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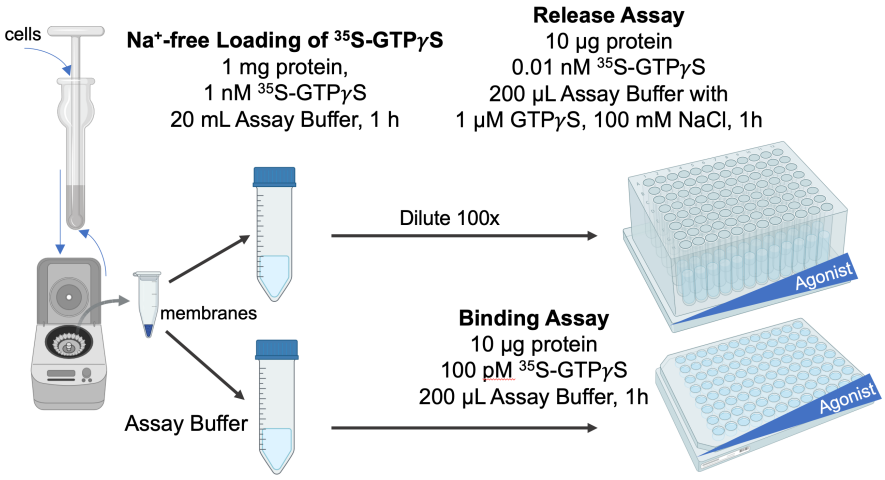
Supplementary information

GTP release-selective agonists prolong opioid analgesic efficacy

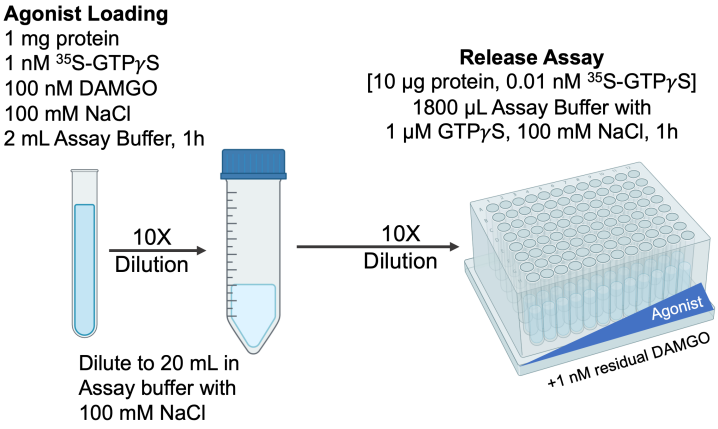
In the format provided by the authors and unedited

Supplemental Information:

Assay methods:

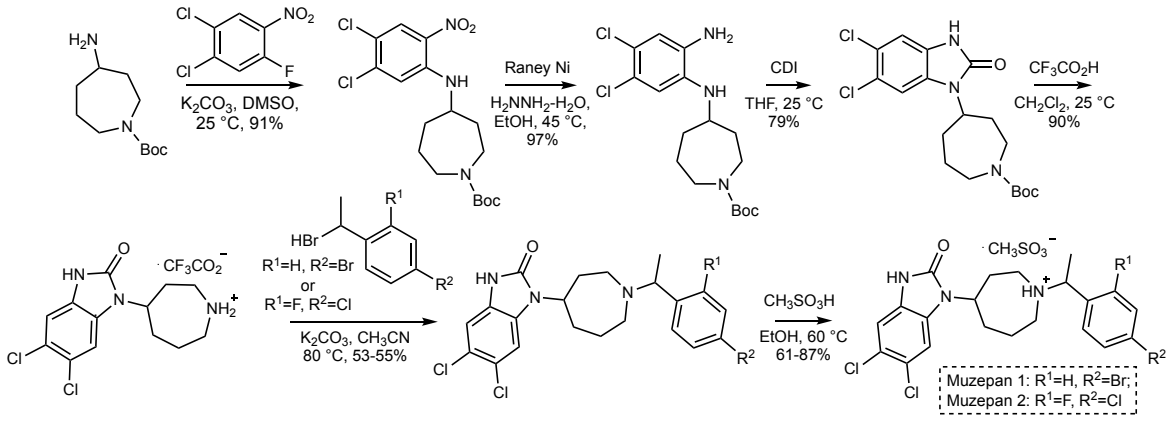


Method Figure 1. Schematic of sodium-free "pulse" loading of ^{35}S-GTPγS for the "chase" release reaction. Images created in BioRender. Bohn, L. (2025) https://BioRender.com/r4cihoy.



Method Figure 2. Schematic of agonist "pulse" loading of ^{35}S-GTPγS for the "chase" release reaction. Images created in BioRender. Bohn, L. (2025) https://BioRender.com/r4cihoy.

Methods for chemical synthesis of Muzepan1 and Muzepan2. The compounds were each prepared in racemic form as a 1:1 mixture of diastereomers in a scheme presented in Supplemental Figure 3.



General Procedures. All reagents, anhydrous solvents, and starting materials were purchased from commercial vendors and used without further purification. All moisture-sensitive reactions were performed under positive pressure argon. Experiments were monitored by LCMS or TLC and visualized using an ultraviolet lamp (254 nm) or by staining with $KMnO_4$. Purification via silica gel flash column chromatography was performed using a Teledyne ISCO Combiflash® Rf+ and Luknova silica gel cartridges. All NMR data was collected at room temperature on a Brüker Ultrashield 400 MHz instrument. Chemical shifts for ^1H NMR spectra are reported in parts per million (ppm) relative to residual solvent signal as an internal standard: DMSO (δ 2.50), $CHCl_3$ (δ 7.26), or MeOH (δ 3.31). Multiplicities are given as: s (singlet), d (doublet), t (triplet), q (quartet), or m (multiplet). Coupling constants are reported as a *J* value in Hertz (Hz). Mass spectra were recorded on a Thermo Scientific 3000 LCQ Fleet system (ESI) using a Discovery® HS C18 HPLC column (10 cm x 2.1 mm, 5 *μ*m) at 35 °C with UV detection at 254 nm. Flow rate was 0.7 mL/min using a solvent gradient of 5-95% B over 4 min (total run time = 6 min), where A = 0.1% formic acid in de-ionized H_2O and B = 0.1% formic acid in CH_3CN. Analytical HPLC was performed on an Agilent 1200 series using an Agilent Eclipse XDB-C18 HPLC column (4.6 x 150 mm, 5 μm) at

40 °C with UV detection at 254 nm. Flow rate was 3.0 mL/min using gradient elution starting with 98:2 (%A/B), where A = 0.1% TFA in de-ionized H_2O and B = 0.1% TFA in CH_3CN. After 1 min, solvent B was increased to 5% and then linearly to 95% B over an additional 5 minutes (total run time = 8 min). The purity of all compounds used in the bioassays was determined to be ≥ 95% by analytical HPLC.

Muzepan1:

1-(1-(1-(4-bromophenyl)ethyl)azepan-4-yl)-5,6-dichloro-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one, mesylate salt.

Step 1. This nucleophilic aromatic substitution step used a variation of a published procedure[35,36]. 1,2-Dichloro-4-fluoro-5-nitrobenzene (0.31 mL, 2.4 mmol) was added to a mixture of *tert*-butyl 4-aminoazepane-1-carboxylate (500 mg, 2.3 mmol) and K_2CO_3 (500 mg, 3.6 mmol) in DMSO (5 mL). The reaction mixture was stirred at room temperature overnight. Upon completion, water was added and the organic layer extracted with EtOAc. The combined organic layers were dried over sodium sulfate and concentrated under reduced pressure. Purification was achieved by flash column chromatography on silica gel using a gradient of EtOAc : hexanes as the eluent. Fractions containing the desired product were pooled and concentrated under reduced pressure to give the product *tert*-butyl 4-((4,5-dichloro-2-nitrophenyl)amino)azepane-1-carboxylate as an orange oil (860 mg, 91% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.28 (s, 1H), 8.06 (d, J = 7.2 Hz, 1H), 6.88 (s, 1H), 3.57 (dd, J = 9.2, 4.0 Hz, 1H), 3.43-3.35 (m, 2H), 2.21-2.14 (m, 1H), 2.01-1.88 (m, 2H), 1.79-1.64 (m, 4H), 1.48 (s, 9H).

Step 2[35]. The product of step 1 (860 mg, 2.1 mmol) was dissolved in ethanol (45 mL) and a 50% aqueous suspension of excess Raney nickel (6 mL) was added. Hydrazine hydrate (0.66 mL, 21 mmol) was then added dropwise to the stirred mixture over 10 min. The mixture was heated to 45°C and maintained at that temperature for 15 min. The mixture was filtered through a pad of

Celite® which was washed with methanol. The solvent was removed under reduced pressure. Purification was achieved by flash column chromatography on silica gel using a gradient of EtOAc : hexanes as the eluent. Fractions containing the desired product were pooled and concentrated under reduced pressure to give the product *tert*-butyl 4-((2-amino-4,5-dichlorophenyl)amino)azepane-1-carboxylate as purple oil (771 mg, 97% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.74 (d, *J* = 5.6 Hz, 1H), 6.56 (s, 1H), 3.55-3.20 (m, 5H), 2.13-1.49 (m, 6H), 1.46 (s, 9H); MS(*m/z*): [M + H] calc'd for $C_{17}H_{26}Cl_2N_3O_2$ is 374.13, found 373.97.

Step 3[35]**.** The product of step 2 (771 mg, 2.1 mmol) was dissolved in THF (21 mL) under argon and CDI, aka 1,1'-carbonyldiimidazole (1 g, 6.2 mmol), was added in one portion. The solution was stirred at room temperature overnight. Upon completion, the solvent was removed under reduced pressure and the residue was dissolved in EtOAc. This reaction mixture was washed with 10% $HCl_{(aq)}$, dried over sodium sulfate, and the solvent was removed under reduced pressure. Purification was achieved by flash column chromatography on silica gel using a gradient of EtOAc : hexanes as the eluent. Fractions containing the desired product were pooled and concentrated under reduced pressure to give the product *tert*-butyl 4-(5,6-dichloro-2-oxo-2,3-dihydro-1*H*-benzo[*d*]imidazol-1-yl)azepane-1-carboxylate as a light-yellow powder (652 mg, 79% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.59 (s, 1H), 7.20 (s, 1H), 7.12 (s, 1H), 4.36 (m, 1H), 3.76-3.49 (m, 3H), 3.26-3.24 (m, 1H), 2.29-2.20 (m, 2H), 2.04-1.75 (m, 4H), 1.53 (s, 9H).

Step 4[35]**.** The product of step 3 (652 mg, 1.6 mmol) was dissolved in a 33% solution of trifluoroacetic acid in dichloromethane (6 mL). The reaction mixture was stirred at room temperature and upon complete conversion the solvent was removed under reduced pressure. The residue was dissolved in a minimal amount of water-CH_3CN (1:1). The mixture was frozen and then was subjected to lyophilization overnight, giving the product 1-(azepan-4-yl)-5,6-

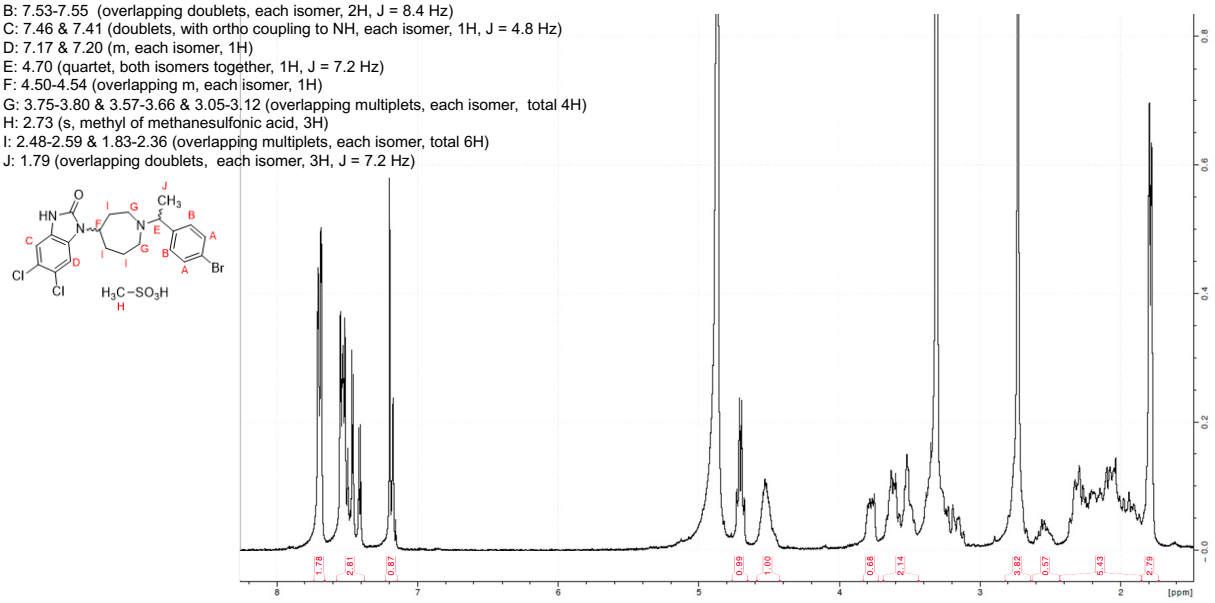
dichloro-1,3-dihydro-2*H*-benzo[*d*]imidazole-2-one a trifluoroacetic acid salt as a light pink solid (609 mg, 90% yield). This material was taken to step 5 without further purification. ^{1}H NMR (400 MHz, CD_3OD) δ 7.47 (s, 1H), 7.20 (s, 1H), 4.50 (tt, *J* = 9.8, 4.6 Hz, 1H), 3.54 (ddd, *J* = 14.2, 6.8, 2.6 H, 1H), 3.42-3.26 (m, 3H), 2.71-2.61 (m, 1H), 2.43-2.34 (m, 1H), 2.25-2.06 (m, 3H), 1.99-1.90 (m, 1H); MS(*m/z*): [M + H] calc'd for $C_{13}H_{16}Cl_2N_3O$ is 300.06, found 300.22.

Step 5. 1-bromo-4-(1-bromoethyl)benzene was prepared by a variation of a published procedure[37] involving the addition of CBr_4 (1.1 g, 3.0 mmol) to an anhydrous dichloromethane (10 mL) solution of 1-(4-bromophenyl)ethan-1-ol (640 mg, 3.2 mmol) and PPh_3 (1.3 g, 5.0 mmol). The mixture was stirred at room temperature overnight. Purification was achieved by flash column chromatography on silica gel using hexanes as the eluent. Fractions containing the desired product were pooled and concentrated under reduced pressure to give the product 1-bromo-4-(1-bromoethyl)benzene as an orange oil. To a solution of 1-bromo-4-(1-bromoethyl)benzene (300 mg, 1.1 mmol) in anhydrous CH_3CN (12 mL) was added the product of step 4 (513 mg, 1.2 mmol) and K_2CO_3 (205 mg, 1.4 mmol). The solution was stirred at 80°C overnight, after which time the solvent was removed under reduced pressure. Purification was achieved by flash column chromatography on silica gel using a gradient of EtOAc : dichloromethane as the eluent. Fractions containing the desired product were pooled and concentrated under reduced pressure to give the product 1-(1-(1-(4-bromophenyl)ethyl)azepan-4-yl)-5,6-dichloro-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one as a white powder (290 mg, 53% yield), which was then suspended in absolute ethanol. Methane sulfonic acid (39 µL, 0.60 mmol) was added and the mixture was heated to 60 ^{o}C for 30 min. The solvent was then removed under reduced pressure and the residue was dissolved in a minimal amount of water-acetonitrile (1:1). The mixture was frozen and then was subjected to lyophilization overnight, giving the final product in the form of a mesylate salt. ^{1}H NMR (400 MHz, CD_3OD) δ 7.70 (dd, *J* = 8.4, 2.0 Hz, 2H), 7.55-7.50 (m, 2H), 7.43 (dd, *J*

= 20.4, 4.8 Hz, 1H), 7.20-7.17 (m, 1H), 4.71 (q, *J* = 6.8 Hz, 1H), 4.55-4.49 (m, 1H), 3.64-3.47 (m, 2H), 3.38-3.12 (m, 2H), 2.80-2.67 (m, 4H), 2.36-1.87 (m, 5H), 1.80 (dd, *J* = 7.2, 2.0 Hz, 3H); MS(*m/z*): [M + H] calc'd for $C_{21}H_{23}BrCl_2N_3O$ is 484.03, found 483.62; HPLC t_R = 5.044 min.

Muzepan1 ^{1}H NMR spectra

A: 7.68-7.71 (overlapping doublets, each isomer, 2H, J = 8.4 Hz)
B: 7.53-7.55 (overlapping doublets, each isomer, 2H, J = 8.4 Hz)
C: 7.46 & 7.41 (doublets, with ortho coupling to NH, each isomer, 1H, J = 4.8 Hz)
D: 7.17 & 7.20 (m, each isomer, 1H)
E: 4.70 (quartet, both isomers together, 1H, J = 7.2 Hz)
F: 4.50-4.54 (overlapping m, each isomer, 1H)
G: 3.75-3.80 & 3.57-3.66 & 3.05-3.12 (overlapping multiplets, each isomer, total 4H)
H: 2.73 (s, methyl of methanesulfonic acid, 3H)
I: 2.48-2.59 & 1.83-2.36 (overlapping multiplets, each isomer, total 6H)
J: 1.79 (overlapping doublets, each isomer, 3H, J = 7.2 Hz)

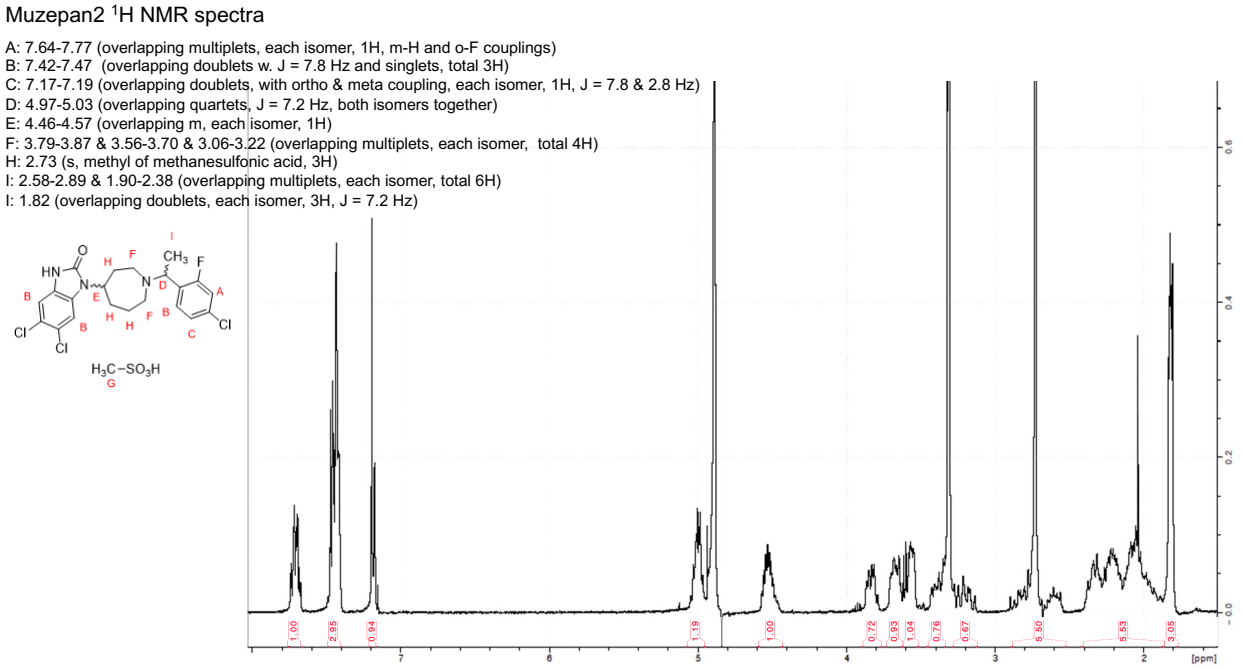


Muzepan2:

1-(1-(1-(4-chloro-2-fluorophenyl)ethyl)azepan-4-yl)-5,6-dichloro-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one, mesylate salt

Following the same protocol used for the synthesis of Muzepan1 but using 1-(1-bromoethyl)-4-chloro-2-fluorobenzene[38] as the alkylating agent in step 5 (prepared as in the synthesis of Muzepan1 from 1-(4-chloro-2-fluorophenyl)ethan-1-ol using CBr_4 and Ph_3P), the title compound was prepared as a mesylate salt in 21% overall yield. ^{1}H NMR (400 MHz, CD_3OD) δ 7.72-7.68 (m, 1H), 7.46-7.42 (m, 3H), 7.19 (d, *J* = 2.8 Hz, 1H), 5.02-4.97 (m, 1H), 4.56-4.50 (m, 1H), 3.79-3.87 (m, 1H), 3.68-3.56 (m, 2H), 3.41-3.19 (m, 2H), 2.71 (s, 3H), 2.34-1.99 (m, 5H), 1.81 (dd, *J*

=6.8, 2.4 Hz, 3H); MS(*m/z*): [M + H] calc'd for $C_{21}H_{22}Cl_3FN_3O$ is 456.07, found 457.56; HPLC t_R = 5.430 min.



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