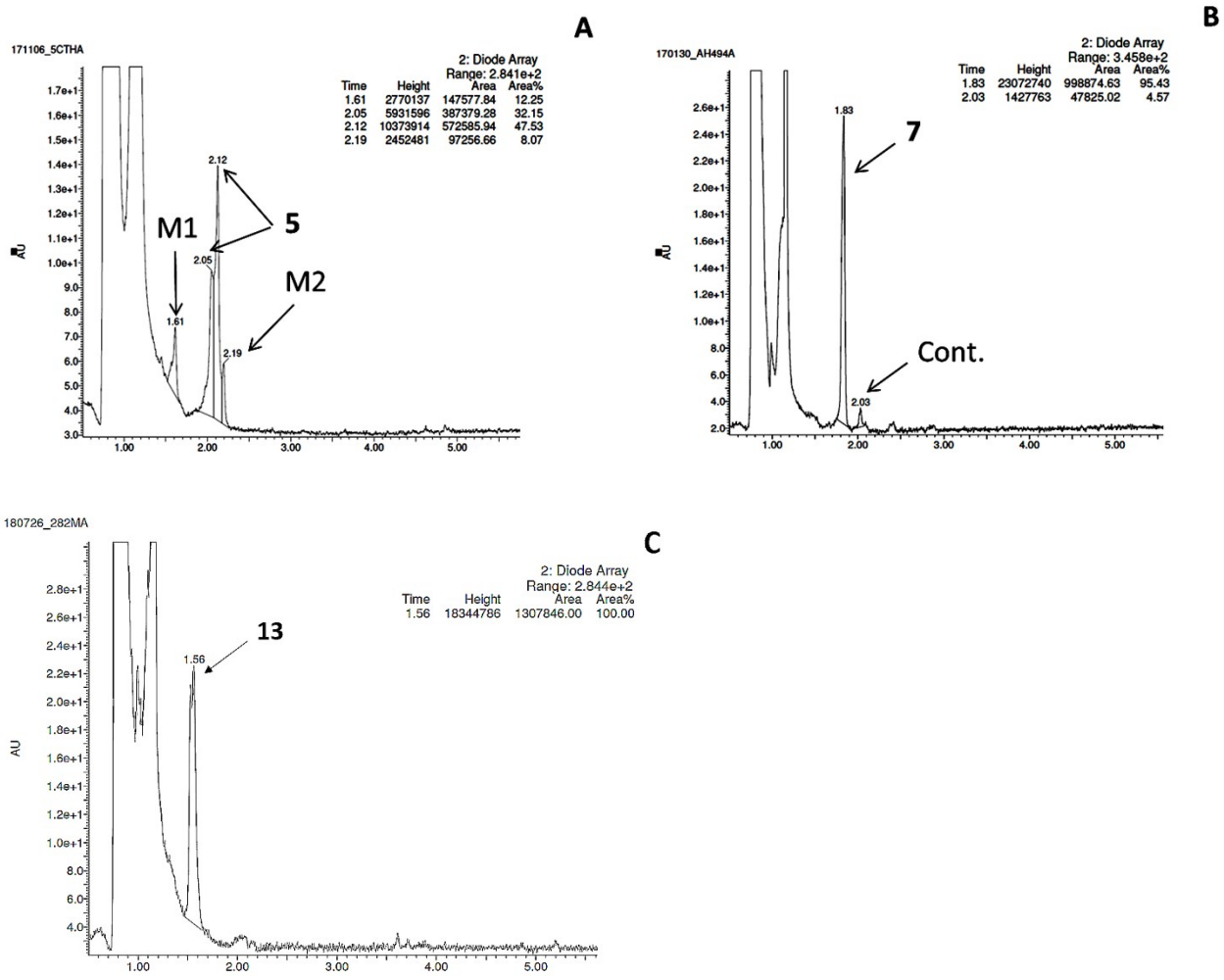


Figure S3 The UPLC spectrum of compound **5** after 120 min incubation with HLMs. Around 20% of **5** was metabolized into two metabolites **M1** and **M2** (A). The UPLC spectrum of compound **7** (B) and **13** (C) after 120 min incubation with HLMs. No metabolites were observed. Peak at $t_R = 2.03$ was identified as a contamination (B).

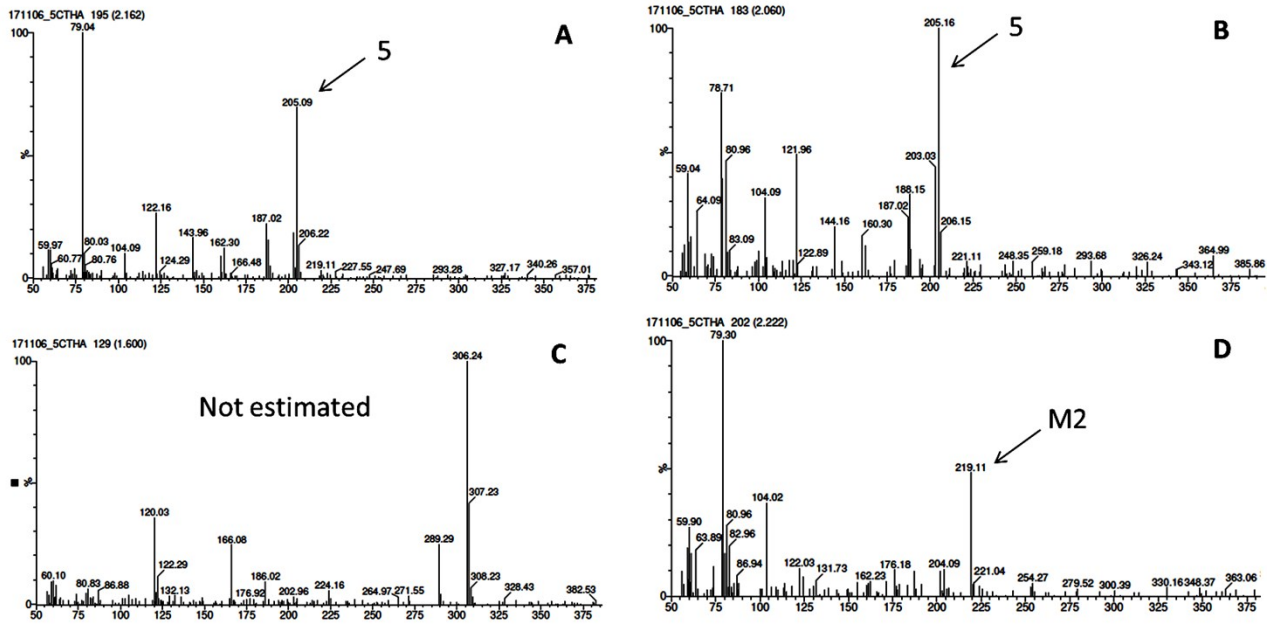


Figure S4 The MS analyses of peaks coming from compound **5** (A-B), and its metabolites **M1** (C) and **M2** (D). Molecular mass of **M1** was not estimated.

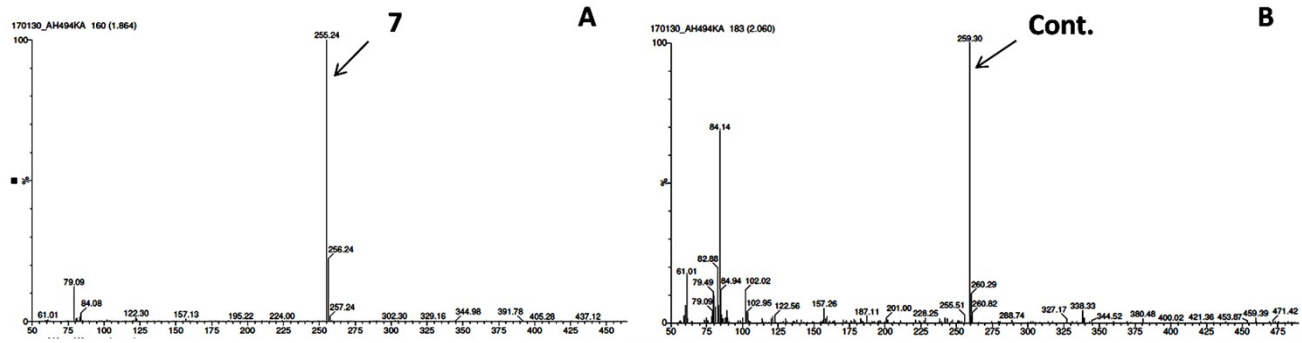
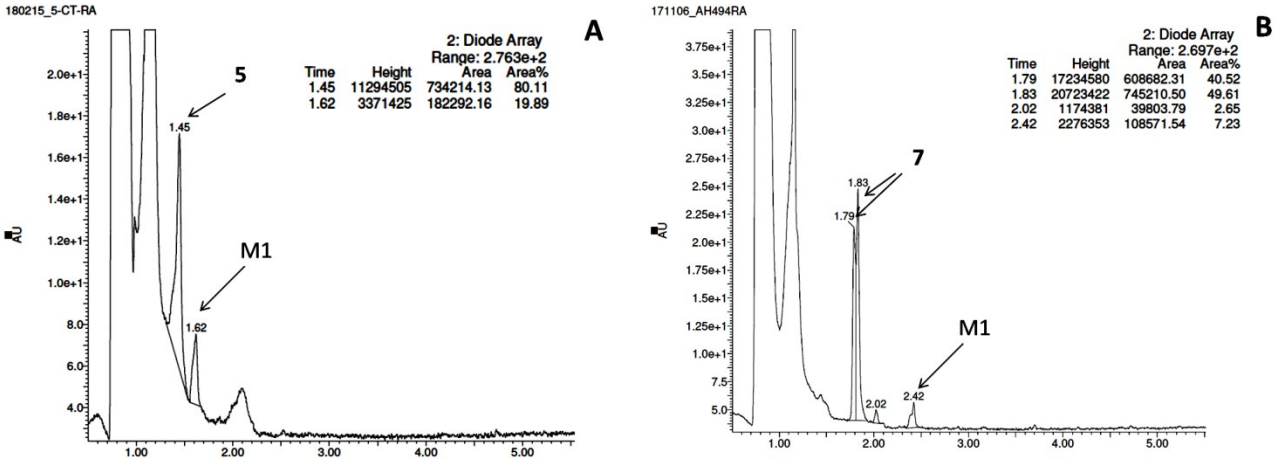


Figure S5 The MS analyses of peaks coming from compound **7** (A) and the contamination (B).

3.3 The in vitro determination of 5,7 and 13 metabolic stability by using rat liver microsomes (RLMs)



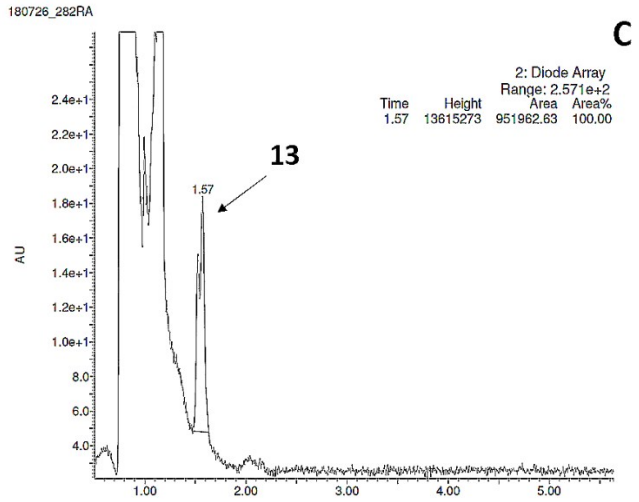


Figure S6 The UPLC spectrum of compound **5** after 120 min incubation with RLMs. Around 20% of **5** was metabolized into one metabolite **M1** (A). The UPLC spectrum of compound **7** after 120 min incubation with RLMs. Around 7% of **7** was metabolized into one metabolite **M1**. Peak at $t_R = 2.02$ was identified as a contamination (B). The UPLC spectrum of compound **13** after 120 min incubation with RLMs. No metabolites were observed (C).

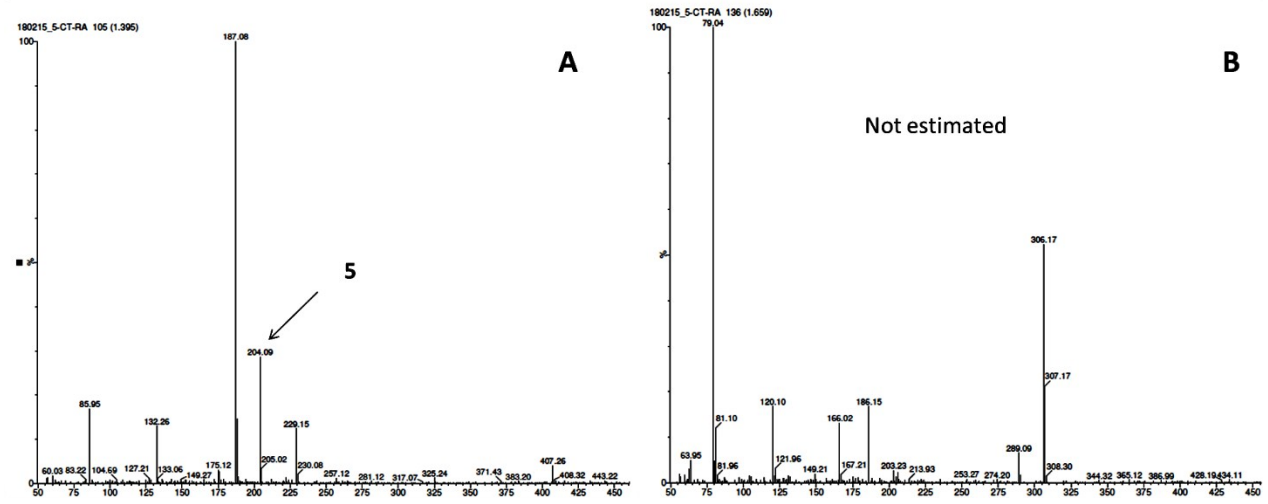


Figure S7 The MS analyses of peaks coming from compound **5** (A) and its metabolite **M1** (B). Molecular mass of **M1** was not estimated.

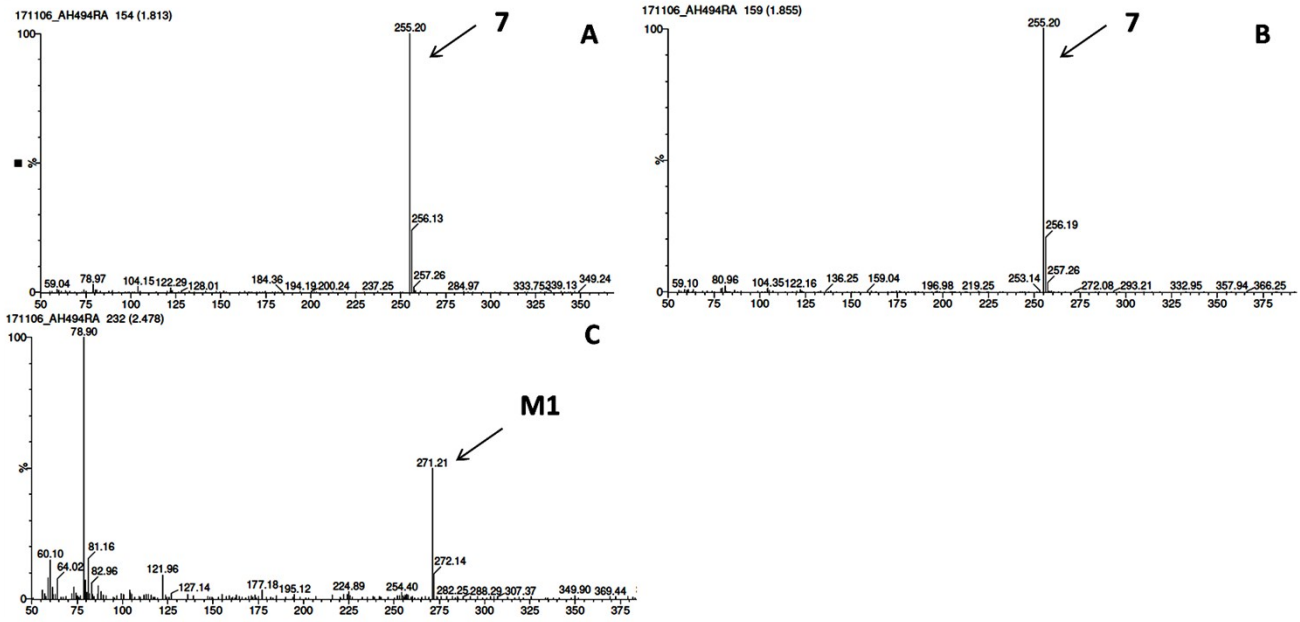


Figure S8 The MS analyses of peaks coming from compound **7** (A-B) and its metabolite **M1** (C)

3.4 The in vitro determination of 5, 7 and 13 metabolic stability by using mouse liver microsomes (MLMs)

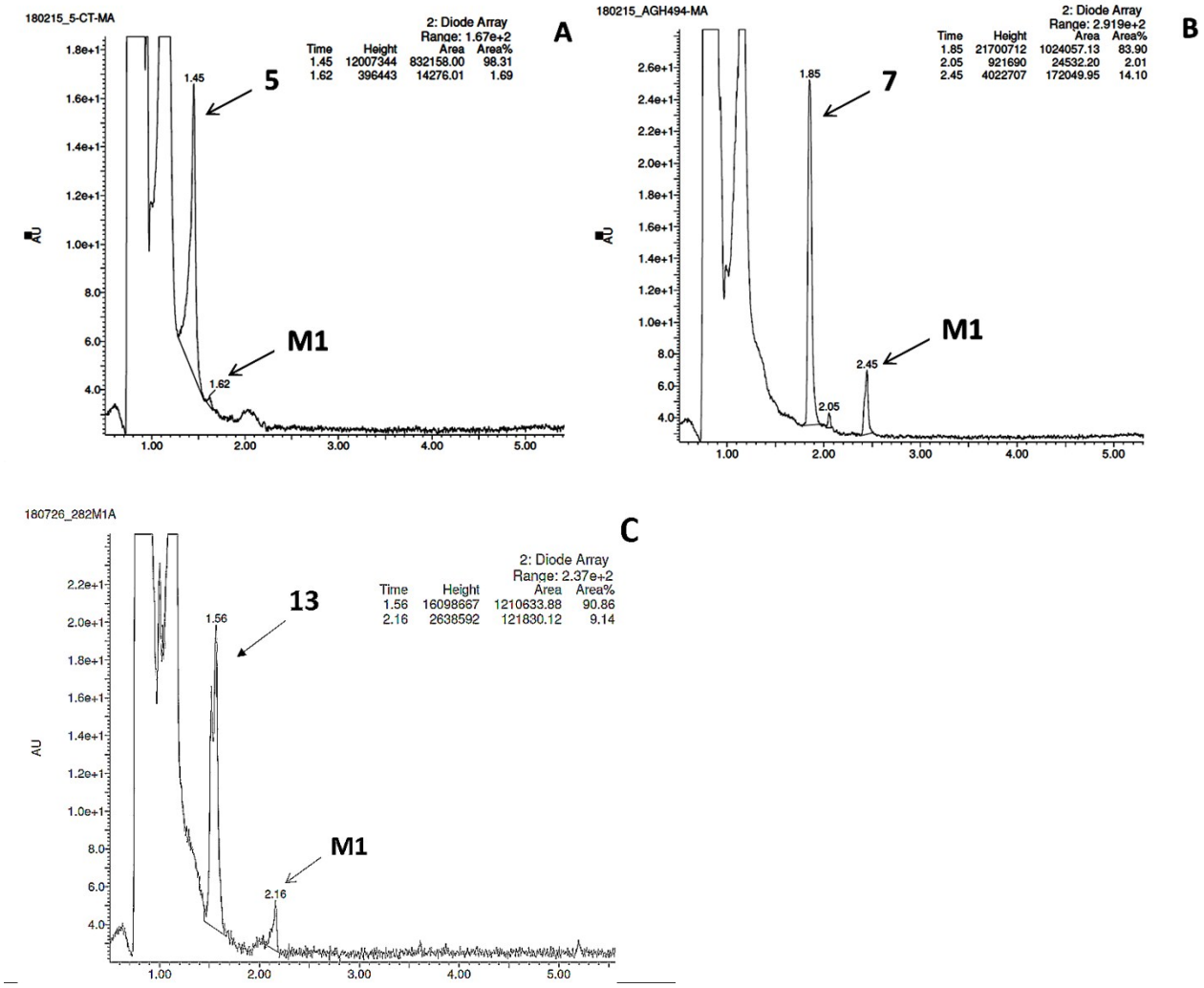


Figure S9 The UPLC spectrum of compound **5** after 120 min incubation with MLMs. Around 1.7 % of **5** was metabolized into one metabolite **M1** (A). The UPLC spectrum of compound **7** after 120 min incubation with MLMs. Around 14% of **7** was metabolized into one metabolite **M1**.

Peak at t_R = 2.05 was identified as a contamination (B). The UPLC spectrum of compound **13** after 120 min incubation with MLMs. Around 9% of **13** was metabolized into one metabolite M1 (C).

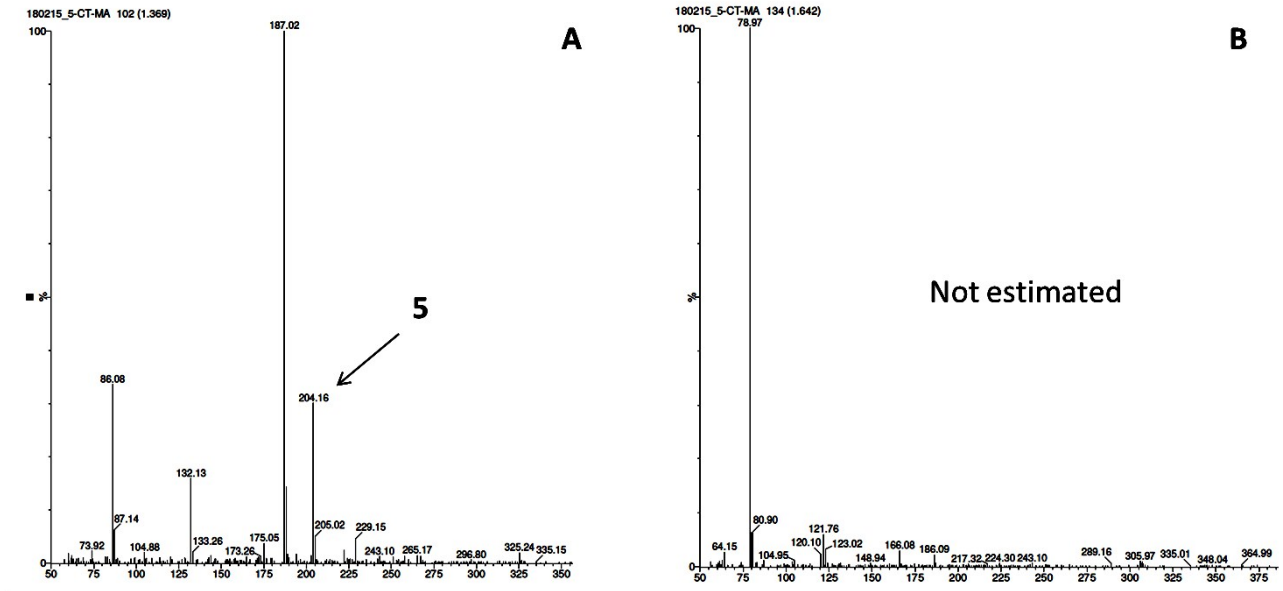


Figure S10 The MS analyses of peaks coming from compound **5** (A) and its metabolite **M1** (B). Molecular mass of **M1** was not estimated.

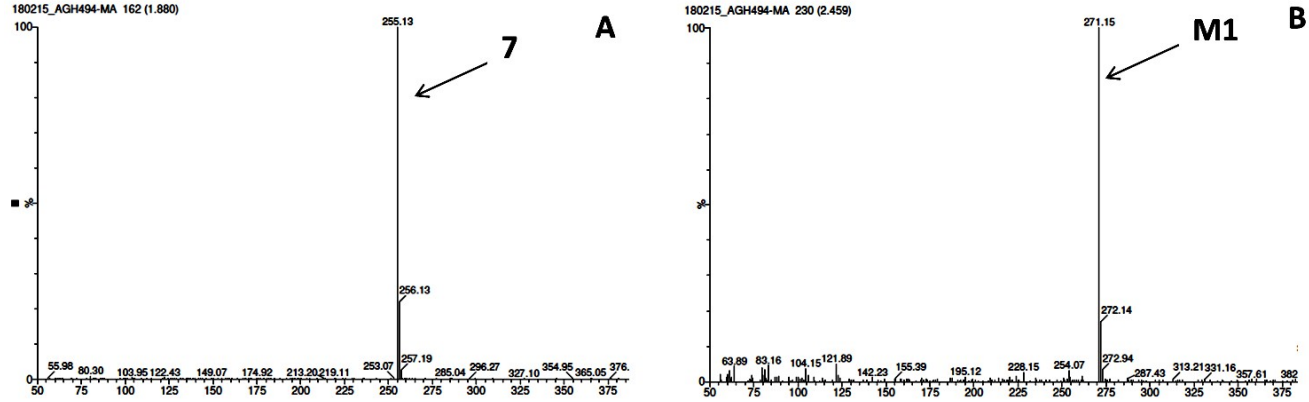


Figure S11 The MS analyses of peaks coming from compound **7** (A) and its metabolite **M1** (B).

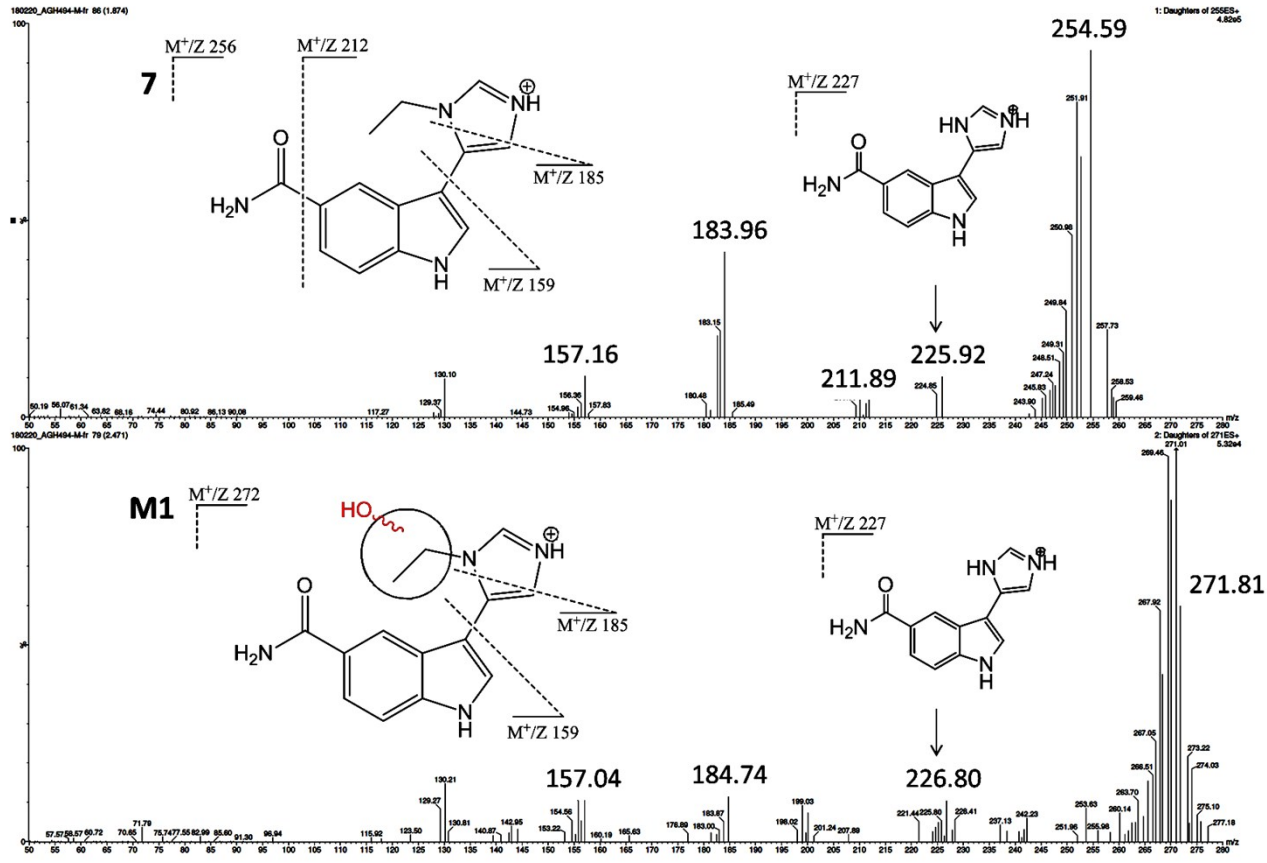


Figure S12 The MS ion fragmentation analysis of compound **7** and its metabolite **M1**.

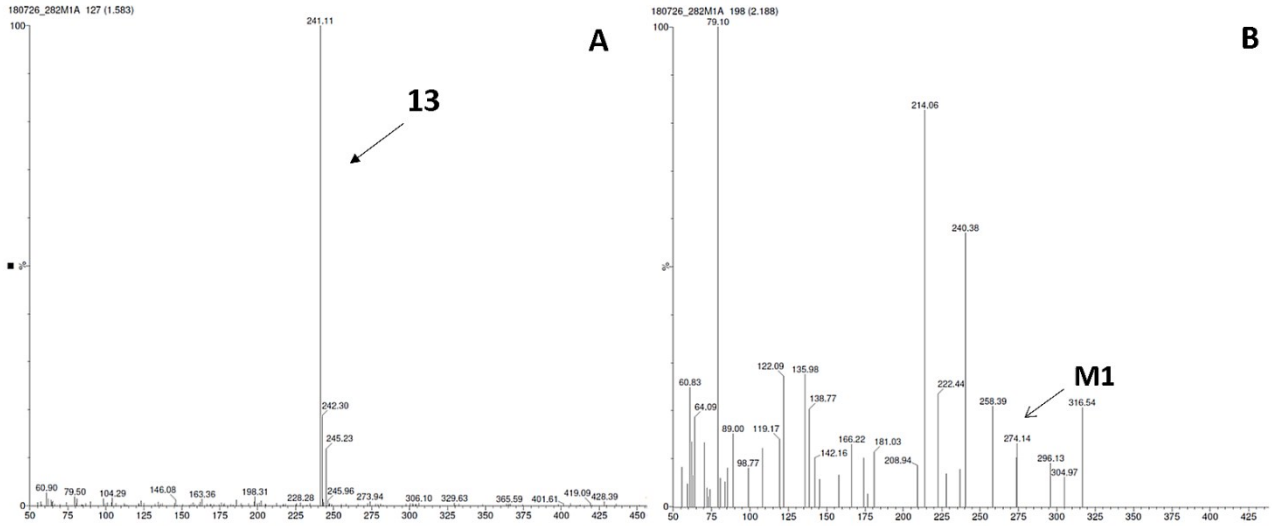


Figure S13 The MS analyses of peaks coming from compound **13** (A) and its metabolite **M1** (B).

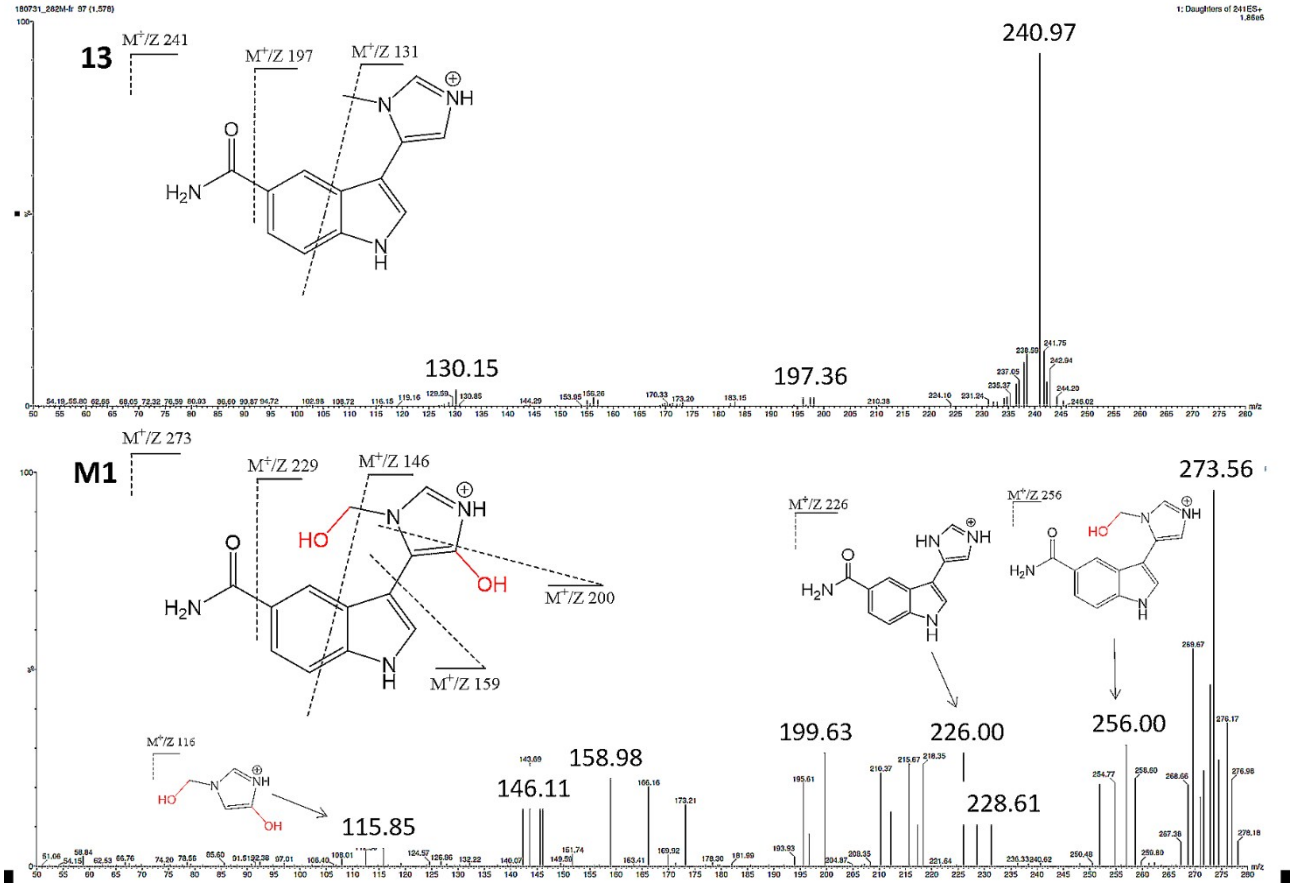


Figure S14 The MS ion fragmentation analysis of compound **13** and its metabolite **M1**.

4. References

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