Discovery of 1,5-Disubstituted Pyridones: A New Class of Positive Allosteric Modulators of the Metabotropic Glutamate 2 Receptor

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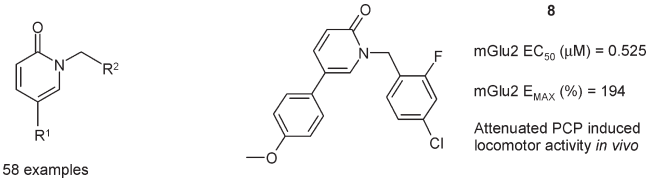
Abstract



A series of 1,5-disubstituted pyridones was identified as positive allosteric modulators (PAMs) of the metabotropic glutamate receptor 2 (mGluR2) via high throughput screening (HTS). Subsequent SAR exploration led to the identification of several compounds with improved *in vitro* activity. Lead compound **8** was further profiled and found to attenuate the increase in PCP induced locomotor activity in mice.

Keywords: mGluR2, metabotropic, glutamate, allosteric, PAM, modulators

Metabotropic glutamate receptors (mGluRs) represent a family of G protein-coupled receptors that are activated by the excitatory neurotransmitter glutamate. Activation of group II metabotropic glutamate receptors (mGluR2 and mGluR3) may provide anxiolytic and/or antipsychotic effects (*1*−*3*). Mixed mGluR2/mGluR3 agonists such as LY354740 (**1**, Figure 1) have shown activity in a range of preclinical animal models of anxiety and schizophrenia. The anxiolytic potential of LY354740 was also confirmed in healthy human volunteers, showing activity in fear-potentiated startle and panic induction after a CO_2 challenge (*4*, *5*). A related prodrug, LY2140023 (**2**), demonstrated improvements in positive and negative symptoms compared to that by the placebo in schizophrenic patients (*6*). In addition, evidence exists from knockout studies that the preclinical antipsychotic effects may be mediated via the mGluR2 receptor (*7*).

Over recent years, there has been increasing interest in trying to identify positive allosteric modulators (PAMs) of mGluR2, which bind at an alternative site to the orthosteric endogenous agonist (*8*). The first mGluR2 PAMs were a series of sulfonamide derivatives discovered by scientists at Lilly, including LY487379 (**3**, Figure 1) (*9*, *10*). We have recently conducted a review of the currently reported mGluR2 PAMs (*11*), and new reports are appearing on a frequent basis (*12*). Recent reports from our laboratories described a series of imidazopyridines identified with the help of computational shape and electrostatic field similarity (*13*). In this article, the discovery of 1,5-disubstituted pyridones as a new series of mGluR2 PAMs is presented. Initial SAR exploration is given along with *in vivo* profiling of a lead compound.

Results and Discussion

High throughput screening (HTS) of the compound collection from Addex Pharmaceuticals in an mGluR2 PAM FLIPR (fluorometric imaging plate reader) assay

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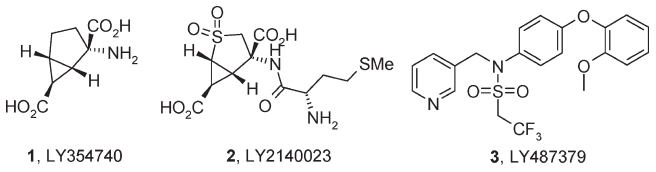

Figure 1. Structures of mixed mGluR2/3 agonists LY354740 and LY2140023, and of the first reported mGluR2 PAM LY487379.

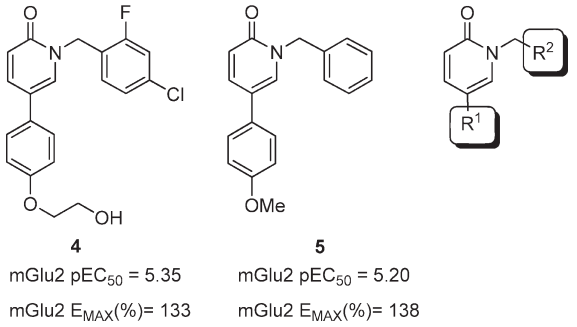

Figure 2. Pyridone hits **4** and **5** identified from an mGluR2 PAM high throughput screen.

led to the discovery of pyridones **4** and **5**. Their potentiating activity was also confirmed in a mGluR2 [^{35}S]-GTPγS assay (Figure 2). The *in vitro* GTPγS assay provided two measures of compound activity, the EC_{50} for the potentiation of glutamate, and the maximal increase in observed glutamate response, the % E_{max} (*14*). Compound **4** displayed an EC_{50} of 4.47 μM and a 133% increase in the maximal glutamate effect, while compound **5** had an EC_{50} of 6.29 μM and an E_{max} of 138%. Despite their low activity, compounds **4** and **5** were selected as viable starting points for a CNS focused exploration given their relatively low molecular weight, 374 and 291, respectively, and polar surface area, 50 and 30, respectively. Our initial efforts focused on building structure−activity relationships (SAR) by sequential modification of both R^1 and R^2 substituents of the pyridone ring.

The initial set of compounds **6**−**31** covered the variation of the R^1 group at position C-5 of the pyridone ring while maintaining the 4-chloro-2-fluoro benzyl substituent in compound **4** constant. The target compounds **6**−**31** were prepared following the synthetic strategies shown in Schemes 1−3.

The majority of the analogues were prepared following the synthetic routes depicted in Scheme 1. N-Alkylation of 5-bromo-2-hydroxypyridine (**32**) with 4-chloro-2-fluorobenzyl bromide afforded compound **33** in 79% yield. Subsequent Suzuki coupling of the 5-bromopyridone **33** with several aryl boronic acids under microwave irradiation led to the corresponding target compounds **4**, **6**, **8**, **9**, **14**−**17**, and **29**−**31** in moderate to good yields. Additionally, **33** was coupled with 4-phenylpiperidine under standard Buchwald−Hartwig coupling conditions to yield the pyridone derivative **26**. Compound **8** was further modified by cleavage of the methoxy ether with BBr_3 to give the phenolic derivative **7**, which was then coupled with different alkylbromides under microwave irradiation resulting in the corresponding *O*-alkylated products **10**−**11** and **19**. Compound **7** was also treated with the commercially available *N*-BOC-hydroxyethanolamine under Mitsunobu reaction conditions followed by cleavage of the BOC group. The subsequent acetylation or sulfonylation of the amino group afforded compounds **12** or **13**, respectively.

Compound **25**, in which position C-5 of the pyridone ring is substituted by a 3-phenylpropyl, was prepared as shown in Scheme 2. Halogen−lithium exchange on commercially available 3-bromo-2-methoxypyridine (**34**) with *n*-butyllithium followed by treatment with 1-bromo-3-phenylpropane led to the methoxypyridine derivative **35** in 17% yield. Treatment of **35** with 4-chloro-2-fluorobenzyl bromide in the presence of NaI afforded the target compound **25** in moderate yield (48%).

The introduction of an oxygen linker between the pyridine core and the aryl substituent at position C-5 proceeded via the reaction of the bromopyridine **34** with phenol under copper-catalyzed Ullmann coupling conditions to afford 2-methoxy-4-phenoxypyridine **36**, which was converted into the final compound **28** under standard N-benzylation conditions (Scheme 2).

Finally, compound **27**, an analogue of **8** which contains a methylene spacer, was synthesized as shown in Scheme 3. In the first step, the addition of 4-methoxybenzylmagnesium bromide to **37** led to the corresponding alcohol **38**, which was then subjected to N-benzylation to give pyridone **39** that was finally transformed into **27** by treatment with Et_3SiH in trifluoroacetic acid.

The variations around the R^1 group and the functional activity of *N*-(4-chloro-2-fluorobenzyl)pyridones **4** and **6**−**31** are listed in Table 1.

The effect of substituents on the C-5 aromatic ring present in **4** was explored with compounds **6**−**24**. The unsubstituted compound **6** had the same level of *in vitro* potency, EC_{50} of 6.31 μM and E_{max} 138%, compared to that of **4**. The 4-hydroxyphenyl derivative **7** and its elongated analogue **18** also displayed similar activities, EC_{50} of 5.01 μM and 5.25 μM and E_{max} of 124 and 145%, respectively. However, a 10-fold increase in potency was obtained with the simple 4-methoxy substituent in compound **8**, which had an EC_{50} of 0.53 μM and an E_{max} of 194%. This compound is the direct analogue of the initial hit compound **5** and demonstrated the beneficial effect of the 4-chloro-2-fluoro decoration on the benzyl R^2 substituent. A decrease in potency was observed with the pyridyl analogue **24**, and the activity was completely lost with the 4-trifluoromethoxy derivative **9**, which indicates that an electron-rich phenyl ring

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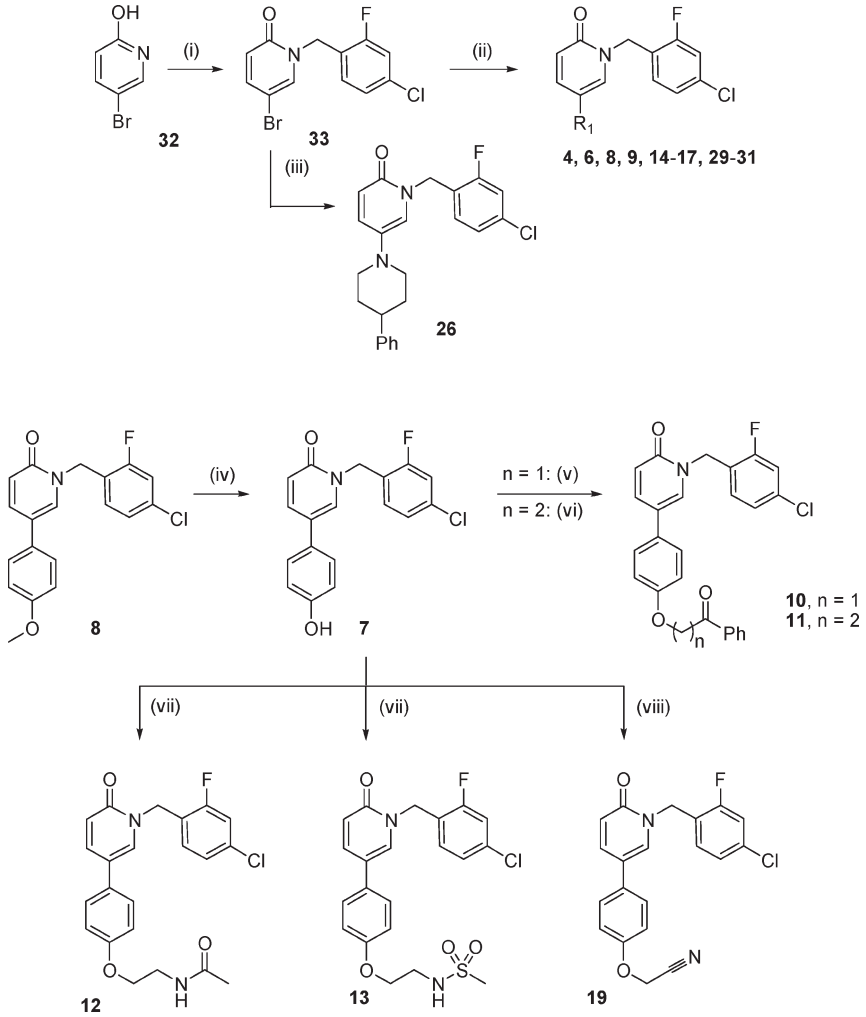
Scheme 1[a]



[a] Reagents and conditions: (i) 4-chloro-2fluorobenzylbromide, K_2CO_3, THF, 60 °C, 17 h, 79%. (ii) $ArB(OH)_2$, 10% $Pd(PPh_3)_4$, $NaHCO_3$ (aq.)/1,4-dioxane, 140 °C, 5 min at μW, 30−75%. (iii) 4-Phenylpiperidine, $Pd(OAc)_2$, BINAP, *t*-BuOK, toluene, 100 °C, 16 h, 77%. (iv) BBr_3, CH_2Cl_2, −40 °C to rt, 24 h, 83%. (v) 2-chloroacetophenone, K_2CO_3, THF, 110 °C, 30 min at μW, 20%. (vi) 2-Chloropropiophenone, K_2CO_3, THF, 110 °C, 30 min at μW, 34%. (vii) (a) *N*-BOC-2-hydroxyethanol, Ph_3P, DEAD, 0 °C to rt, 16 h, 66%; (b) HCl/1,4-dioxane, MeOH, 80 °C, 2 days, 100%; (c) AcCl (**12**) or $MeSO_2Cl$ (**13**), Et_3N, CH_2Cl_2, rt, 1 h, 47−65%, respectively. (viii) 2-Bromoacetonitrile, K_2CO_3, acetonitrile, 180 °C, 5 min at μW, 42%.

at position C-5 of the pyridone core might be preferred. The effect of a H-bond acceptor was also explored with the introduction of several alkyl chains at the *para*-position of the C-5 phenyl ring (**10**, **11**, and **19**). The results obtained suggested that although an acceptor function is tolerated it does not result in an increase in activity. Amino substitution either at the *meta*- or *para*-position (**14**, **15**) was detrimental for the E_{max} activity, with values of 67% and 102%, respectively. However, improved potency was observed with amide derivatives **16** and **17**, which had EC_{50} values of 0.81 μM and 1.74 μM, respectively. The interesting activity seen for compound **8** prompted us to study the 4-methoxy substitution in combination with other small substituents on the same phenyl ring (**20**−**23**). Unfortunately, none of these analogues showed better activity than **8**.

Several different spacers were introduced between the pyridone core and the phenyl ring (compounds **25**−**28**), although only the 4-piperidinyl spacer (**26**) was somewhat tolerated. The introduction of tetrazole substituents commonly found in series of mGluR2 PAM indanones from Merck (*15*) resulted in weakly active compounds (**29**, **30**) in this class. In contrast, the 4-pyridylthiomethyl (**31**), also found in Merck's indanone series (*16*), gave rise to the most potent compound identified in this R^1 exploration. This finding supports the hypothesis that an electron-rich phenyl ring in combination with groups that introduce moderate polarity such as a H-bond acceptor is beneficial for activity.

We then focused our attention on exploring substitution at the pyridone nitrogen of the initial hit **5** while maintaining the 4-methoxyphenyl substituent constant

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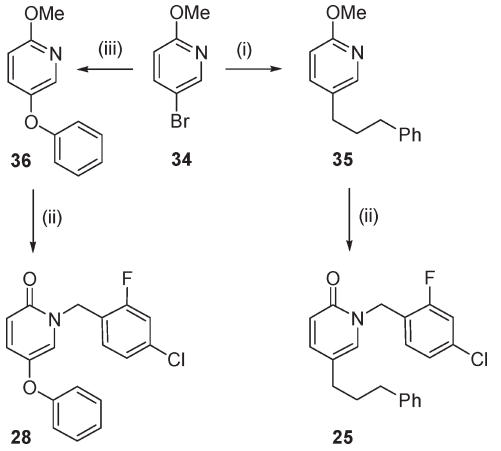
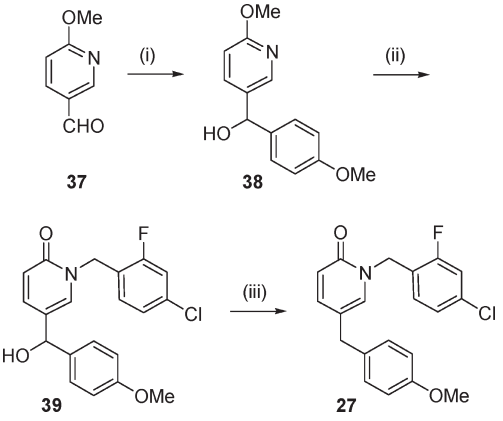
Scheme 2[a]



[a] Reagents and conditions: (i) 1-(3-Bromopropyl)benzene, *n*-BuLi, THF, −78 °C to rt, 1 h, 17%. (ii) 4-Chloro-2fluorobenzyl bromide, acetonitrile, NaI, 90 °C, 14 h, 48−58%. (iii) Phenol, CuI, Cs_2CO_3, Me_2NCH_2COOH, 1,4-dioxane, 150 °C, 25 min at μW, 44%.

Scheme 3[a]



[a] Reagents and conditions: (i) 4-Methoxybenzylmagnesium bromide, THF, −78 °C to rt, 14 h, 68%. (ii) 4-Chloro-2fluorobenzylbromide, acetonitrile, NaI, 90 °C, 14 h, 48−58%. (iii) Et_3SiH, TFA, rt, 1 h, 79%.

at the C-5 position of the pyridone ring. A straightforward synthesis route that allowed the rapid preparation of a set of 27 compounds (**41**−**68**) is shown in Scheme 4. Suzuki coupling between 4-methoxyphenylboronic acid and 4-bromo-2-methoxypyridine **34** afforded the corresponding coupling product **40**, which was transformed into the final compounds **41**−**68** by treatment with the appropriate alkylating agent in the presence of NaI.

As shown in Table 2, lipophilic decoration on the aryl via halogen atoms or halogenated alkyl groups (**41**−**43**, **45**−**49**) was beneficial for potency. Compound **45** was the most active one identified during this R^2 exploration, with an EC_{50} of 0.24 μM and an E_{max} of 228%. The increase in potency is obvious in comparison with those of analogues having hydrophilic substitutions such as the phenol analogue **44**, which was inactive up to 10 μM. The same trend in SAR was seen with analogues **51**−**56**, where the aryl ring is replaced by different heterocycles such as pyridyl, thiazolyl, benzimidazolyl, or benzothiazolyl, which only showed weak activity. The unsubstituted pyridyl **51** was inactive; however, the introduction of additional lipophilic substituents such as trifluoromethyl or methoxy resulted in weakly active compounds (**52** and **53**). Lengthening the distance between the pyridone and the aryl through an ethyl- or methyloxy-chain (**57**, **58**) did not have an effect on activity. Lipophilic mapping on these elongated analogues again increased activity (**59**, **60**). Finally, the importance of having an aromatic ring as a substituent on the pyridone nitrogen was investigated with several analogues having *N*-aliphatic groups (**61**−**68**). Compounds showed comparable potency indicating that aromaticity played no particular role in potency. However, these data confirm the importance of having lipophilic substituents in this area of the molecule, with the most lipophilic alkyl groups displaying better activities (**67**, **68**). In summary, the exploration of the R^2 substituents revealed that neither polarity nor weak basicity are well tolerated at R^2, while hydrophobicity is essential to maintain reasonable potency levels.

To further profile the series, a representative set of compounds was tested for single point microsomal stability in human liver microsomes (HLM). The results are shown in Table 3, with most of the compounds suffering from extensive metabolism. The 4-methoxyphenyl compound **8** was completely metabolized after 15 min of incubation with HLM, with the major metabolite identified as the corresponding des-methyl analogue (**7**). Somewhat decreased metabolism was found with the 4-hydroxy analogue **7** (70%) and its elongated version **18** (82%). Aniline derivative **15** and its *N*-acetyl analogue **16** were also somewhat more stable than **8** (64% and 83% metabolized, respectively). A remarkable improvement was found for compounds **12** and **13** where the 4-methoxy group was replaced by bulkier alkoxy groups (24% and 35%, respectively). The presence of either electron donating or withdrawing ortho-substituents to the 4-methoxy group (compounds **20**, **22**) did not prevent extensive degradation. Improved stability was observed with the tetrazole derivative **30** (59%), which suggests that introduction of polar functional groups may increase metabolic stability in this series. Finally, the two most interesting examples with respect to their primary activity, compounds **31** and **45**, suffered from extensive metabolism with 73% and 98% metabolized in the HLM assay, respectively.

Despite its high metabolic turnover, compound **8** was chosen as a prototype to further evaluate the potential of this novel mGluR2 PAM series.

The ability of compound **8** to shift the *in vitro* concentration−response curve (CRC) of glutamate was

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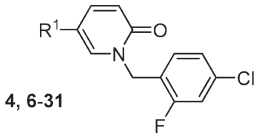
Table 1. Functional Activity of Representative mGluR2 PAMs **4** and **6**−**31**[a]



4, 6-31

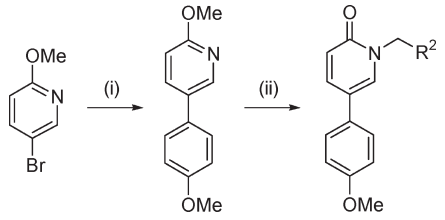
compound	R^1	mGlu2 EC_{50} (μM)[a]	mGlu2 E_{max} (%)
4	$HOCH_2CH_2O$-Ph-	4.47	133
6	Ph-	6.31	138
7	4-OH-Ph-	5.01	124
8	4-MeO-Ph-	0.53	194
9	4-CF_3OPh-	> 10	
10	4-(PhC(O)CH_2O)-Ph-	11.5	182
11	4-(PhC(O)CH_2CH_2O)-Ph-	1.26	196
12	4-(AcNHCH_2CH_2O)-Ph-	0.79	102
13	4-($MeSO_2NHCH_2CH_2O$)-Ph-	0.68	120
14	3-NH_2-Ph-	3.98	67
15	4-NH_2-Ph-	9.33	102
16	4-AcNH-Ph-	0.81	48
17	4-(pyrrolidinone-1-yl)-Ph-	1.74	112
18	$HOCH_2$Ph-	5.25	145
19	4-($CNCH_2O$)-Ph-	3.39	184
20	3-F-4-MeO-Ph-	0.71	241
21	3,4-diMeO-Ph-	3.31	152
22	3-Me-4-MeO-Ph-	1.05	185
23	5,3-diMe-4-MeO-Ph-	1.00	116
24	3-(6-MeO)pyridyl-	9.33	149
25	$PhCH_2CH_2CH_2$-	0.56	33
26	4-phenylpiperidinyl-	3.24	101
27	4-MeO-$PhCH_2$-	1.93	70
28	Ph-O-	> 10	--
29	3-(5-tetrazolyl)-Ph-	7.59	65
30	4-(5-tetrazolyl)-Ph-	1.00	32
31	4-(4-pyridylthiomethyl)-Ph-	0.18	168

[a] Values are means of three experiments.

investigated further. As shown in Figure 3, the CRC of glutamate shifts to the left and upward with increasing concentrations of compound **8**. A 7-fold shift in the glutamate EC_{50} was seen in the presence of 3 μM of **8**, with the glutamate pEC_{50} shifted from 4.9 to 5.8. These results are in line with a positive allosteric interaction between glutamate and compound **8**.

Targeting the allosteric site of mGluRs is expected to improve the chance of identifying selective ligands. Possible agonist or antagonist effects of compound **8** against mGluR3, mGluR5, mGluR7, and mGluR8 were first evaluated (*17*). Compound **8** was inactive in all assays except for mGluR7 antagonism where it had an EC_{50} of 4.94 μM and an E_{max} of 78%. In addition, compound **8** was profiled against 50 targets in a selectivity screen performed at CEREP (*18*). Selectivity was

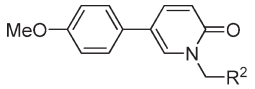
Scheme 4[a]



34 40 41-68

[a] Reagents and conditions: (i) 4-Methoxyphenylboronic acid, 10% $Pd(PPh_3)_4$, $NaHCO_3$ (aq.)/1,4-dioxane, 140 °C, 5 min at μW, 75%. (ii) R^1CH_2X, acetonitrile, NaI, 150 °C, 30 min at μW, 30−65%.

Table 2. Functional Activity of Representative mGluR2 PAMs **5**, **41**−**68**[a]



5, 41-68

compound	R^2	mGlu2 EC_{50} (μM)[a]	mGlu2 E_{max} (%)
5	Ph-	6.29	138
41	2-F-Ph-	1.00	44
42	3-F-Ph-	1.91	96
43	4-Cl-Ph-	1.41	189
44	4-OH-Ph-	> 10	
45	4-CF_3O-Ph-	0.24	228
46	2,3-diF-Ph-	10	131
47	2,4-diF-Ph-	4.79	128
48	2,4,6-triF-Ph-	2.96	118
49	3-F-4-Cl-Ph-	0.48	180
50	2-F-4-CF_3−Ph-	3.16	124
51	3-Py-	> 10	
52	3-(6-MeO)Py-	6.03	66
53	3-(6-CF_3)Py-	6.76	144
54	4-(2-Me)-triazolyl-	> 10	
55	2-benzimidazolyl-	> 10	
56	2-benzothiazolyl-	5.62	79
57	$PhCH_2CH_2$-	3.71	117
58	$PhOCH_2$-	3.98	87
59	3-Cl-$PhOCH_2$-	3.16	124
60	4-Cl-$PhOCH_2$-	1.91	115
61	*n*-propyl-	7.24	53
62	*n*-butyl-	9.28	122
63	*i*-butyl-	0.54	75
64	*s*-butyl-	12.88	97
65	$CF_3CH_2CH_2$-	8.71	97
66	cyclopentyl-	9.33	122
67	cyclohexyl-	0.43	120
68	cyclohexylmethyl-	1.08	161

[a] Values are means of three experiments.

good overall, and the only interaction greater than 50% effect at a 10 μM concentration was with the norepinephrine transporter.

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Table 3. Metabolic Stability in Human Liver Microsomes (HLM) of Some Representative Compounds

compound	HLM[a] (%)
7	70
8	100
12	24
13	35
15	64
16	83
18	82
20	100
22	87
30	59
31	73
45	98

[a] HLM data refer to the percentage of compound metabolized after 15 min at 5 μM concentration.

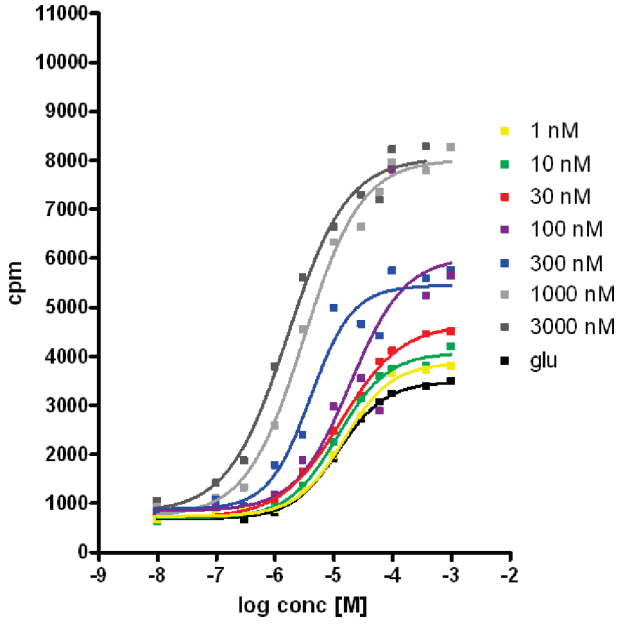

Figure 3. Glutamate (Glu) concentration response curve in presence of varying concentration of compound **8,** demonstrating an approximate 7-fold-shift in Glu EC_{50} at 3 μM concentration of **8** (experiment performed with CHO cells expressing cloned human mGluR2).

Compound **8** was further evaluated *in vivo* in the phencyclidine (PCP)-induced hyperlocomotion model in mice. This model is based on the evidence that the noncompetitive NMDA receptor channel blocker PCP induces schizophrenia-like behavior in humans (*19*). Hence, PCP is widely used in animal models of psychosis. In rodents, PCP causes an increase in motor activity. As this induced behavior may be linked to increased glutamate release, it is speculated that mGlu2 receptor activation may block these specific PCP-mediated effects (*20*). As shown in Figure 4A, no alteration in

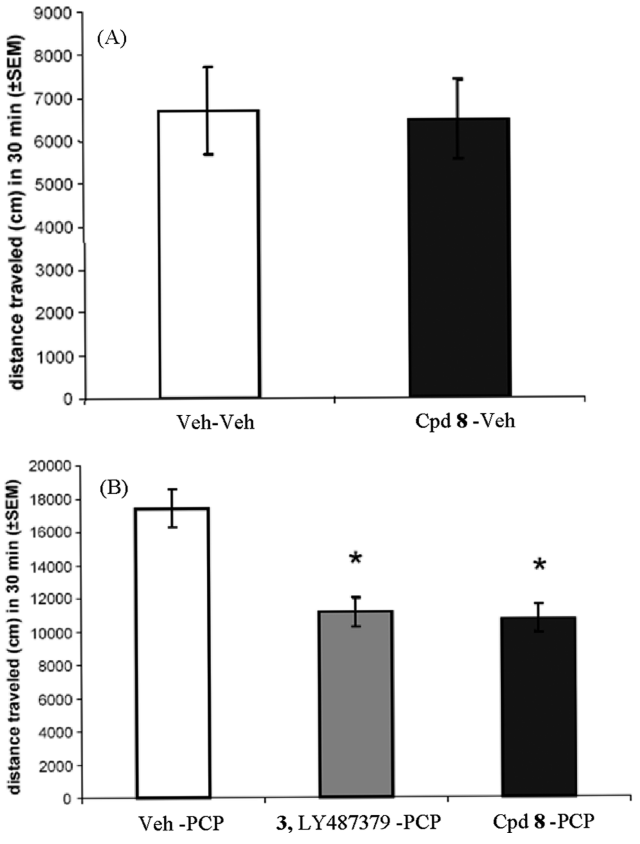

Figure 4. Effect of compound **8** (200 mg/kg ip) on PCP-induced (5 mg/kg sc) locomotor activity in mice. (A) Compound **8** shows no effect on locomotor activity in the absence of PCP. (B) Compound **8** and LY487379 (**3**) both reduce PCP-induced activity (same dose for both compounds). * indicates $p < 0.001$.

locomotor activity was observed in the absence of PCP indicating that compound **8** has no effect on locomotion per se. However, from Figure 4B it can be seen that compound **8** (200 mg/kg intraperitoneal (ip)) significantly attenuated the increase in locomotor activity induced by PCP (5 mg/kg ip). The effect level was comparable to that of the Lilly reference PAM, LY487379, **3**, administered at the same dose and by the same route (*20*). Monitoring of plasma and brain levels of **8** showed concentrations of 3270 ng/mL and 4478 ng/g, respectively, 30 min after dosing, resulting in a brain to plasma ratio of 1.4.

In summary, a novel series of 1,5-disubstituted pyridones with mGluR2 PAM activity have been presented. Systematic SAR studies from the initially available hits **4** and **5** have resulted in compounds with better potency in a GTPγS assay. Although those analogues showed poor *in vitro* metabolic stability in HLM, alternative compounds were identified with reduced metabolic turnover. Compound **8** displayed good brain levels after IP administration and comparable activity to a reference PAM, LY487379, in the *in vivo* PCP induced locomotor activity test. Taken together, these results suggest promise

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for the 1,5-pyridone series. However, the challenge remains to combine potency with metabolic stability and identify compounds suitable for *in vivo* administration by alternative routes. These studies are underway and will be the subject of future reports from our laboratories.

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10. In our in house GTPγS assay, compound **3** displayed an mGluR2 PAM E_{max} of 169%; however, a definitive EC_{50} could not be determined because, prior to reaching a plateau, the GTPγS response gradually decreased, likely due to the solubility limits for the compound.

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14. The effect of these compounds on glutamate-induced [^{35}S]-GTPγS binding induced by 4 μM glutamate (~EC_{20}) was characterized using a CHO cell line expressing the human mGluR2 receptor. For this, membranes prepared from human mGluR2 CHO cells were preincubated with compound alone (for determination of agonist effects) or together with an EC_{20} concentration (f.c. 4 μM) of glutamate (for determination of PAM effects) for 30 min at 30 °C. [^{35}S]GTPγS was added at a final concentration of 0.1 nM and incubated for another 30 min at 30 °C. Final assay mixtures contained 7 μg of membrane protein, and the total reaction volume was 200 μL. Reactions were terminated by rapid filtration through Unifilter-96 GF/B filter plates (Packard, Meriden, CT) using a 96-well Packard filtermate harvester, and filter-bound radioactivity was counted on a Microplate Scintillation and Luminescence Counter from Packard. Z-Primes were ~0.7. Data were calculated as % of the control agonist challenge. Sigmoid concentration−response curves plotting these percentages versus the log concentration of the test compound were analyzed using nonlinear regression analysis.

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17. Compound **8** was tested for agonist or antagonist activity on mGlu receptors in fluorescent Ca^{2+} assays using HEK293 cells expressing human mGluR3, mGluR5, mGluR7, or mGluR8.

18. The CEREP selectivity screen was performed on the following targets: 5HT1A, 5HT2A, 5HT3, 5HT5A, 5HT6, 5HT7, A1, A2A, A3, AT1, Beta1, BK2, CCKA, CCR1, D1, D2, DAT, ETA, GAL2, H1, H2, IL8B, CXCR2, M1, M2, M3, MC4, NET, NK2, NK3, NPY1, NPY2, NT1, OP1, OP3, ORL1, V1A, VIP, SST, 5HT1B, Alpha1, Alpha2, BZD, CaCH, CICH, GABA, KCH, NaCH, and SKCaCH.

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20. Details of the protocol for the *in vivo* assay: Phencyclidine (PCP)-induced hyperlocomotion in mice Male C57BL6/j mice (Charles River, France) were housed 5 per cage and kept under a 12-h light/dark cycle under constant temperature and humidity conditions. All experimental procedures were performed in full compliance with the French and European legislation governing the protection of vertebrate animals used in scientific research. Tested mGluR2 PAM compounds, (cpd **8**, LY487379) were synthesized in house, and phencyclidine (PCP) was purchased from Sigma-Aldrich (France). Nonhabituated animals were treated with the vehicle, or 200 mg/kg ip of either cpd **8** or LY487379, and immediately challenged with either PCP (5.0 mg/kg, ip) or vehicle and individually placed into open-fields for a 30-min period. The distance traveled by animals was measured using video tracking and computerized analysis systems (Viewpoint, France) and the data analyzed using GraphPad Prism (v. 4.01, GraphPad, San Diego, CA, USA).

Note Added after ASAP Publication

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