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Exploration of Synthetic Approaches and Pharmacological Evaluation of PNU-69176E and Its Stereoisomer as 5-HT$_{2C}$ Receptor Allosteric Modulators

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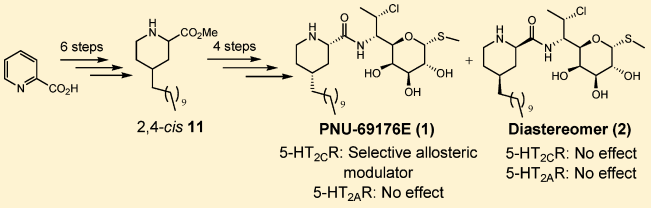
Supporting Information

ABSTRACT: Allosteric modulators of the serotonin (5-HT) 5-HT$_{2C}$ receptor (5-HT$_{2C}$R) present a unique drug design strategy to augment the response to endogenous 5-HT in a site- and event-specific manner with great potential as novel central nervous system probes and therapeutics. To date, PNU-69176E is the only reported selective positive allosteric modulator for the 5-HT$_{2C}$R. For the first time, an optimized synthetic route to readily access PNU-69176E (**1**) and its diastereomer **2** has been established in moderate to good overall yields over 10 steps starting from commercially available picolinic acid. This synthetic approach not only enables a feasible preparation of a sufficient amount of **1** for use as a reference compound for secondary pharmacological studies, but also provides an efficient synthesis of key intermediates to develop novel and simplified 5-HT$_{2C}$R allosteric modulators. Compound **1** and its diastereomer **2** were functionally characterized in Chinese hamster ovary (CHO) cells stably transfected with the 5-HT$_{2C}$R using an intracellular calcium (Ca_i^{2+}) release assay. Compound **1** demonstrated efficacy and potency as an allosteric modulator for the 5-HT$_{2C}$R with no intrinsic agonist activity. Compound **1** did not alter 5-HT-evoked Ca_i^{2+} in CHO cells stably transfected with the highly homologous 5-HT$_{2A}$R. In contrast, the diastereomer **2** did not alter 5-HT-evoked Ca_i^{2+} release in 5-HT$_{2A}$R-CHO or 5-HT$_{2C}$R-CHO cells or exhibit intrinsic agonist activity.



KEYWORDS: *PNU-69176E, diastereomer, synthesis, allosteric modulator, 5-HT$_{2C}$ receptor*

The serotonin (5-HT) 2C receptor (5-HT$_{2C}$R) is implicated in a diversity of physiological functions, such as nociception, motor behavior, endocrine secretion, thermoregulation, appetite modulation, and the control of exchanges between the central nervous system (CNS) and the cerebrospinal fluid.[1] This receptor has also been implicated in numerous psychiatric pathologies, and the modulation of 5-HT$_{2C}$R function holds a tremendous amount of therapeutic promise for the treatment of diseases of significant unmet medical need, including addiction, anxiety, depression, obesity/eating disorders, Parkinson's disease, and schizophrenia.[1b,d,2] Successful development of 5-HT$_{2C}$R ligands requires selectivity versus the highly homologous 5-HT$_{2A}$R and 5-HT$_{2B}$R, as 5-HT$_{2A/2B}$R agonists can result in significant CNS (5-HT$_{2A}$R) and cardiovascular (5-HT$_{2B}$R) adverse effects.[3] Allosteric modulators of 5-HT$_{2C}$R present a novel and attractive drug design strategy to augment the response to endogenous 5-HT and to achieve high receptor subtype selectivity and specificity with ligand binding to an allosteric site rather than to the orthosteric binding site that binds the endogenous agonist.[4] PNU-69176E [(2*S*,4*R*)-*N*-((1*S*,2*S*)-2-chloro-1-((2*R*,3*R*,4*S*,5*R*,6*R*)-3,4,5-trihydroxy-6-(methylthio)tetrahydro-2*H*-pyran-2-yl)propyl)-4-undecylpiperidine-2-carboxamide (**1**); Figure 1)] is the only reported 5-HT$_{2C}$R selective positive allosteric modulator, which was identified via screening of a chemical library of Pharmacia (now Pfizer).[5] Structurally, **1** consists of two moieties, a piperidinyl ring with a long alkyl chain (undecyl) and a polar moiety including the α-D-galactopyranoside, and can be viewed as an analogue of pirlimycin with a long alkyl chain (Figure 1). Although the preparation of pirlimycin and its analogues is available in the literature,[6] to our knowledge neither the synthesis nor the chemical characterization of **1** and its diastereomer **2** have been reported.

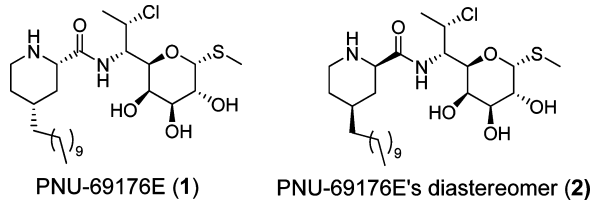


Figure 1. Structures of PNU-69176E (**1**) and its diastereomer (**2**).

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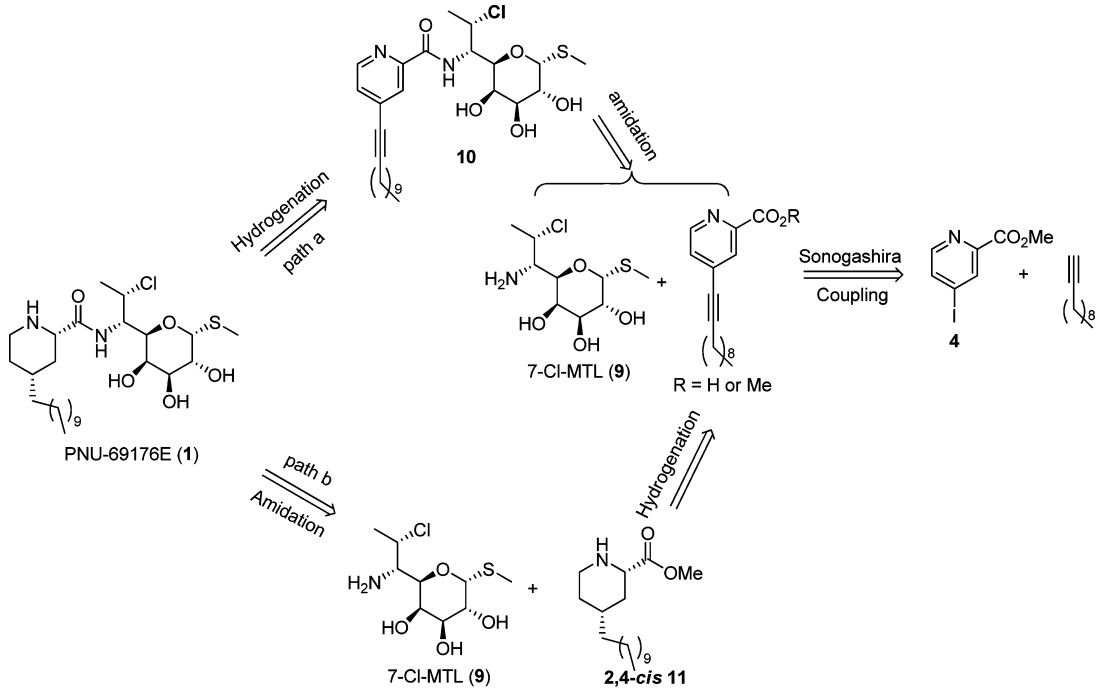
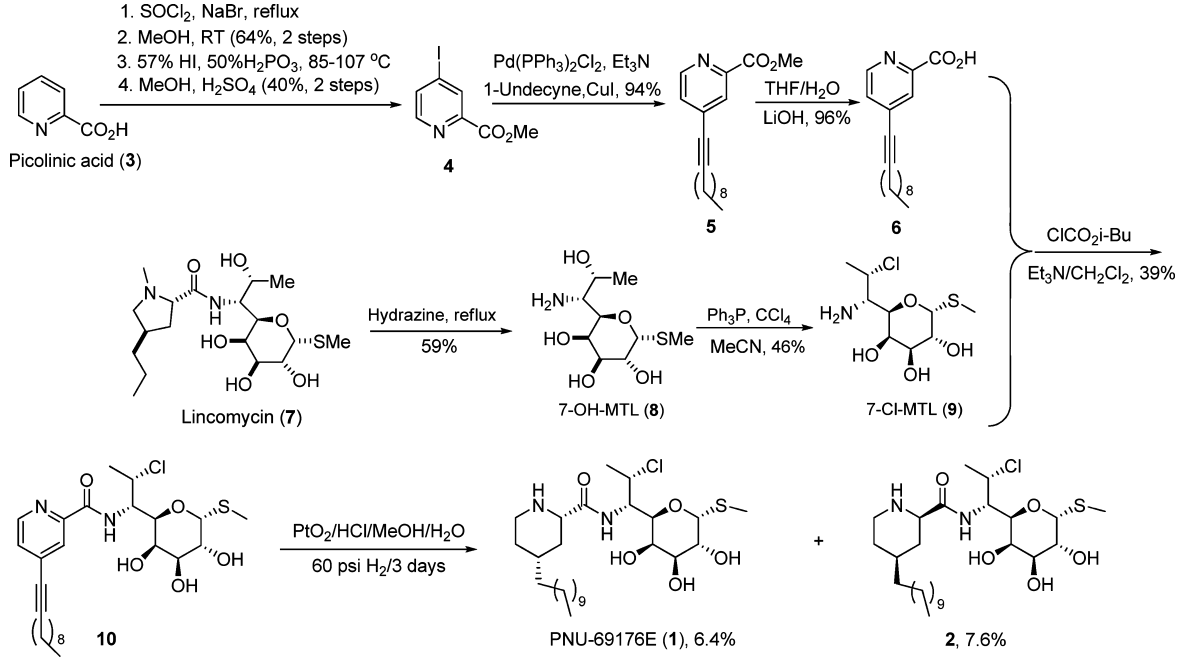


Figure 2. Retrosynthetic analysis of PNU-69176E (**1**). Path a and path b are outlined in more detail in Schemes 1 and 2, respectively.

Scheme 1. Synthesis of PNU-69176E (1) and Its Diastereomer (2) Based on Path a



The goal of our drug discovery efforts is to identify novel, positive allosteric modulators of the 5-HT_{2C}R with high potency, specificity, and druglike properties based on **1**. Thus, it is imperative to establish a practical method to readily access **1**, a fairly complex molecule, on a large scale as a reference compound as well as to evaluate pharmacological and biological properties of the parent molecule and its derivatives. In the current report, we investigated two synthetic routes and established a feasible method to readily access **1** and its diastereomer **2** for the first time. Meanwhile, we have evaluated and compared the allosteric modulation of **1** and its diastereomer **2** employing an intracellular calcium (Ca_i^{2+}) release assay in two cell lines stably transfected with either the 5-HT_{2C}R or 5-HT_{2A}R. The exploration of the synthetic approaches elucidates an efficient synthesis of key building blocks which can be used to develop novel and simplified 5-HT_{2C}R positive allosteric modulators.

RESULTS AND DISCUSSION

The retrosynthetic analysis of **1** is outlined in Figure 2, showing the two possible approaches to achieve this objective. Both path a and path b included Sonogashira coupling as one of the key steps to introduce the long alkyl chain. In path a, amidation of pyridine acid with methyl (7*S*)-7-chloro-7-deoxythiolincosa-

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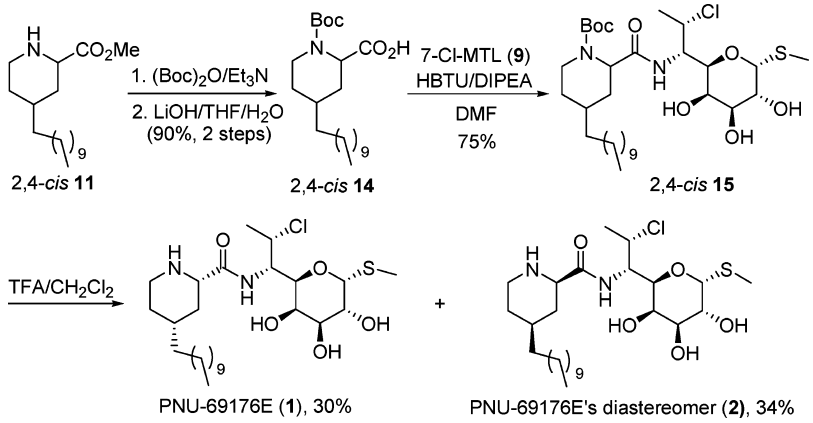
Scheme 2. Synthesis of PNU-69176E (1) and Its Diastereomer (2) Based on Path b



mide (7-Cl-MTL) occurred prior to hydrogenation of pyridyl and alkynyl moieties, while in path b hydrogenation of pyridyl and alkynyl moieties was carried out prior to amidation of piperidine acid with 7-Cl-MTL.

Initially, we attempted path a (Scheme 1) to synthesize **1**, because of the similarity of this approach to the synthesis of pirlimycin analogues.[6] Starting from the commercially available and inexpensive picolinic acid (**3**), methyl 4-iodopicolinate (**4**) was prepared in a four-step sequence that included chlorination, methyl esterification, iodination, and another methyl esterification according to literature procedures.[7] Methyl 4-iodopicolinate (**4**) was then subjected to Pd-catalyzed Sonogashira coupling reaction with undec-1-yne followed by ester hydrolysis to afford 4-(undec-1-ynyl)picolinic acid (**6**) as a key intermediate. Hydrazinolysis of lincomycin (**7**) using hydrazine yielded methyl (7*R*)-7-hydroxylthiolincosamide (7-OH-MTL, **8**), which was further chlorinated in the presence of CCl_4/PPh_3 to give 7-Cl-MTL (**9**).[8] Following a procedure similar to that reported by Birkenmeyer et al.,[6] the picolinic acid derivative **6** was reacted with isobutyl chloroformate to give a mixed anhydride, which was further coupled with 7-Cl-MTL (**9**) to afford 4-(undec-1-ynyl)-2-pyridinecarboxamide (**10**) in 39% yield.

Hydrogenation of alkynyl and pyridyl moieties in the unsaturated amide **10** is crucial to the preparation of **1**. The hydrogenation of 4-ethyl-2-pyridinecarboxamide on a Parr hydrogenator at 50 psi in the presence of 3.7 equiv of PtO_2 was reported to result in pirlimycin and its isomer.[6] Because of the similarity of the hydrogenation substrates, we initially utilized the same conditions to prepare **1** and its isomer **2**. However, no reductive product was detectable on silica gel TLC even after 18 h of reaction time. Therefore, we increased hydrogen pressure, amount of PtO_2, and reaction time (60 psi, 7 equiv of PtO_2, and 120 h, respectively), leading to a mixture of two isomeric products, which have substantially different mobilities upon silica gel TLC. After the purification with silica gel column, two diastereomers of PNU-69176E were obtained as a colorless gel in yields of 7.6% and 6.4%, respectively. Given the similarity in the chemical structures of PNU-69176E and pirlimycin, and because our synthetic approach followed that of the literature,[6] we assigned the stereochemistry of these two products analogously to that of pirlimycin and its diastereomer; these assignments were confirmed by X-ray analysis.[6] The configuration of the more polar isomer was assigned as *cis*-2*S*,4*R* and named PNU-69176E (**1**), while the less polar product was assigned as *cis*-2*R*,4*S*, the diastereomer **2** of PNU-69176E. The lipophilicity of undecyl long alkyl chain is problematic to the growth of single crystals from either PNU-69176E or its diastereomer to unambiguously confirm their configurations.

In summary, following synthetic path a, we succeeded in preparation of **1** and **2** through eight steps in 0.6% and 0.7% overall yields, respectively, starting from picolinic acid. Nevertheless, the low overall yields hampered us from achieving sufficient amounts of **1** and **2** for further pharmacological evaluation. We presumed that the low yields and harsh reaction conditions of the hydrogenation were ascribed to inactivation of the catalyst PtO_2, which was poisoned by the sulfur in the 7-Cl-MTL moiety. Therefore, to establish a more practical synthesis of **1**, we decided to explore an alternative synthetic route via path b in which the hydrogenation reaction proceeds prior to the coupling with 7-Cl-MTL to avoid inactivation of the catalyst by sulfur.

The synthesis of PNU-69176E based on path b (Scheme 2) was initiated by hydrogenation of the intermediate picolinate **5** prepared in path a. Since the hydrogenation of 4-alkynyl substituted picolinate has been reported,[9] we first followed the published method to attempt this hydrogenation, which was carried out on a Parr hydrogenator at 50 psi in the presence of 0.4 equiv of PtO_2 and 1 equiv of HOAc. Based upon our findings, the reduction reaction was still incomplete after 12 h, and the reductive product was a complex reaction mixture of several components. After purification with silica gel column, the desired amino carboxylate **11** product was obtained in 25% yield. We also isolated the incompletely hydrogenated product **13** and the excessively hydrogenated product **12** in 35% and 15% yields, respectively (Table 1, entry 1). Extending the reaction time from 12 to 48 h led to **12** as a sole product in 90% yield (Table 1, entry 2). Because the effect of Brønsted acids on the reduction of pyridine may vary, several Brønsted acids were screened to optimize the hydrogenation conditions to avoid the excess hydrogenation of the carboxyl group. In the case of formic acid, no excessively hydrogenated product **12** was detected on silica gel TLC, but the reaction was still incomplete after a reaction time of 24 h under 60 psi of hydrogen (Table 1, entry 3). The strong protonic acid HCl was also tested in this hydrogenation reaction. To our delight, the reaction was complete after 24 h and the desired product **11** was obtained as the sole product in nearly quantitative yield (Table 1, entry 4); it is of note that no excessively hydrogenated product **12** was detected even when the reaction time was prolonged to 36 h (Table 1, entry 5). When H_2SO_4

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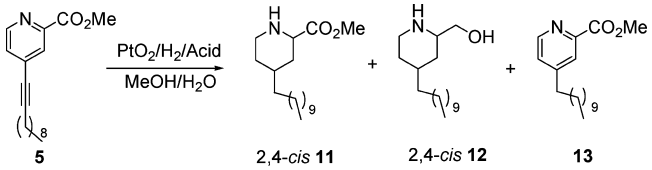
Table 1. Optimization of the Hydrogenation Conditions of Picolinate 5[a]



entry	Brønsted acid	H_2 (psi)	time (h)	products (yield[b])
1	HOAc	50	12	**11** (25%), **12** (15%), **13** (35%)
2	HOAc	50	48	**12** (90%)
3	HCOOH	60	24	**11** (38%), **13** (45%)
4	HCl	60	24	**11** (97%)
5	HCl	60	36	**11** (97%)
6	H_2SO_4	60	24	**11** (80%)
7	H_3PO_4	60	24	**11** (75%)

[a]A mixture of **5** (1.74 mmol, 1 equiv), PtO_2 (0.4 equiv), acid (1 equiv) in MeOH (9 mL) and H_2O (6 mL) was shaken under H_2 on a Parr hydrogenator. [b]Isolated yield.

and H_3PO_4 were used, the reactions were complete but in slightly lower yields (Table 1, entries 6 and 7). Therefore, optimized conditions to prepare the desired key intermediate **11** were achieved by adopting HCl. The stereochemistry of 2,4-disubstituted piperidinyl carboxylate **11** was examined by ^{1}H NMR decoupling and nuclear Overhauser effect (NOE) analysis. The carboxylate **11** displayed a coupling of 11.4 Hz between H-2 and H-3_{ax}; the resonances of H-4 and H-3_{ax} were overlapped and cannot be specifically assigned; however, there was NOE observed from H-2 and H-4 (Figure 3, see the

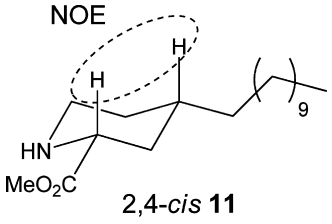


Figure 3. Configuration determination of **11** by NOE analysis.

Supporting Information). These data indicated that the *cis*-configuration was present in **11** and that all axial orientations were occupied by H-2 and H-4. This result was in full agreement with observations on a similar substrate reported by Shuman et al.[10] The carboxylate **11** generated by this synthetic protocol was racemic (DL-*cis*) due to the nonstereoselective nature of these hydrogenation conditions. It was expected that the coupling of **11** with 7-Cl-MTL would result in a pair of diastereomers, which were confirmed to be separable by silica gel column in later steps.

With the amino carboxylate **11** in hand, Boc-protection of the free amino functionality followed by hydrolysis of the methyl ester afforded the *N*-Boc acid **14** in 90% yield, which was further coupled with 7-Cl-MTL in the presence of HTBU and DIPEA to give the amide **15** in 75% yield. ^{1}H NMR displayed that the amide **15** was a mixture of two diastereomers, which were not separable on silica gel TLC. Finally, the removal of the Boc group from the amide **15** using TFA in dichloromethane resulted in **1** and its diastereomer **2**, which were separable on silica gel TLC. After purification with silica gel column, PNU-69176E and its diastereomer **2** were obtained in yields of 30% and 34%, respectively (Scheme 2). We have also fully characterized these two products produced from path b, and the spectroscopic data were in complete agreement with those obtained from path a. Therefore, using the synthetic route based on path b, **1** and its diastereomer **2** were successfully synthesized through 10 steps in 4.6% and 5.2% overall yields, respectively, starting from picolinic acid. Taken together, a substantially improved and more efficient synthetic route to readily access PNU-69176E and its diastereomer **2** has been established.

The best-characterized intracellular signaling pathway of the 5-HT_{2C}R is the activation of phospholipase C (PLCβ) via $G_{\alpha q/11}$ proteins and the production of diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3), leading to increased Ca_i^{2+} release from intracellular stores.[11] Functional characterization of our synthetic PNU-69176E and its diastereomer **2** was determined by utilizing an Ca_i^{2+} release assay in live cells in which Ca_i^{2+} levels can be regarded as an outcome measure of activation of the 5-HT_{2C}R signaling pathway.[12] Biological analyses conducted in Chinese hamster ovary cells (CHO) stably expressing physiological levels of the human 5-HT_{2C}R (5-HT_{2C}R-CHO)[13] showed that compound **1** potentiated the Ca_i^{2+} release induced by 0.3 nM 5-HT (~5-HT EC_{20}) from 23.9% of a maximal 5-HT-induced Ca_i^{2+} release (5-HT_{max}; determined at 1 μM 5-HT) to 48.5% of 5-HT_{max} [$F_{(10,51)}$ = 9.01, $p < 0.01$; Figure 4]. A priori comparisons using Dunnett's

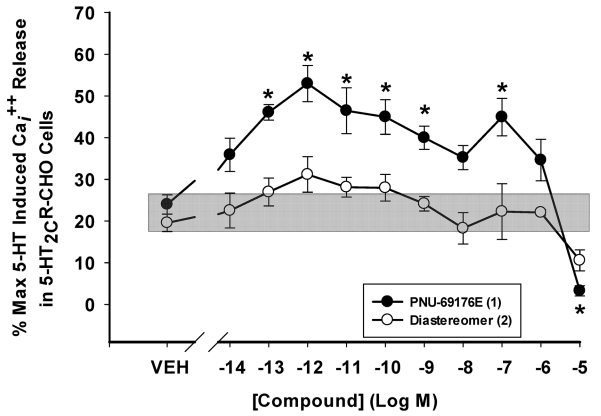


Figure 4. In vitro Ca_i^{2+} release assay in live 5-HT_{2C}R-CHO cells. PNU-69176E (**1**; ●) potentiated 5-HT (0.3 nM)-induced Ca_i^{2+} release in 5-HT_{2C}R-CHO cells, while its diastereomer (**2**; ○) had no effect. Data represent mean ± SEM of four wells per concentration over at least three independent experiments and are expressed as % 5-HT_{max} Ca_i^{2+} response determined at 1 μM 5-HT. *$p < 0.05$ versus vehicle (VEH). Shaded area indicates the range of VEH response.

procedure revealed that compound **1** significantly enhanced Ca_i^{2+} release above that of 0.3 nM 5-HT alone at concentrations in the range of 10^{-13}–10^{-7} M and reduced Ca_i^{2+} release at the highest concentration utilized (10^{-5} M) ($p < 0.05$). In contrast, the diastereomer **2** did not alter Ca_i^{2+} release evoked by 0.3 nM 5-HT [$F_{(9,32)}$ = 2.04, n.s.; Figure 4]. Neither compound **1** [$F_{(10,68)}$ = 0.81, n.s.] nor the diastereomer **2** [$F_{(10,34)}$ = 0.76, n.s.] in concentrations up to 10^{-5} M induced Ca_i^{2+} release in the 5-HT_{2C}R-CHO cells in the absence of 5-HT (Figure 5). In addition, 1 nM of compound **1** enhanced the Ca_i^{2+} response at low concentrations of 5-HT [10^{-11}–3×10^{-10} M; $F_{(15,55)}$ = 16.73, $p < 0.01$; Figure 6]. This profile for compounds **1** and **2** in 5-HT_{2C}R-CHO cells was distinguished

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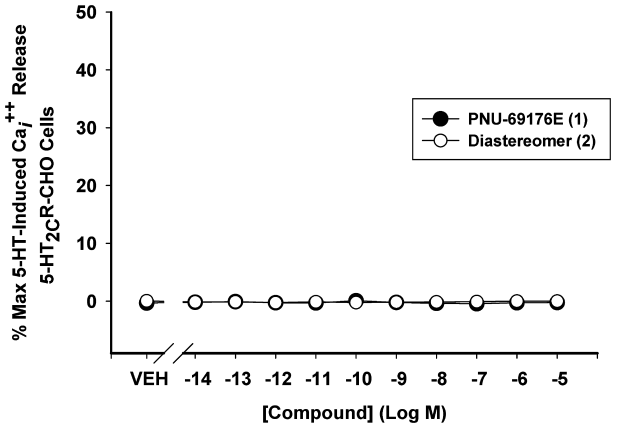

Figure 5. In vitro Ca_i^{2+} release assay in live 5-HT$_{2C}$R-CHO cells. In the absence of 5-HT, neither PNU-69176E (**1**; ●) nor its diastereomer (**2**; ○) affected Ca_i^{2+} release in 5-HT$_{2C}$R-CHO cells. Data represent mean ± SEM of four wells per concentration over at least three independent experiments and are expressed as % 5-HT$_{max}$ Ca_i^{2+} response determined at 1 μM 5-HT.

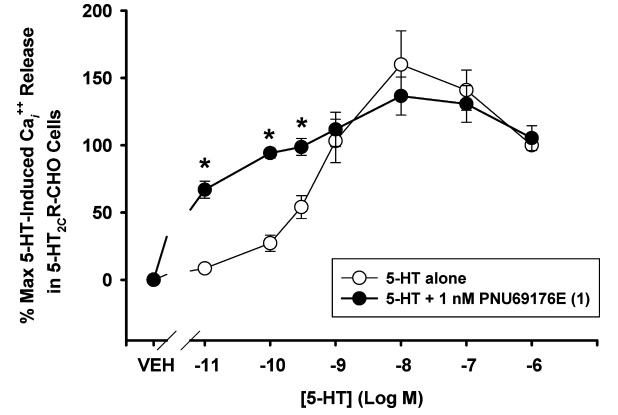

Figure 6. In vitro Ca_i^{2+} release assay in live 5-HT$_{2C}$R-CHO cells. PNU-69176E (**1**; ●) (1 nM) enhanced the Ca_i^{2+} release induced by low concentrations of 5-HT (○). Data represent mean ± SEM of four wells per concentration over at least three independent experiments and are expressed as % 5-HT$_{max}$ Ca_i^{2+} response determined at 1 μM 5-HT. *p < 0.05 versus 5-HT alone.

from that seen in 5-HT$_{2A}$R-CHO cells in which neither compound alone or in the presence of 5-HT (compound **1**, $F_{(10,43)}$ = 0.78; compound **2**, $F_{(10,55)}$ = 1.27; Figure 7) altered Ca_i^{2+} release.

Multiple allosteric modulators of G-protein-coupled receptors have been developed and predicted to have robust effects in a variety of CNS disorders. Our preliminary data with the lead compound **1** demonstrate our ability to detect positive, and perhaps negative, allosteric activity (Figure 4) selectively at the 5-HT$_{2C}$R versus the highly homologous 5-HT$_{2A}$R. In our hands, compound **1** produced the anticipated characteristics based upon a previous study[5] which identified positive allosteric modulation by PNU-69176E in the presence of 5-HT at concentrations less than 10 μM and negative allosteric modulation at higher concentrations. These investigators also detected intrinsic activation of GTPγS binding and inositol 1,4,5-triphosphate (IP_3) release/[^{3}H]IP accumulation by PNU-69176E in the absence of 5-HT; in contrast, we did not detect intrinsic agonist activity for compound **1** in the 5-HT$_{2C}$R-induced Ca_i^{2+} release assay (Figure 5). Such differences may be

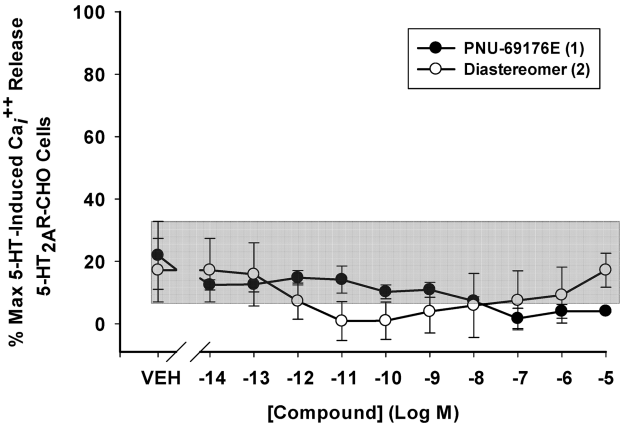

Figure 7. In vitro Ca_i^{2+} release assay in live 5-HT$_{2A}$R-CHO cells. Neither PNU-69176E (**1**; ●) nor its diastereomer (**2**; ○) altered 5-HT-induced Ca_i^{2+} release in 5-HT$_{2A}$R-CHO cells. Data represent mean ± SEM of four wells per concentration over at least three independent experiments and are expressed as % 5-HT$_{max}$ Ca_i^{2+} response determined at 1 μM 5-HT. Shaded area indicates the range of VEH response.

attributable to the choice of expression system and the protein expression level for the 5-HT$_{2C}$R. In the present studies, we employed a stably transfected CHO cell line (~250 fmol/mg protein) which expresses vastly lower levels of the 5-HT$_{2C}$R protein relative to the stably transfected HEK293 cell line (~45 pmol/mg protein) used in the previous report.[5] These technical aspects highlight the nuances that have hampered GPCR allosteric modulator drug discovery in the past, but also present new prospects for preclinical lead discovery.[14]

Compound **1** potently enhanced 5-HT-induced Ca_i^{2+} release in the 5-HT$_{2C}$R-CHO cells with modest efficacy. This disconnect between potency and efficacy has previously been observed for both glutamate and acetylcholine receptor allosteric modulators;[14] this observation may be related to the ability of compound **1** to alter the affinity for the orthosteric ligand as well as saturability of the endogenous orthosteric ligand-induced effect.[14] Limits to the pharmacological effect are achieved when the allosteric site is saturated, allowing the allosteric modulator to fine-tune intracellular signaling activated by the endogenous orthosteric ligand, thereby reducing adverse effects of enhanced and prolonged activation of the 5-HT$_{2C}$R (e.g., receptor desensitization).[14] Thus, site-specific augmentation of the 5-HT$_{2C}$R response offers pharmacological, and perhaps therapeutic, advantages to an orthosteric agonist approach. Further pharmacological studies on **1**, including in-depth characterization of the distinct 5-HT$_{2C}$R-linked intracellular signaling pathways potentiated by **1**, as well as in vivo behavioral and bioavailability analyses are currently underway.

CONCLUSION

In summary, two synthetic routes to synthesize PNU-69176E and its diastereomer **2** starting from the commercially available and inexpensive picolinic acid (**3**) have been explored. Compounds **1** and **2** were successfully achieved over 10 steps in moderate to good overall yields through the optimized synthetic route based on path b with a complete characterization by ^{1}H NMR, ^{13}C NMR, MS and HRMS spectrometry, and optical rotation analysis. The crucial step of hydrogenation catalyzed by PtO_2 was optimized by utilizing HCl as the Brønsted acid, affording the key intermediate piperidyl

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carboxylate **11** in a satisfactory yield. The biological characterization of the synthesized compounds conducted using a Ca_i^{2+} release assay in 5-HTR-CHO cells revealed that **1** demonstrated selectivity for the 5-HT_{2C}R versus the highly homologous 5-HT_{2A}R as a potent allosteric modulator of 5-HT_{2C}R-induced Ca_i^{2+} release with modest efficacy and no intrinsic agonist activity in the absence of 5-HT. In contrast, the diastereomer **2** did not alter 5-HT evoked Ca_i^{2+} release in either 5-HT_{2A}R- or 5-HT_{2C}R-CHO cells nor did it display intrinsic agonist activity. Further pharmacological evaluation of **1**, including in vivo behavioral studies, and development of novel and simplified analogues based on key building blocks such as **11** and the synthetic methodologies established here are in progress. These analogues will be crucial for obtaining meaningful structure−activity relationship (SAR) data, and the findings will be reported in due course.

METHODS

4-Undec-1-ynyl-pyridine-2-carboxylic Acid Methyl Ester (5). Compound **4** (2.77 g, 10.55 mmol, 1 equiv), triphenylphosphine (0.276 g, 1.05 mmol, 0.1 equiv), copper(I) iodide (0.2 g, 1.05 mmol, 0.1 equiv), palladium acetate (0.118 g, 0.53 mmol, 0.05 equiv), and triethylamine (37 mL) were added to a dry flask. The mixture was degassed with nitrogen, and 1-undecyne (4.16 mL, 21.1 mmol, 2.0 equiv) was added. The reaction mixture was stirred at RT for 12 h. The insoluble solid was filtered, the filtrate was concentrated under vacuum, and the dark residue was purified with silica gel chromatography; elution with 1:3 ethyl acetate−hexane provided the desired product **5** as a brown oil (2.85 g, 94%). ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.65 (d, 1H, J = 4.8 Hz), 8.08 (s, 1H), 7.41 (d, 1H, J = 4.2 Hz), 4.00 (s, 3H), 2.44 (t, 2H, J = 7.2 Hz), 1.62 (m, 2H), 1.44 (m, 2H), 1.29 (m, 10H), 0.88 (t, 3H, J = 7.2 Hz).^{13}C NMR (150 MHz, $CDCl_3$): δ 165.4, 149.6, 147.9, 133.8, 128.7, 127.3, 97.7, 77.8, 52.9, 31.8, 29.4, 29.2, 29.1, 28.9, 28.3, 22.7, 19.5, 14.1. MS (-ESI): m/z = 286.1512 $[M - H]^-$

4-Undec-1-ynyl-pyridine-2-carboxylic Acid (6). Lithium hydroxide monohydrate (313 mg, 13.6 mmol, 1.5 equiv) was added to a solution of **5** (2.5 g, 8.7 mmol, 1 equiv) in tetrahydrofuran (THF; 12 mL) and H_2O (3 mL). The reaction mixture was stirred at RT overnight, and TLC indicated that the reaction was incomplete. Another portion of lithium monohydrate (627 mg, 27.2 mmol, 3 equiv) was added into the reaction mixture. The reaction was stirred for another 8 h, and TLC showed the starting material had been completely consumed. The solvent was removed under vacuum to give a solid residue, which was taken up with 5% HCl (10 mL) and extracted with EtOAc (three times). The organic layers were combined, washed with brine, and dried over anhydrous Na_2SO_4. The solvent was evaporated to afford the desired product **6** (2.3 g, 96%) as a white solid; mp 93−94 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ 10.05 (br s, 1H), 8.62 (br s, 1H), 8.25 (br s, 1H), 7.56 (m, 1H), 2.44 (t, 2H, J = 7.2 Hz), 1.63 (m, 2H), 1.45 (m, 2H), 1.30 (m, 10H), 0.88 (t, 3H, J = 7.2 Hz).^{13}C NMR (150 MHz, $CDCl_3$): δ 135.6, 127.0, 99.3, 77.8, 31.8, 29.4, 29.2, 29.0, 28.8, 28.1, 22.6, 19.5, 14.0.

***N*-((1*S*,2*S*)-2-Chloro-1-((2*R*,3*R*,4*S*,5*R*,6*R*)-3,4,5-trihydroxy-6-(methylthio)tetrahydro-2*H*-pyran-2-yl)propyl)-4-(undec-1-ynyl)picolinamide (10).** A solution of **6** (201 mg, 0.73 mmol) and triethylamine (186 mg, 1.84 mmol) dissolved in 10 mL of acetonitrile was cooled to 10 °C, and isobutylchloroformate (100 mg, 0.73 mmol) was added in one portion. The mixture was stirred at 10 °C for 1 h. A separate solution of 7-Cl-MTL (200 mg, 0.73 mmol) dissolved in 3 mL of acetone and 3 mL of H_2O was added into the previous mixture. The reaction mixture was stirred at RT for 18 h, and the solvent was then removed under vacuum to give an oil residue. The residue was purified by silica gel column; elution with 10% MeOH in CH_2Cl_2 afforded the desired amide **10** (110.0 mg, 39%) as a colorless solid; mp 83−84 °C; silica gel TLC R_f = 0.30 (1:15 MeOH/CH_2Cl_2). ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.57 (d, 1H, J = 9.0 Hz), 8.50 (d, 1H, J = 4.8 Hz), 8.15 (s, 1H), 7.39 (d, 1H, J = 4.2 Hz), 5.45 (d, 1H, J = 4.2 Hz), 5.00 (m, 1H), 4.57 (m, 1H), 4.49 (m, 1H), 4.23 (m, 1H), 4.19 (s, 1H), 3.89 (d, 1H, J = 8.4 Hz), 2.66 (br s, 2H), 2.46 (m, 2H), 2.17 (s, 3H), 1.74 (br s, 1H), 1.64 (m, 2H), 1.46 (m, 2H), 1.29 (m, 10H), 1.22 (d, 3H, J = 6.6 Hz), 0.90 (t, 3H, J = 6.0 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ 163.8, 149.3, 148.1, 134.0, 128.1, 124.5, 97.5, 87.6, 78.0, 77.4, 75.6, 71.7, 69.7 (2C), 53.6, 31.8, 29.4, 29.2, 29.0, 28.8, 28.2, 22.6, 19.4, 17.0, 14.0, 13.5. MS (-ESI): m/z = 525.2155 $[M - H]^-$.

Methyl 4-Undecylpiperidine-2-carboxylate (11). Platinum oxide (158.0 mg, 0.69 mmol) was added to a solution of **5** (500 mg, 1.74 mmol) in a mixture of MeOH (9 mL), water (6 mL), and hydrochloric acid (0.144 mL, 1.74 mmol). The reaction mixture was purged and charged with hydrogen (60 psi) for 24 h. The platinum oxide was removed by filtration, and the filtrate was concentrated to give an oily residue. The residue was diluted with CH_2Cl_2 and washed with saturated $NaHCO_3$ aqueous solution. After drying over anhydrous Na_2SO_4, the solvent was removed under vacuum to give a colorless oily residue. The residue was purified with the silica gel column; elution with 1:20 methanol/dichloromethane gave the desired product **11** (500 mg, 97%) as a colorless oil. ^{1}H NMR (600 MHz, $CDCl_3$): δ 3.72 (s, 3H), 3.32 (dd, 1H, J = 11.4 and 1.8 Hz), 3.15 (d, 1H, J = 11.4 Hz), 2.61 (dt, 1H, J = 12.0 and 1.8 Hz), 2.04 (d, 1H, J = 12.6 Hz), 1.65 (d, 1H, J = 13.2 Hz), 1.29 (br s, 1H), 1.26 (s, 20H), 1.03 (q, 2H, J = 12.0 Hz), 0.88 (t, 3H, J = 7.2 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ 173.8, 59.0, 51.7, 45.8, 36.9, 36.1(2C), 32.7, 31.8, 29.7, 29.5 (4C), 29.2, 26.3, 22.6, 14.0. MS (-ESI): m/z = 296.2100 $[M - H]^-$.

(4-Undecyl-piperidin-2-yl)methanol (12). Platinum oxide (318.0 mg, 1.4 mmol) was added to a solution of **5** (1.0 g, 3.5 mmol) in a mixture of MeOH (12 mL), water (12 mL), and acetic acid (0.218 mL, 3.5 mmol). The reaction mixture was purged and charged with hydrogen (50 psi) for 2 days. The platinum oxide was removed by filtration, and the filtrate was concentrated to give an oily residue. The residue was dissolved in methanol and basified with saturated $NaHCO_3$ aqueous solution. The resulting solution was concentrated again under vacuum to give a white solid residue. The residue was purified with silica gel column; elution with 1:10 methanol/dichloromethane gave the title product **12** as a colorless gel (843.8 mg, 90%). ^{1}H NMR (600 MHz, $CDCl_3$): δ 3.59 (d, 1H, J = 7.8 Hz), 3.39 (t, 1H, J = 8.4 Hz), 3.11(m, 3H), 2.64 (m, 2H), 1.69 (d, 1H, J = 10.8 Hz), 1.61 (d, 1H, J = 12.0 Hz), 1.37 (m, 1H), 1.26 (s, 20H), 1.05 (m, 1H), 0.89 (t, 3H, J = 6.6 Hz), 0.78 (m, 1H).^{13}C NMR (150 MHz, $CDCl_3$): δ 66.5, 58.1, 46.3, 37.3, 36.0, 35.4, 33.2, 32.0, 30.0, 29.9, 29.8, 29.7, 29.5, 27.8, 26.6, 22.8, 14.2. MS (-ESI): m/z = 269.1853 $[M - H]^-$

1-(*tert*-Butoxycarbonyl)-4-undecylpiperidine-2-carboxylic acid (14). Et_3N (0.87 mL, 6.06 mmol) and $(Boc)_2O$ (850 mg, 3.94 mmol) were added to a solution of **11** (900 mg, 3.02 mmol) in methanol (10 mL. The mixture was stirred at room temperature overnight. The solvent was removed under vacuum to give an oily residue. The residue was purified with silica gel column; eluting with 6:1 hexane/ethyl acetate gave the Boc-protected product (1.08 g, 90%) as a colorless oil. Lithium hydroxide monohydrate (514 mg, 12.24 mmol) was added to a mixture of the Boc-protected product (1082 mg, 2.72 mmol) in 12 mL of THF and 4 mL of water. The mixture was stirred at room temperature for 48 h. THF was removed under vacuum. The aqueous layer was taken up in ethyl acetate and partitioned with 10% $NaHSO_4$ aqueous solution. The organic layer was washed with water and brine, and then dried over anhydrous Na_2SO_4 and concentrated under vacuum to give the desired product **14** (1043 mg, 99%) as a colorless gel. ^{1}H NMR (600 MHz, $CDCl_3$): δ 9.50 (br s, 1H) 4.26 (s, 1H), 3.50 (br s, 1H), 3.39 (m, 1H), 2.00 (m, 1H), 1.74 (m, 2H), 1.57 (s, 1H), 1.43 (s, 9H), 1.33 (m, 1H), 1.24 (s, 20H), 0.87 (t, 3H, J = 7.2 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ 177.1, 175.1, 80.5, 34.0, 31.8 (2C), 31.4 (2C), 29.6 (3C), 29.5 (3C), 29.3, 29.1, 28.2 (3C), 27.0, 22.6, 14.0. MS (-ESI): m/z = 382.2231 $[M - H]^-$.

PNU-69176E (1) and PNU-69176E Diastereomer (2). *Path a (Scheme 1).* PtO_2 catalyst (329 mg, 1.47 mmol) was added to a solution of **10** (110 mg, 0.21 mmol) in a mixture of methanol (6 mL), H_2O (4 mL), and 37% HCl (150 μL. The resulting

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mixture was reduced on a Parr hydrogenator at 60 psi for 5 days. Analysis of the reaction mixture by TLC in a system composed of methanol/$CHCl_3$ (1:6) showed that no starting material remained. The platinum solid was filtered through Celite, and the filtrate was vacuum concentrated to afford an oily residue. The residue was purified by silica gel column; elution with 15% MeOH in CH_2Cl_2 afforded PNU-69176E (**1**) (more polar isomer, 7.0 mg, 6.4%) and the diastereomer (**2**) (less polar isomer, 9.0 mg, 7.6%) as a colorless gel.

Path b (Scheme 2). HBTU (306 mg, 0.80 mmol) and DIPEA (200 mg, 1.56 mmol) were added to a solution of **14** (240 mg, 0.62 mmol) and 7-Cl-MTL (**9**) (170 mg, 0.62 mmol) in 6 mL of DMF. The resulting mixture was stirred at room temperature for 16 h. TLC showed that no starting material remained. The solvent DMF was removed under vacuum to give a dark oil residue, which was then partitioned between CH_2Cl_2 (50 mL) and 10% citric aqueous solution (10 mL). The organic layer was separated and washed with saturated aqueous $NaHCO_3$ (10 mL). After drying over anhydrous Na_2SO_4, the solvent was removed under vacuum to give an oily residue. This crude product was purified with silica gel column; elution with 10% MeOH in CH_2Cl_2 afforded the amide **15** (299 mg, 75%). The amide **15** (170 mg, 0.26 mmol) was dissolved in CH_2Cl_2 (1 mL), followed by the addition of TFA (250 μL). The resulting mixture was stirred at room temperature for 2 h, and monitored by TLC. The solvent was removed under vacuum to give an oily residue, which was then partitioned between CH_2Cl_2 (30 mL) and saturated $NaHCO_3$ aqueous solution (10 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated to give an oily residue, which was then purified with silica gel column; elution with 15% MeOH in CH_2Cl_2 afforded PNU-69176E (**1**) (more polar isomer, 42 mg, 30%) and the diastereomer **2** (less polar isomer, 48 mg, 34%).

1 (Colorless Gel). Silica gel TLC R_f = 0.21 (1:10 MeOH/CH_2Cl_2); $[\alpha]_D^{27}$ +167.2 (c 0.1, MeOH). ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.22 (d, 1H, J = 9.0 Hz), 5.35 (d, 1H, J = 4.2 Hz), 4.77 (m, 1H), 4.43 (d, 1H, J = 3.6 Hz), 4.35 (m, 1H), 4.16 (m, 1H), 4.08 (s, 1H), 3.83 (d, 1H, J = 7.8 Hz), 3.25 (d, 1H, J = 10.8 Hz), 3.14 (d, 1H, J = 12.0 Hz), 2.67 (t, 1H, J = 11.4 Hz), 2.41 (br s, 4H), 2.13 (s, 3H), 2.06 (d, 1H, J = 12.6 Hz), 1.68 (d, 1H, J = 11.4 Hz), 1.38 (br s, 1H), 1.25 (s, 20H), 1.14 (d, 3H, J = 6.0 Hz), 1.07 (m, 1H), 0.93 (m, 1H), 0.88 (t, 3H, J = 6.6 Hz).^{13}C NMR (150 MHz, $CDCl_3$): δ 174.0, 87.5, 77.3, 75.3, 71.6, 69.5 (2C), 60.7, 52.8, 45.8, 37.2, 36.8, 35.9, 32.2, 31.8, 29.7, 29.6 (2C), 29.5 (2C), 29.2, 26.4, 22.6, 16.9, 14.0, 13.5. MS (-ESI-LR): m/z = 535.3 $[M - H]^+$. HRMS calcd for $C_{26}H_{50}ClN_2O_5S$ $[M - H]^-$, 535.2972; found, 535.2982.

Diastereomer 2 (Colorless Gel). Silica gel TLC R_f = 0.42 (1:10 MeOH/CH_2Cl_2); $[\alpha]_D^{27}$ +103.6 (c 0.1, MeOH). ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.13 (d, 1H, J = 9.0 Hz), 5.35 (d, 1H, J = 5.4 Hz), 4.79 (m, 1H), 4.44 (dd, 1H, J = 2.7 Hz, 4.8 Hz), 4.34 (m, 1H), 4.15 (m, 1H), 4.08 (m, 1H), 3.83 (m, 1H), 3.23 (dd, 1H, J = 3.0 Hz, 11.4 Hz), 3.15 (d, 1H, J = 11.4 Hz), 2.66 (m, 1H), 2.12 (br s, 4H), 2.13 (s, 3H), 2.06 (m, 1H), 1.67 (d, 1H, J = 12.6 Hz), 1.40 (br s, 1H), 1.30 (s, 20H), 1.14 (d, 3H, J = 6.6 Hz), 1.02 (m, 1H), 0.89 (m, 1H), 0.88 (t, 3H, J = 6.6 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ 174.2, 87.6, 77.4, 75.3, 71.6, 69.8 (2C), 61.0, 53.0, 46.0, 37.4, 37.2, 36.0, 32.7, 32.0, 29.9, 29.8 (2C), 29.7 (2C), 29.4, 26.5, 22.8, 17.2, 14.2, 13.6. MS (+ESI-LR): m/z = 537.3 $[M + H]^+$. HRMS calcd for $C_{26}H_{50}ClN_2O_5S$ $[M + H]^+$, 537.3129; found, 537.3104.

In Vitro Pharmacological Assessment of PNU-69176E (1) and Its Diastereomer 2. The Chinese hamster ovary (CHO) cell lines stably transfected with 5-HT_{2C}R or 5-HT_{2A}R were a generous gift of K. Berg and W. Clarke (University of Texas Health Science Center, San Antonio).[13] Cells containing approximately 250 fmol/mg protein of either human 5-HT_{2C}R or 5-HT_{2A}R were grown at 37 °C, 5% CO_2, and 85% relative humidity in GlutaMax α-MEM (Invitrogen, Carlsbad CA), 5% fetal bovine serum (Atlanta Biologicals, Atlanta GA), and 100 μg/mL hygromycin (Mediatech, Manassas, VA) and were passaged when they reached 80% confluence.

Changes in Ca_i^{2+} release were determined using the calcium sensitive dye Calcium 4 (FLIPR No-wash kit, Molecular Devices, Sunnyvale, CA, part #R8142). Cells were plated in serum-replete medium at 20 000 cells/well in black-sided, clear bottom 96-well tissue culture plates and were fed ~24 h later with serum-free medium. Following a 3 h incubation, medium was removed and replaced with 40 μL of fresh serum-free medium plus 40 μL Calcium 4 dye solution in Hank's balanced saline solution (HBSS, without $CaCl_2$ or $MgCl_2$) supplemented with 2.5 mM water-soluble probenicid (Invitrogen) to inhibit extracellular transport of the dye. Plates were incubated for 60 min at 37 °C and 60 min at RT in the dark. Fluorescence (λ_{ex} = 485 nm, λ_{em} = 525 nm) was measured with a FlexStation3 instrument (Molecular Devices). A baseline was established for each well during the initial segment of each run. Addition of 20 μL of 5× concentrated **1** or its diastereomer **2** occurred at 17 s, and fluorescence was recorded every 1.7 s for 90 s to determine intrinsic agonist activity. This first round of 90 s recordings was also used to provide a 15 min preincubation period for test compounds in some experiments, while for other experiments this preincubation step was eliminated and **1** was added simultaneously with 5-HT. Following another 17 s baseline recording, 25 μL of 5× concentrated 5-HT was added and fluorescence was again measured every 1.7 s for 90 s. Maximum peak heights were determined by the FlexStation software (SoftMax Pro 5.2) for each well. Data from each well were normalized to total cell mass as determined with crystal violet staining and then expressed as a percent of the maximum Ca_i^{2+} response obtained with 1 μM 5-HT. The main effects of treatment (**1** or its diastereomer **2**) and 5-HT concentration were analyzed by either two-way or one-way analysis of variance (ANOVA) as appropriate using the general linear model (GLM) procedure (SAS for Windows, Version 8.2). Subsequent a priori comparisons were made using Dunnett's procedure with vehicle (0.3 nM 5-HT) as the comparator (**1** concentration–response curve, Figure 4) or Newman–Keuls comparing the response at each 5-HT concentration to that in the presence of 1.0 nM **1** (5-HT fold-shift curve, Figure 6).

■ ASSOCIATED CONTENT

Supporting Information

Copies of spectra and analytical results. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

C.D. carried out all the synthesis, purification, and chemical characterization of PNU-69176E (**1**) and its diastereomer **2**. N.M.B., T.D.S., P.K.S., and N.C.A. carried out all the pharmacological evaluation of the allosteric modulation effects. K.A.C. directed the pharmacological work. J.Z. directed the synthetic efforts. J.Z. and K.A.C. conceived the project and wrote the paper based upon drafts written by C.D. and T.D.S.

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Notes

The authors declare no competing financial interest.

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