**Supplementary Data 1. PsychLight1 and psychLight2 protein sequences. Related to STAR Methods.**

psychLight1.prot (695 aa)

```
MKTIIALSYIFCLVFADYKDDDDAMDILCEENTSLSSTTNSLMQLNDDTRLYSND
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FNSGEANTSDAFNWTVDSENRTNLSCEGCLSPSCLSLLHLQEKNWSALLTAVVII
      60        70        80        90       100       110

LTIAGNILVIMAVSLEKKLQNATNYFLMSLAIADMLLGFLVMPVSMLTILYGYRW
         120       130       140       150       160

PLPSKLCAVWIYLDVLFSTASIMHLCAISLDRYVAIQNPAHHSRFNSRTKAFLKI
     170       180       190       200       210       220

IAVWTISVGISMPIPVFGLQDDSKVFKEGSCLLADDNFVLIGSFVSFFIPLTIMV
         230       240       250       260       270

ITYFLTIKSLQKQLSSGYNVYIKADKQKNGIKANFKIRHNIEDGGVQLAYHYQQN
     280       290       300       310       320       330

TPIGDGPVLLPDNHYLSVQSKLSKDPNEKRDHMVLLEFVTAAGITLGMDELYKGG
         340       350       360       370       380

TGGSMVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTT
     390       400       410       420       430       440

GKLPVPWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYIQERTIFFKDDGN
         450       460       470       480       490

YKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNMHDQLNEQKACKVLGI
     500       510       520       530       540       550

VFFLFVVMWCPFFITNIMAVICKESCNEDVIGALLNVFVWIGYLSSAVNPLVYTL
         560       570       580       590       600

FNKTYRSAFSRYIQCQYKENKKPLQLILVNTIPALAYKSSQLQMGQKKNSKQDAK
     610       620       630       640       650       660

TTDNDCSMVALGKQHSEEASKDNSDGVNEKVSCV*
         670       680       690       695
```

```
MKTIIALSYIFCLVFADYKDDDDAMDILCEENTSLSSTTNSLMQLNDDTRLYSND
1         10        20        30        40        50

FNSGEANTSDAFNWTVDSENRTNLSCEGCLSPSCLSLLHLQEKNWSALLTAVVII
      60        70        80        90       100       110

LTIAGNILVIMAVSLEKKLQNATNYFLMSLAIADMLLGFLVMPVSMLTILYGYRW
         120       130       140       150       160

PLPSKLCAVWIYLDVLFSTASIMHLCAISLDRYVAIQNPKHHSRFNSRTKAFLKI
     170       180       190       200       210       220

IAVWTISVGISMPIPVFGLQDDSKVFKEGSCLLADDNFVLIGSFVSFFIPLTIMV
         230       240       250       260       270

ITYFLTIKSLQKQLSSGYNVYIKADKQKNGIKANFKIRHNIEDGGVQLAYHYQQN
     280       290       300       310       320       330

TPIGDGPVLLPDNHYLSVQSKLSKDPNEKRDHMVLLEFVTAAGITLGMDELYKGG
         340       350       360       370       380

TGGSMVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTT
     390       400       410       420       430       440

GKLPVPWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYIQERTIFFKDDGN
         450       460       470       480       490

YKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNFNMHDQLNEQKACKVL
     500       510       520       530       540       550

GIVFFLFVVMWCPFFITNIMAVICKESCNEDVIGALLNVFVWIGYLSSAVNPLVY
         560       570       580       590       600

TLFNKTYRSAFSRYIQCQYKENKKPLQLILVNTIPALAYKSSQLQMGQKKNSKQD
     610       620       630       640       650       660

AKTTDNDCSMVALGKQHSEEASKDNSDGVNEKVSCVFCYENEV*
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```
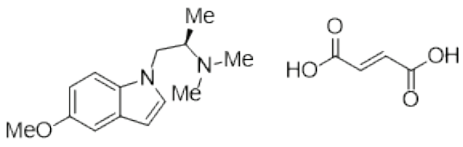
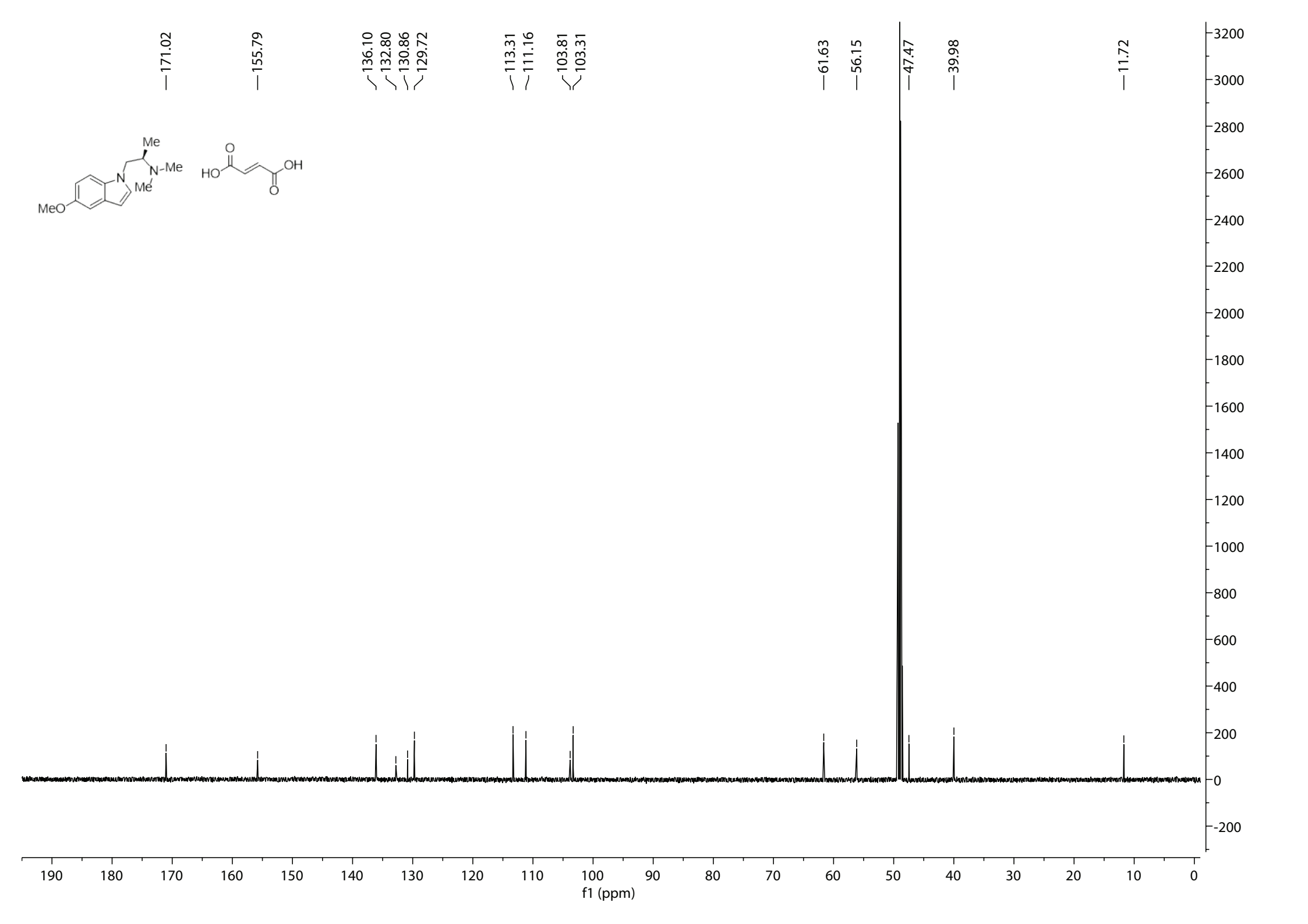

**Supplementary Data 2. Novel compound characterizations. Related to STAR Methods.**

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103.31
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47.47
39.98
11.72
Me
N-Me
Me
N
MeO
HO
O
OH
O
3200
3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
-200
190
180
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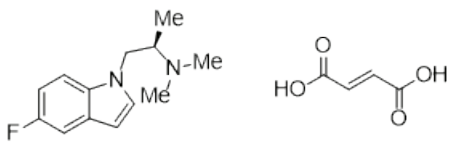
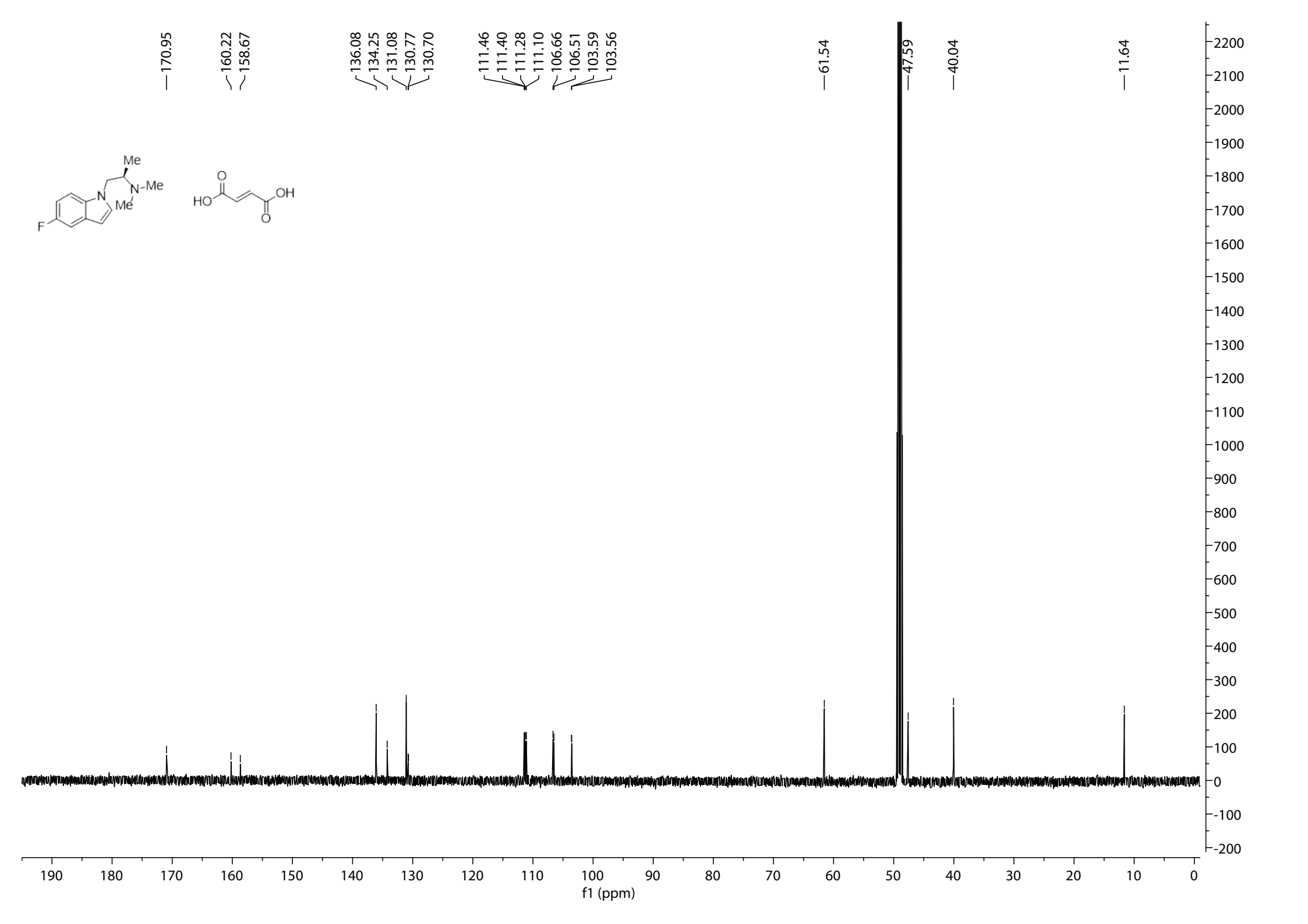
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)


Me
N-Me
Me
N
F


O
HO
OH
O


1.00
0.96
0.88
0.95
2.16
0.87
0.97
1.02
1.02
5.90
2.95
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
-500
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
f1 (ppm)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160.22
158.67
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134.25
131.08
130.77
130.70
111.46
111.40
111.28
111.10
106.66
106.51
103.59
103.56
61.54
47.59
40.04
11.64
Me
N-Me
Me
N
F
O
HO
OH
O
f1 (ppm)

**Supplementary Table 1. Overview of experimental design and results. Related to all Figures.**

| Question | Approach | Results | Figures |
|---|---|---|---|
| **Development of psychLight for imaging serotonin and hallucinogens** | | | |
| Can we design a sensor to probe ligand-induced conformational changes of 5-HT2A receptor? | We replaced the third intracellular loop of the 5-HT2A receptor with cpGFP followed by linker screening and membrane localization optimization | psychLight responded to serotonin, but not to 5-HT2AR antagonists. Serotonin displayed an $EC_{50}$ = 26.3 nM | Figure 1A–D |
| What is the sensitivity and kinetics of psychLight? | Two-photon uncaging and imaging of serotonin in cultured cortical slices | psychLight displayed fast off kinetics (5.4 ms) in response to single pulse uncaging (10ms) | Figure 1E–H |
| | Two-photon imaging of serotonin release triggered by electrical stimuli in acute slices | psychLight was able to detect electrically-evoked serotonin release in BNST acute slices. The fluorescence response can be modulated by a SSRI and abolished by a sodium channel blocker TTX and a serotonin receptor antagonist granisetron | Figure 1I–M |
| **In vivo imaging of serotonin release in multiple brain regions with fiber photometry** | | | |
| Can psychLight detect behaviorally relevant serotonin release? | We applied fiber photometry to study endogenous serotonin release in DRN, BNST, BLA, and OFC triggered by auditory fear conditioning | psychLight can faithfully detect the serotonin dynamics across the full-course of fear-learning in single trials (d'>12). | Figure 2 |
| **Imaging hallucinogenic conformations of 5-HT2AR** | | | |
| Can psychLight be used to detect activation of 5-HT2AR by hallucinogens in vivo? | In vivo fiber-photometry recording in mPFC | Fluorescence increased upon administration of 5-MeO-DMT and the onset of increase correlated with the head-twitch response | Figure 3A–C |
| How effectively do hallucinogenic compounds activate psychLight? | Concentration-response curve in 293T cells | Determined $EC_{50}$ values for a panel of hallucinogens | Figure 3D–G |
| | | PsychLight $EC_{50}$ values correlate with human hallucinogenic potency | Figure 3H |
| | | PsychLight response is not equivalent to other measures of 5-HT2AR activation | Figure 3I |
| **Medium-throughput pharmacological assay based on psychLight** | | | |
| Can we use psychLight to establish a cell-based assay for determining hallucinogenic potentials of library compounds? | | Z-score of the assay is 0.6 | Figure S3D |
| | Engineered HEK293T cell line stably expressing psychLight2 | The assay is sensitive to compounds with similar molecular structures | Figure 4B–C |
| | Screened a library consisting of hallucinogens, non-halluci-nogens, and a panel of compounds with unknown hallucinogenic potentials | Defined a ligand score to predict the pahrmacological features of compounds:<br>Ligand score >0: 5-HT2AR-activating hallucinogens<br>Ligand score <0: non-hallucinogenic 5-HT2AR ligands<br>Ligand score ~0: not 5HT2AR ligands | Figure 4D–E |
| | | Schild regression analysis defined non-hallucinogenic ligands as competative antagonists | Figure 5D<br>Figure S6 |
| **Identification of new hallucinogenic and non-hallucinogenic compounds** | | | |
| Will predicted hallucinogens produce hallucinogenic behaviors? | Performed a three-point dose-response study of 5-halo-DMT family measuring head-twitch response and locomotion | 5-F-DMT and 5-Cl-DMT produce robust HTR as predicted by psychLight<br>5-Br-DMT did not produce a HTR as predicted by psychLight | Figure 5A–C |
| What are the pharmacological features and behavioral effects of predicted non-hallucinogens, such as AAZ-A-154 ? | Performed Schild regression analysis<br>Performed a three-point dose-response study measuring head-twitch response and locomotion | AAZ-A-154 is a competative 5-HT2AR ligand that did not produce a HTR as predicted by psychLight | Figure 5D–F |
| **Characterizing antidepressant-like effects of a novel non-hallucinogenic compound** | | | |
| Can AAZ-A-154 promote dendritic growth in cultured neurons?<br>If yes, is the effect 5-HT2R dependent? | Performed Sholl analysis in cultured cortical neurons treated with AAZ-A-154 or vehicle in the presence and absence of 5HT2R antagonist ketanserin. | AAZ-A-154 promoted dendritic growth. This effect can be blocked by ketanserin, implicating 5-HT2Rs in the mechanism of action | Figure 6A–C |
| Does AAZ-A-154 have antidepressant potential?<br>If yes, is the effect comparable to Ketamine? | Performed forced swim test | AAZ-A-154 produces both rapid (30 min) and long-lasting (1 week) antidepressant-like effects after a single administration comparable to ketamine | Figure 6D |
| | Measured anhedonia in a genetic animal model relevant to depression | VMAT2-HET mutants exhibited a sucrose preference that was indistinguishable from WT controls after a single-adminis-tration and the effect was long-lasting (12-days) | Figure 6E |