JOURNAL OF NATURAL PRODUCTS

Article

pubs.acs.org/jnp

Modulating the Serotonin Receptor Spectrum of Pulicatin Natural Products

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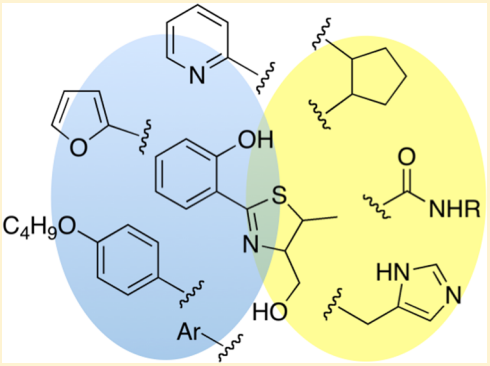
Supporting Information

ABSTRACT: Serotonin (5-HT) receptors are important in health and disease, but the existence of 14 subtypes necessitates selective ligands. Previously, the pulicatins were identified as ligands that specifically bound to the subtype 5-HT_{2B} in the 500 nM to 10 μM range and that exhibited *in vitro* effects on cultured mouse neurons. Here, we examined the structure−activity relationship of 30 synthetic and natural pulicatin derivatives using binding, receptor functionality, and *in vivo* assays. The results reveal the 2-arylthiazoline scaffold as a tunable serotonin receptor-targeting pharmacophore. Tests in mice show potential antiseizure and antinociceptive activities at high doses without motor impairment.



Siderophores in the yersiniabactin family are widely distributed among diverse bacterial phyla, where they are known to be responsible for iron import and for interspecies competition for iron.[1] Among the probable breakdown products of yersiniabactin-like siderophores is a family of small heterocycles, such as aerugine, pulicatins, and relatives (Figure 1), which are sometimes found in great abundance as

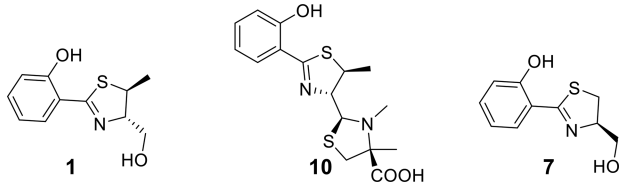


Figure 1. Structures of key metabolites pulicatin B (**1**), its probable precursor siderophore (**10**), and aerugine (**7**).

fermentation products.[2] The proposal that these are breakdown products of larger siderophores recently gained support from biosynthetic work on a methyltransferase that installs the thiazoline 5-methyl group.[3]

Previously, we reported that pulicatins and aerugine bind to serotonin (5-HT) receptors, with micromolar to nanomolar affinity.[4] In addition, aerugine relatives have other demonstrated activities on neurons.[5] Interestingly, 5-HT is also a critical component of the gut−brain axis and is regulated in part by the human microbiome.[6] While research has largely focused on bacteria that directly or indirectly regulate the metabolism of serotonin itself, bacteria inhabiting the gut produce secondary metabolites with the potential to affect the serotonin system.[7]

Beyond the gut−brain axis, the 14 serotonin receptor subtypes play central roles in human physiology.[8] For example, the 5-HT_{2B} subtype is found in many tissues, including the brain, gastrointestinal tract, cardiovascular system, and dorsal root ganglion (DRG) neurons.[9] In the brain, the 5-HT_{2B} receptor is the downstream target of selective serotonin reuptake inhibitors (SSRIs), since it is the major serotonin-responsive modulator of depression.[10] In the gut and in the DRG, 5-HT_{2B} signaling promotes hyperalgesia, at least in part by regulating the capsaicin receptor, TRPV1.[11]

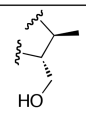
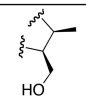
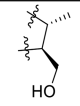
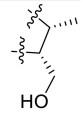
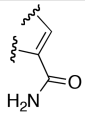
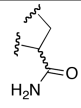
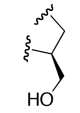
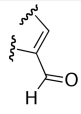
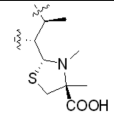
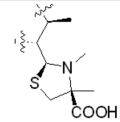
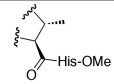
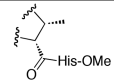
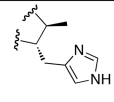
5-HT_{2B} receptors regulate aspects of cardiovascular function, and agonists can cause the negative side effect of damage to the heart.[12] Cardiovascular damage is associated with specific kinetics of ligand−protein interaction.[12] In addition to 5-HT_{2B}, 14 other receptor subtypes also contribute to many different physiological functions.[13,14] For example, 5-HT_3 antagonists control nausea and vomiting.[15] 5-HT_{1B} agonists are useful in treating migraine.[16] Because of the many different functions of serotonin receptors, it is important to develop agents with different spectra of selectivity, different kinetics, and different tissue distribution.

Here, we further investigate pulicatin activity *in vivo* and *in vitro*. Previously, ligand displacement assays with natural materials showed that natural pulicatin derivatives **2** and **5**−

Received: May 15, 2017
Published: July 26, 2017

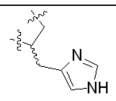
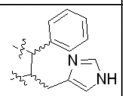
DOI: 10.1021/acs.jnatprod.7b00317
J. Nat. Prod. 2017, 80, 2360−2370

Table 1. Thiazoline Ring Substituent Pulicatin Derivative Binding Affinity in Ligand Displacement Assays Using a Series of Serotonin Receptors, with K_i Shown in nM[a]

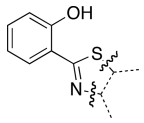
#	R[b]	ee	1B	1D	1E	2A	2B	2C	3	5a	6	7
1		50	>	>	>	>	**130**	1600	>	>	>	>
2		100	>	>	>	>	**510**	>	>	>	>	>
3		*rac*	>	>	>	>	**620**	2200	>	>	>	>
4		50	>	>	>	>	**400**	>	>	>	>	>
5		NA	>	>	>	>	**1000**	>	>	>	>	>
6		*rac*	>	>	>	>	**1300**	>	>	>	>	>
7		100	>	>	>	>	**1400**	>	>	>	>	>
8		NA	>	>	>	>	**1500**	>	>	>	>	>
9		100	>	>	>	>	**3900**	>	>	>	>	>
10		100	>	>	>	>	**4700**	>	>	>	>	>
11		100	-	3300	1400	**310**	1000	820	-	-	-	-
12		100	-	**530**	4600	-	1400	880	3100	>	>	>
13		*rac*	-	2000	>	1400	**47**	140	240	>	2600	3600

DOI: 10.1021/acs.jnatprod.7b00317
J. Nat. Prod. 2017, 80, 2360−2370

Table 1. continued

#	R[b]	ee	1B	1D	1E	2A	2B	2C	3	5a	6	7
14		*rac*	>	1900	>	>	**100**	>	950	>	>	7400
15		*rac*	-	2200	2400	1100	**100**	770	860	4700	2200	>
16		*rac*	>	>	>	>	>	>	>	>	>	>

[a]Data for **2** and **5**−**10** were previously reported[18] and are provided here for comparison. Bold: most potent binding target. In this and ensuing figures, affinity is rounded to two significant figures. Note: ">" means >10 μM; "-" indicates not tested; *rac*, racemic. [b]R is positioned on the following scaffold:



10 exhibited selective binding to the 5-HT$_{2B}$ receptor, with K_i from 510 to 4700 nM (Table 1).[4] The assays were performed at the Psychoactive Drug Screening Program (PDSP). Test compounds compete with radiolabeled lysergic acid diethylamide (LSD) or with other ligands for other serotonin receptor subtypes, and therefore constants are reported as inhibition of LSD binding. Subsequently, additional functional tests were performed for some compounds. Comparative binding constants for serotonin and a large number of serotonin receptor-binding compounds are provided at the PDSP Web site (https://kidbdev.med.unc.edu/databases/pdsp.php). In the pulicatin series, because binding affinity and other bioassay outcomes sometimes gave different answers, here we sought in part to use synthesis to generate sufficient compounds for further biological evaluation.

We also sought to create analogues exhibiting greater potency and selectivity. While the initial analogues were not exceptionally potent, they are very small molecules, exhibit excellent solubility in organic and aqueous solvents, and were quite selective in initial assays; in short, they are good initial hit compounds for a medicinal chemistry campaign. Based upon our initial structure−activity relationship (SAR) findings from naturally occurring compounds, we expected that subtle changes in pulicatin structure could yield potent and selective serotonin receptor modulating ligands.[4] Most importantly, we noted that addition of a methyl group to the thiazoline ring (e.g., compounds **1**−**4** in comparison to **7**) improved binding affinity by >3-fold. Moreover, greater sp^2 substitution did not substantially affect potency. Finally, configuration led to subtle differences in binding affinity. However, none of these changes modified potency or selectivity by 10-fold or more. Therefore, we aimed to explore more significant changes to either the thiazoline ring or the aromatic moiety to determine which factors were most important in determining selectivity. We systematically explored changes to each ring using synthetic methods.

Here, we synthesized enantiomerically enriched **1**, **3**, and racemic **4** and generated an additional set of synthetic analogues **11**−**30** for SAR studies (Tables 1−3). The results show that simple thiazoline natural products are tunable and selective ligands for serotonin receptors.

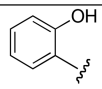
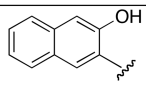
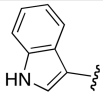
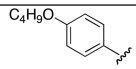
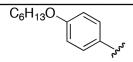
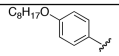
RESULTS AND DISCUSSION

Synthesis of Pulicatin B. A simple synthetic strategy was generated based upon the previously published method[17] that efficiently provides pulicatin derivatives with high enantiomeric excess (ee), although in our hands ee was modest (Scheme 1). We synthesized pulicatin B (**1**) in three steps from salicylic acid and L-threonine methyl ester in 35% yield (ee 50%), enabling us to provide **1** in gram scale for animal testing. D-Threonine methyl esters were used in the synthesis of the enantiomer of pulicatin B (**3**) (racemic). In the formation of 4,5-substituted Δ^2-thiazolines by Lawesson's reagent, the *trans*-Δ^2-thiazoline dominated. Very little *cis* form (~2%) was produced. Moreover, the subsequent reduction reaction of methyl ester using $NaBH_4$ further enriched the *trans*-Δ^2-thiazoline isomer. Hence, the racemic *cis* isomer, pulicatin A (**4**), could be synthesized in very low yield (<1%), and most synthetic work focused on generating *trans* isomers. This racemization can be rationalized by a proposed reaction mechanism for Lawesson's reagent in related transformations.[17]

Effect of Thiazoline Ring Substituent Modifications on Receptor Binding Affinity. Because of the apparent sensitivity of the binding affinity to slight changes in thiazoline ring configuration and substituents, we synthesized analogues **11**−**16** for further SAR studies (Table 1). The idea was based on preliminary molecular modeling suggesting that the primary OH group might bind to the 5-HT$_{2B}$ receptor in the same location as the amine in serotonin (data not shown).[18] All derivatives lost the selective 5-HT$_{2B}$ binding observed for the natural compounds, and instead binding to a variety of different 5-HT receptors was observed. Intriguingly, a compound linked to histidine (**11**) showed a change in selectivity to the 5-HT$_{2A}$ receptor at 310 nM. The most striking shift was observed for imidazole-linked pulicatin B derivatives (**13**−**15**). For example, **13** exhibited ligand displacement at the 5-HT$_{2B}$ receptor at 47 nM, while the best other analogue, pulicatin B (**1**), exhibited binding affinity at 130 nM. However, this increased affinity

DOI: 10.1021/acs.jnatprod.7b00317
J. Nat. Prod. 2017, 80, 2360−2370

Table 2. Aromatic Ring Substituent Effects on Ligand Displacement Assays Using a Series of Serotonin Receptors, with K_i Shown in nM

Cmpd	R[b]	Conf.[b]	1B	1D	1E	2A	2B	2C	3	5a	6	7
1		*trans*	>	>	>	>	**130**	1600	>	>	>	>
2		*cis*	>	>	>	>	**500**	>	>	>	>	>
17		*trans*	>	5300	>	>	3000	**35000**	6700	>	>	>
18		*trans*	>	8500	>	>	**1200**	1900	3600	>	>	>
19		*trans*	>	>	>	>	**810**	2400	1800	>	>	>
20		*trans*	>	**2700**	>	>	2400	5400	2800	>	>	>
21		*trans*	>	>	>	>	>	>	>	>	>	>
22		*trans*	>	>	>	>	**1400**	2200	>	>	>	>
23		*trans*	>	8600	>	>	**1500**	>	6700	8200	>	>
24		*trans*	>	9700	>	**2200**	7500	3200	>	>	>	>
25		*trans*	>	>	>	3900	4300	**1700**	>	>	>	>
26		*trans*	>	>	>	6900	>	**3600**	>	>	>	>

[a]Bold: most potent binding target. Note: ">" means >10 μM. [b]R is positioned on the following scaffold, with configurations at 1 and 2 positions as shown.

came at the expense of selectivity, since nanomolar to low micromolar affinity was observed for **13** at the 1B, 2A, 2B, 2C, 3, 6, and 7 receptors. By contrast, the loss of hydrophilic functional groups at the thiazoline ring substituent (**16**) totally abolished affinity. These results indicated that addition of basic nitrogen to the pulicatin skeleton tended to decrease selectivity in binding assays.

Effect of Aromatic Ring Substitution Modifications. Because the natural pulicatin derivatives exhibited the greatest selectivity, using methods optimized for synthesis of **1**, we modulated the aromatic ring substitution of **1** to generate different aryl derivatives **17**–**26** (Table 2). Although all aryl derivatives had less affinity than the natural products at the 5-HT_{2B} receptor, all derivatives retained binding affinity at 2B at <10 μM except for **21** and **26**. In addition, all compounds showed affinity for at least one serotonin receptor except for furan **21**, which showed no affinity for any receptors tested at concentrations of <10 μM. All compounds except for **24**–**26** retained the greatest affinity for the 5-HT_{2B} receptor; **24**–**26** had the highest affinity for 5-HT_{2C}, while **24** had preferential binding at the 5-HT_{2A} receptor. These results showed that the natural aromatic ring substitutions provided the greatest selectivity for the 5-HT_{2B} receptor.

SAR of Imidazole Group. Because of the potent binding affinity of imidazole derivatives **13**–**15**, we synthesized additional analogues **27**–**30** with differing aryl groups (Table 3). In this series, we observed a greater amount of the *cis* isomer than in other syntheses, enabling the purification and testing of both *cis* and *trans* racemates. The resulting derivatives had reduced affinity for the 5-HT_{2B} receptor compared to **13**; however, they exhibited a shift in spectrum of binding affinity. Compounds **27** and **28**, containing benzoate in place of salicylate, had greater affinity at the 5-HT_3 receptor. Compounds **29** and **30**, containing the biphenyl group, bound preferentially at the 5-HT_{2A} reeptor. This latter result is reminiscent of the shift to 2A binding observed with **24**–**26**. In sum, modest changes to the pulicatin structure did not improve selective binding to the 5-HT_{2B} receptor, but instead reduced the selectivity or altered the preferred binding partner.

DOI: 10.1021/acs.jnatprod.7b00317
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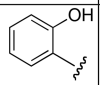
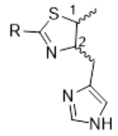
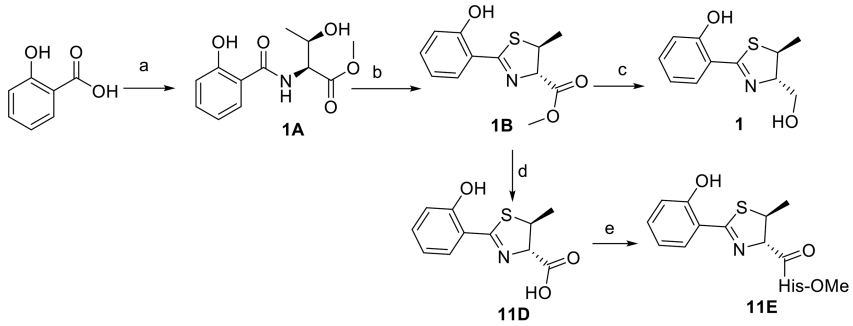
Table 3. Imidazole Derivative Binding Affinity in Ligand Displacement Assays Using a Series of Serotonin Receptors, with K_i Shown in nM[a]

Cmpd	R[b]	Conf.[b]	1B	1D	1E	2A	2B	2C	3	5a	6	7
13		*trans*	>	2000	>	1400	**47**	140	240	>	2600	3600
27		*trans*	-	800	>	5800	380	1200	**150**	>	>	>
28		*cis*	-	1600	4000	-	690	750	**77**	6800	>	3300
29		*trans*	-	-	-	**240**	1100	1400	-	-	-	-
30		*cis*	-	-	-	**310**	1400	2300	-	-	-	-

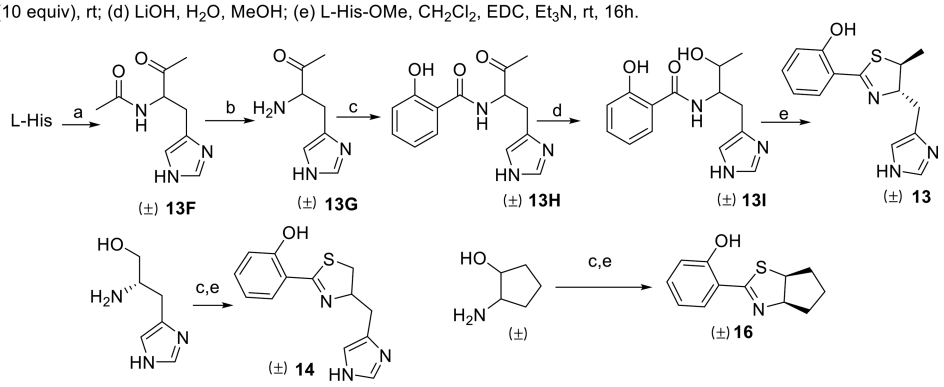
[a]Bold: most potent binding target. Note: ">" means >10 μM; "-" indicates not tested. [b]R is positioned on the following scaffold, with configurations at 1 and 2 positions as shown.



Scheme 1. Synthetic Strategy



(a) L-Thr-OMe, CH_2Cl_2, EDC, Et_3N, rt, 16h; (b) Lawesson's reagent (1 equiv), toluene, reflux 16h; (c) MeOH, $NaBH_4$ (10 equiv), rt; (d) LiOH, H_2O, MeOH; (e) L-His-OMe, CH_2Cl_2, EDC, Et_3N, rt, 16h.



(a) Ac_2O or C_6H_5COCl, pyridine, reflux 2h; (b) 6M HCl reflux 12 h; (c) ArCOCl, pyridine, rt 2h; (d) MeOH, $NaBH_4$ (3 equiv), 0 °C, 30s, H_2O; (e) Lawesson's reagent (1 equiv), toluene, pyridine, reflux 16h.

By modulating pulicatins, a library of 5-HT receptor-binding ligands with different selectivities was generated.

Functional Assays with Pulicatin Derivatives. Selected compounds were used in functional assays against various serotonin receptors. The experiments revealed that ligand displacement assays with pulicatin derivatives provided results that were grossly different than those from functional assays (Table 4). Compounds **1** and **2** did not exhibit agonist activity at 5-HT_{2B}, but were antagonists at 18 and 30 μM, respectively. These values differ by about 50−140× from the ligand displacement assay binding affinities. Imidazoles **13** and **15** were more active, with **13** showing 0.8 μM antagonism in Ca-release assays, but no activity in Tango assays. Strikingly, **24** and **25** were Tango partial agonists (50%) of the 5-HT_{1B} receptor at 29 and 180 nM, respectively, and antagonists at 2 μM (100%), despite their lack of ligand displacement affinity at the same receptor at 10 μM.

Mouse *in Vivo* Assays. We examined the pulicatins in a series of assays aimed to examine their potential 5-HT receptor activity *in vivo* based upon some of the known activities.[19,20]

DOI: 10.1021/acs.jnatprod.7b00317
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Table 4. Summary of Receptor Antagonist Assays with Pulicatin Derivatives in Specific 5-HT Receptor Subtypes

	antagonist IC_{50} (μM)			Tango antagonist IC_{50} (μM)				
compound	2A	2B	2C	7A	1A	1B	4	1D
1		18						
2		30						
11		10	>10		>10			
12		>10			>10			
13		0.8	6.8		>10			
15	13	3.8	>10	>10	>10			>10
24		>10			>10	2.1	>10	>10
25		>10			>10	1.9	>10	>10
26					>10			
27		10		>10	>10			
28		>10		>10	>10			

The antiseizure activity of pulicatin B (**1**) was tested in mice at the NIH/NINDS Epilepsy Therapy Screening program contract site at the University of Utah. Four mice each were used for initial screening in the maximal electroshock seizure (MES)[21,22] and 6 Hz psychomotor seizure tests[23] (Table 5).

Table 5. Summary of *in Vivo* Epilepsy and Rotarod Minimal Motor Impairment Studies[a]

		time (h)				
		0.25	0.5	1.0	2.0	4.0
test	dose (mg/kg)	N/F[b]	N/F	N/F	N/F	N/F
6 Hz (32 mA)	100	NT	3/4	NT	1/4	NT
6 Hz (44 mA)	100	2/4	1/4	1/4	0/4	0/4
MES	100	0/4	0/4	0/4		
motor impairment	100	0/4	0/4	0/4	0/4	0/4

[a]Doses of 10, 30, and 100 mg/kg were used; only the highest dose evaluated is shown. [b]N/F = number of animals active or impaired over the number tested.

Using intraperitoneal dosing using four mice in each condition, **1** was found to be inactive at doses up to 100 mg/kg in the MES test; in the 6 Hz test at 32 mA, at the highest dose of 100 mg/kg, three of four mice were protected from seizure for 30 min, while one out of four mice was still protected after 2 h. No acute motor impairment was observed in any of the 24 mice used in the initial testing, as defined using the rotorod test as previously described.[24] The effect on the 6 Hz test was evaluated by repeating the test with four mice at the 100 mg/kg dose at 44 mA. After 0.25 h, two out of four mice were protected, with one out of four protected at 0.5 and 1 h; no mice were protected at the 2 or 4 h time point. No overt impairment of neurological or motor function was observed in the four mice on the rotorod, on which a mouse can normally maintain its equilibrium for extended periods as the rod rotates at a fixed speed of 6 rpm. If the animal falls off the rotorod three times in a 1 min observation period, it is considered to cause minimal motor impairment. These experiments indicate a potentially modest protection in the 6 Hz test at a relatively high, but nonimpairing, dose. In the 6 Hz test, the compound was active at both the 32 and 44 mA current stimulation intensities. This is significant because seizures stimulated at 44 mA are more recalcitrant to many established antiseizure drugs, such as phenytoin, lamotrigine, and ethosuximide.[23] For example, in a previous report from the same lab that tested our sample,[23] while ethosuximide, levetiracetam, and valproic acid exhibit ED_{50}s of 167, 19.4, and 126 mg/kg respectively at 32 mA, at 44 mA the ED_{50}s increase to >600, 1089, and 310 mg/kg, respectively (generally, well above toxic dose levels). Thus, **1** has a somewhat favorable profile in this initial assay.

Because of the association of 5-HT_{2B} and pain,[20] we further evaluated **1** in the tail flick and hot plate assays of acute thermal nociception (Figure 2).[25−27] By one-way ANOVA, there was not a significant effect on the latency of response at 100 mg/kg in the tail flick assay. By contrast, a statistically significant result was detected in the hot plate assay. These results should be considered as an initial indication that synthetic **1** might have antinociceptive efficacy in mice. In identical assays performed in the same lab, antinociceptive compounds generally act at doses in the range of ∼1 (i.e., morphine) to ∼300 (i.e., aspirin) mg/kg.[28]

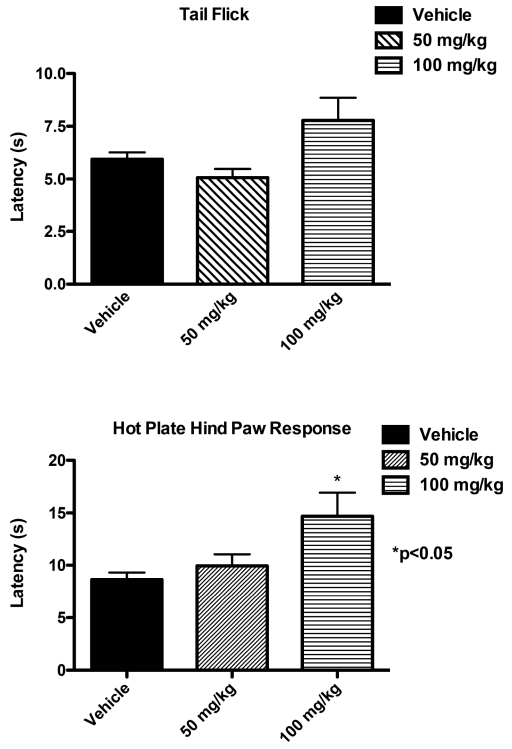

Figure 2. Results of compound **1** in the tail flick and hot plate assays.

CONCLUSIONS

Here, we describe the synthesis and pharmacological properties of 30 natural and synthetic pulicatin derivatives. Despite

DOI: 10.1021/acs.jnatprod.7b00317
J. Nat. Prod. 2017, 80, 2360−2370

substantial attempts at synthetic optimization, no derivative exhibited a more favorable potency/selectivity profile for the 5-HT_{2B} receptor than the initially identified pulicatins A (**2**) and B (**1**), which were highly selective for the 5-HT_{2B} receptor in ligand binding assays. However, several ligands displayed greater potency with a decrease in selectivity, and in several cases we changed the selectivity from 5-HT_{2B} to other receptors, such as 5-HT_3 or 5-HT_{1B}. These studies define functional groups of importance to selective binding of serotonin receptors, which are readily apparent in Tables 1−4.

Binding values did not correlate well with results from functional agonist/antagonist assays. Although radiolabeled lysergic acid diethylamide was readily displaced from the receptors, a much higher concentration of ligand was required to antagonize the action of serotonin. Some ligands that did not score in binding assays were active in functional assays. This could have to do with the different kinetics or conditions in the assays or with the known differences in binding between serotonin and LSD.[29]

We sought to determine whether binding data would translate to *in vivo* effects. Pulicatin B (**1**) was selected for initial testing because of its selectivity and ease of synthesis and because it was previously shown to be active in mouse neurons.[4] As a caveat for all of the work described, it is important to note that we do not directly examine the effects of **1** on 5-HT_{2B} in animals. We considered this of lesser importance in this initial work because of the complex pharmacology surrounding the aminergic receptors *in vivo*, where many useful compounds are more complex than thought from initial assays. Instead, here we sought *in vivo* data that might suggest further use or abandonment of this compound series. Surprisingly, we found that the compound **1** exhibited no motor impairment, but was modestly effective at reducing limbic seizures induced by 6 Hz at 32 and 44 mA. This is potentially significant because 44 mA seizures are considered to be more resistant to therapeutics. Moreover, initial results suggested that **1** may be effective in reducing acute thermal pain. By contrast, we did not achieve statistical significance for an effect on pain in the tail flick test, and the compound was inactive at 100 mg/kg in the MES test.

5-HT_{2B} is especially important in hyperalgesia and neuralgia.[30] These effects are mediated largely through pain sensory neurons in the GI and in the dorsal horn. Therefore, we performed preliminary testing to demonstrate that pulicatins may be effective at reducing acute thermal pain. Both the tail flick and the hot-plate tests evaluate *in vivo* sensitivity to acute heat-induced pain stimuli.[31] The tail flick is a spinally mediated flexor withdrawal reflex, whereas the hot plate test is supraspinally mediated. This may explain why the compound is more effective in one model compared to the other.[32]

The major application of 5-HT_{2B} antagonists is in chronic pain conditions, where the 5-HT_{2B} receptor is greatly upregulated.[33] It may be that pulicatin B (**1**) is more effective at a lower dose under this condition, where 5-HT_{2B} is one of the dominant determinants of pain. The lack of observed impairment caused by **1** and the simplicity of synthesis are promising in this regard. Because of the *in vivo* activity observed in this initial study, in future work, we plan to test these in spinal nerve ligation models, where the upregulation of 5-HT_{2B} may be more important in mediating pain, and a significant effect might be clearer.

Finally, the pulicatins were initially isolated from bacteria associated with an animal (cone snail). It would be interesting to determine whether pulicatins or their relatives ever reach sufficient local concentration to impact seritonergic signaling in humans or other animals. The ready tunability of these ligands to different 5-HT receptor subtypes would then indicate the potential for complex interactions between microbe and host.

EXPERIMENTAL SECTION

General Experimental Procedures. UV spectra were obtained using a PerkinElmer Lambda2 UV/vis spectrometer. NMR data were collected using either a Varian INOVA 500 (^{1}H 500 MHz, ^{13}C 126 MHz) NMR spectrometer with a 3 mm Nalorac MDBG probe or a Varian INOVA 600 (^{1}H 600 MHz, ^{13}C 150 MHz) NMR spectrometer equipped with a 5 mm ^{1}H[^{13}C,^{15}N] triple resonance cold probe with a *z*-axis gradient, utilizing residual solvent signals for referencing ($CDCl_3$: δ_H 7.26, δ_C 77.36; acetone-d_6: δ_H 2.09, δ_C 30.60; CD_3CN: δ_H 1.96, δ_C 1.79; CD_3OD: δ_H 3.34, δ_C 49.86; DMSO-d_6: δ_H 2.54, δ_C 40.45). High-resolution mass spectra (HRMS) were obtained using a Bruker APEXII FTICR mass spectrometer equipped with an actively shielded 9.4 T superconducting magnet (Magnex Scientific Ltd.), an external Bruker APOLLO ESI source, and a Synrad 50W CO_2 CW laser. Supelco Discover HS (4.6 × 150 mm) and semipreparative (10 × 150 mm) C_{18} (5 μm) columns were used for analytical and semipreparative HPLC, respectively, as conducted on a Hitachi Elite Lachrom System equipped with a diode array L-2455 detector. The enantiomeric excess was calculated from the area of the HPLC peak using a silica-based protein phase enantiomer separation column, RESOLVOSIL BSA-7, eluting with 0.1 M phosphate buffer pH 7.5 and 2% 1-propanol. Unless stated otherwise, all reagents and solvents were purchased from commercial suppliers, and all reactions were carried out under anhydrous conditions with an argon atmosphere. Yields were calculated by HPLC or ^{1}H NMR spectroscopy.

Representative Procedures. *Methyl N-(2-hydroxybenzoyl)-L-threoninate (1A).* Salicylic acid (138 mg, 1 mmol) was dissolved in CH_2Cl_2 (15 mL). To the solution were added in order ethylene dichloride (EDC) (178 μL), Et_3N (140 μL), and L-threonine methyl ester hydrochloride (169 mg, 1 mmol). The mixture was stirred for 18 h at room temperature (rt). The mixture was concentrated by rotary evaporation at 25 °C. To the residue were added H_2O (15 mL) and EtOAc (20 mL). The EtOAc layer was then washed with brine, dried over $CaCl_2$, and evaporated to dryness to afford the desired amide (**1A**, 200 mg, 79%). Spectroscopic data agreed with published results.[34]

Heterocyclization of 1A. Compound **1A** (200 mg) and 2,4-bis(*p*-methoxyphenyl)-1,3,2,4-dithiaphosphetane 2,4-disulfide (320 mg, 1 equiv) were dissolved in anhydrous toluene (150 mL). The solution was heated to reflux under an argon atmosphere for 18 h. After removal of the solvent, the residue was purified by silica gel column chromatography with hexane−EtOAc (25:1) to yield **1B** (100 mg, 50%): ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 7.42 (1H, d, J = 7.9 Hz), 7.38 (1H, dd, J = 7.9, 7.9 Hz), 7.04 (1H, d, J = 7.9 Hz), 6.88 (1H, dd, J = 7.9, 7.9 Hz), 5.21 (1H, d, J = 7.7 Hz), 4.22 (1H, dq, J = 7.7, 6.9 Hz), 3.84 (3H, s), 1.31 (3H, d, J = 6.9 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ_C 174.5, 159.6, 134.0, 130.9, 119.2, 117.7, 79.8, 52.7, 45.9, 18.3; ESIMS m/z 252 $[M + H]^+$.

Methyl Ester Reduction of 1B. Compound **1B** (100 mg) was dissolved in methanol (70 mL) with stirring at rt, and $NaBH_4$ (150 mg) was added to the solution. After 1 min, the reaction was quenched with trifluoroacetic acid (TFA), and the solvent was removed by rotary evaporation. The residue was extracted with H_2O (20 mL) and $CHCl_3$ (40 mL). The organic layer was dried to yield compound **1** (80 mg, 90%). Spectroscopic data agreed with natural pulicatin B.[4]

For the synthesis of pulicatin A, the *cis* isomer of compound **1B** (6 mg) was dissolved in precooled MeOH (−10 °C, 2 mL), and $NaBH_4$ (15 mg) was added to the solution. After 10 s, the reaction was quenched with TFA, and the solvent was removed by rotary evaporation to yield compound **2** (2.1 mg, 39%). Spectroscopic data agreed with natural pulicatin A.[4]

DOI: 10.1021/acs.jnatprod.7b00317
J. Nat. Prod. 2017, 80, 2360−2370

*Preparation of Compounds **11** and **12**.* Compound **1B** (*trans:cis* 2:1, 5 mg) was dissolved in 10% LiOH solution in H_2O–MeOH (1:1, 2 mL). The mixture was stirred for 4 h at rt. The MeOH was removed under vacuum, EtOAc (4 mL) was added, and the organic layer was concentrated. After drying overnight on a lyophylizer, the residue containing **11D** was dissolved in anhydrous CH_2Cl_2 (4 mL). After adding L-histamine methyl ester (3.5 mg), EDC (8 μL), and Et_3N (3 μL), the mixture was stirred for 18 h at rt. The solution was concentrated by rotary evaporation at 25 °C. To the residue were added H_2O (5 mL) and EtOAc (10 mL), and the resulting suspension was mixed well. The EtOAc layer was then washed with brine, dried over $CaCl_2$, and then evaporated to dryness to afford the desired amide mixture (**11** and **12**), which was further purified by C_{18} HPLC with 40% MeCN in H_2O to obtain the pure isomers **11** (*trans*, 2 mg, 40%) and **12** (*cis*, 1.0 mg, 40%).

11D: ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 7.47 (brs 1H), 7.44 (d, J = 7.8 HZ, 1H), 7.16 (brs, 1H), 6.89 (dd, J = 7.8, 7.5 Hz), 5.05 (brs 1H), 4.28 (brs, 1H), 1.61 (d, J = 6.0 Hz); ESIMS m/z 238 $[M + H]^+$.

11: ^{1}H NMR (600 MHz, acetone-d_6) δ_H 8.91 (1H, brs), 8.36 (1H, d, J = 8.3 Hz), 7.53 (1H, brs), 7.46–7.48 (2H, m), 6.96–7.01 (2H, m), 5.03 (1H, d, J = 5.7 Hz), 4.92 (1H, m), 4.30 (1H, dq, J = 7.0, 5.6 Hz), 3.77 (3H, s), 3.50 (1H, dd, J = 16.0, 4.3 Hz), 3.29 (1H, dd, J = 16.0, 9.5 Hz), 1.55 (3H, d, J = 6.8 Hz); HSQC (600 MHz, acetone-d_6) δ_C 135.1 (CH), 132.5 (CH), 120.7 (CH), 118.6 (CH), 85.6 (CH), 53.7 (CH_3), 53.4 (CH_3), 48.5 (CH), 27.7 (CH_2), 22.4 (CH_3); HRESIMS m/z 389.1268 $[M + H]^+$ (calcd for $C_{18}H_{20}N_4O_4S$, 389.1278).

12: ^{1}H NMR (600 MHz, acetone-d_6) δ_H 8.89 (1H, brs), 8.30 (1H, d, J = 8.3 Hz), 7.57 (1H, brs), 7.45–7.48 (2H, m), 6.96–7.00 (2H, m), 5.05 (1H, d, J = 5.9 Hz), 4.90 (1H, m), 4.33 (1H, dq, J = 7.0, 5.9 Hz), 3.73 (3H, s), 3.49 (1H, dd, J = 16.0, 4.3 Hz), 3.35 (1H, dd, J = 16.0, 9.5 Hz), 1.53 (3H, d, J = 6.8 Hz); HSQC (600 MHz, acetone-d_6) δ_C 135.4 (CH), 132.6 (CH), 120.9 (CH), 118.9 (CH), 85.6 (CH), 53.7 (CH_3), 53.6 (CH_3), 48.8 (CH), 28.0 (CH_2), 22.9 (CH_3); HRESIMS m/z 389.1268 $[M + H]^+$ (calcd for $C_{18}H_{20}N_4O_4S$, 389.1278).

*Preparation of Compound **13F**.* L-Histidine (15.5 g) was dissolved in anhydrous pyridine (100 mL), and Ac_2O (15 mL) was added (note: in the preparation of compound **15**, benzoyl chloride was used instead of Ac_2O) into the solution under stirring in an ice bath. The mixture was heated to reflux for 2 h. The reaction was quenched with 20% Na_2CO_3 (100 mL), and pyridine was removed by rotary evaporation. The residue was partitioned with EtOAc (200 mL) to yield **13F** (19.0 g, 80%): ^{1}H NMR (500 MHz, CD_3OD) δ_H 7.74 (1H, s), 6.93 (1H, s), 4.65 (1H, dd, J = 8.5, 5.2 Hz), 3.12 (1H, dd, J = 15.1, 5.2 Hz), 2.92 (1H, dd, J = 15.1, 8.5 Hz), 2.16 (3H, s), 1.97 (6H, s); ^{13}C NMR (126 MHz, CD_3OD) δ_C 207.1, 171.7, 134.9, 133.1, 116.7, 59.0, 27.2, 26.0, 21.0; ESIMS m/z 196 $[M + H]^+$.

*Preparation of Compound **13G**.* Compound **13F** (19.0 g) was dissolved in 6 M HCl (100 mL), and the solution was heated to reflux for 12 h. The mixture was dried under blowing air to yield compound **13G** (12.0 g, 99%): ^{1}H NMR (500 MHz, CD_3OD) δ_H 9.04 (1H, s), 7.67 (1H, s), 4.71 (1H, m), 3.72 (1H, dd, J = 15.1, 5.2 Hz), 3.39 (1H, dd, J = 15.1, 8.5 Hz), 2.47 (3H, s); ^{13}C NMR (126 MHz, CD_3OD) δ_C 202.1, 134.8, 126.8, 118.8, 58.1, 26.3, 24.3; ESIMS m/z 154 $[M + H]^+$.

*Preparation of Compound **13H**.* Compound **13G** (153 mg) was dissolved in anhydrous pyridine (50 mL). The solution was cooled in an ice bath. Aspirin acyl chloride (198 mg) was added to the cooled solution, and the mixture was stirred for 2 h in an ice bath. The reaction was quenched by adding H_2O (10 mL). The pyridine was removed by rotary evaporation. The residue was purified by a silica gel column with hexane–EtOAc (20:1) to yield compound **13H** (204 mg, 75%): ^{1}H NMR (500 MHz, DMSO-d_6) δ_H 7.90 (1H, s), 7.86 (1H, d, J = 8.6 Hz), 7.41 (1H, dd, J = 8.6, 8.6 Hz), 7.11 (1H, s), 6.90 (2H, m), 4.86 (1H, m), 3.24 (2H, brs), 2.17 (3H, s); ^{13}C NMR (125 MHz, DMSO-d_6) δ_C 205.7, 171.2, 161.3, 136.6, 135.2, 134.8, 134.2, 127.3, 119.1, 118.7, 115.9, 60.7, 28.1, 27.4; ESIMS m/z 274 $[M + H]^+$.

*Preparation of Compound **13I**.* Compound **13H** (204 mg) was dissolved in MeOH (10 mL), $NaBH_4$ (85 mg) was added to the solution, and the mixture was stirred for 1 min and then quenched by 1% TFA in H_2O (10 mL). The MeOH was removed by rotary evaporation. The residue was washed with EtOAc (40 mL), and the organic layer was dried to yield compound **13I** (143.5 mg, 70%): ^{1}H NMR (600 MHz, CD_3OD) δ_H 7.84 (1H, d, J = 8.1 Hz), 7.32 (1H, m), 6.85 (1H, d, J = 8.1 Hz), 6.78 (1H, dd, J = 8.1, 8.0 Hz), 4.49 (1H, m), 4.01 (1H, m), 3.33 (1H, m), 3.15 (1H, m), 1.26 (3H, d, J = 6.5 Hz); key HSQC (600 MHz, CD_3OD) δ_C 136.7 (CH), 130.8 (CH), 121.5 (CH), 120.4 (CH), 71.9 (CH), 57.7 (CH), 27.6 (CH_2), 22.5 (CH_3); ESIMS m/z 276 $[M + H]^+$.

*Preparation of Compound **13**.* Compound **13I** (143 mg) and 2,4-bis(*p*-methoxyphenyl)-1,3,2,4-dithiaphosphetane 2,4-disulfide (210 mg, 1 equiv) were dissolved in anhydrous toluene (150 mL). The solution was heated to reflux under an argon atmosphere for 18 h. After removal of the solvent, the residue was purified by a silica gel column with hexane–EtOAc (20:1) followed by C_{18} HPLC (35% MeCN in H_2O with 1% TFA) to yield **13** (5 mg, 3.5%): ^{1}H NMR (600 MHz, DMSO-d_6) δ_H 8.90 (1H, brs), 7.83 (1H, brs), 7.73–7.76 (2H, m), 7.25–7.28 (2H, m), 5.21 (1H, brs), 4.28 (1H, brs), 3.62 (1H, dd, J = 11.0, 3.4 Hz), 3.52 (1H, dd, J = 11.0, 6.4 Hz), 1.79 (3H, d, J = 6.7 Hz); ^{13}C NMR (126 MHz, acetone-d_6) δ_C 171.3, 159.1, 133.9, 133.2, 130.9, 130.6, 118.9, 117.0, 116.8, 116.2, 81.2, 48.0, 28.6, 21.2; HRESIMS m/z 274.1004 $[M + H]^+$ (calcd for $C_{14}H_{15}N_3OS$, 274.1014).

*Preparation of Compound **14**.* L-Histidinol dihydrochloride (214 mg) and aspirin acyl chloride (198 mg) were suspended in anhydrous pyridine (100 mL). The mixture was stirred for 10 h at rt and dried by rotary evaporation followed by lyophilizing overnight. The residue was directly subjected to the heterocyclization described in the preparation of **1B** to yield compound **14** (4.6 mg 1.7%): ^{1}H NMR (600 MHz, DMSO-d_6) δ_H 9.03 (1H, brs), 7.53 (1H, brs), 7.42 (2H, m), 6.95 (2H, m), 5.05 (1H, m), 3.26 (1H, dd, J = 11.5. 7.2 Hz), 3.16 (1H, m), 3.10 (2H, m); ^{13}C NMR (126 MHz, acetone-d_6) δ_C 171.9, 158.9, 133.9, 133.3, 130.9, 130.6, 119.0, 117.1, 116.8, 116.1, 110.0, 74.9, 35.3; HRESIMS m/z 260.0846 $[M + H]^+$ (calcd for $C_{13}H_{13}N_3OS$, 260.0858).

*Compound **15**:* ^{1}H NMR (500 MHz, acetone-d_6) δ_H (*cis*) 8.88 (1H, s), 7.31–7.55 (8H, m), 7.00 (2H, m), 5.40 (1H, brs), 5.39 (1H, brs), 3.01 (1H, m), 2.93 (1H, m); key HSQC (500 MHz, acetone-d_6) δ_C 78.1 (CH), 54.7 (CH), 26.8 (CH_2); (*trans*) ^{1}H NMR (500 MHz, acetone-d_6) δ_H 8.88 (1H, s), 7.31–7.55 (8H, m), 7.00 (2H, m), 5.29 (1H, brs), 5.13 (1H, brs), 3.43 (2H, m); key HSQC (500 MHz, acetone-d_6) δ_C 82.6 (CH), 56.3 (CH), 38.6 (CH_2); ^{13}C NMR (125 MHz, acetone-d_6) δ_C 171.7, 158.9, 140.8, 137.4, 133.5, 130.6, 128.9, 128.6, 128.5, 128.3, 128.0, 127.4, 119.1, 119.1, 116.9, 116.9, 115.9, 115.9, 82.6, 78.1, 56.3, 54.7, 28.6, 26.8, 18.0, 16.2; HRESIMS m/z 336.1160 $[M + H]^+$ (calcd for $C_{19}H_{17}N_3OS$, 336.1165).

The same procedure was used to synthesize compound **16**: ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 7.45 (1H, dd, J = 7.9, 7.9 Hz), 7.40 (1H, d, J = 7.9 Hz), 7.16 (1H, d, J = 7.9 Hz), 6.90 (1H, dd, J = 7.9, 7.9 Hz), 5.31 (1H, ddd, J = 8.6, 8.6, 2.8 Hz), 4.34 (1H, ddd, J = 8.3, 8.3, 3.3 Hz), 2.14 (3H, m), 1.96 (1H, m), 1.78 (2H. m); HSQC (500 MHz, $CDCl_3$) δ_C 135.6 (CH), 131.2 (CH), 117.9 (CH), 119.6 (CH), 78.7 (CH), 49.4 (CH), 34.2 (CH_2), 36.7 (CH_2), 23.4 (CH_2); HRESIMS m/z 220.0784 $[M + H]^+$ (calcd for $C_{12}H_{13}NOS$, 220.0796).

*Compound **17**:* ^{1}H NMR (500 MHz, acetone-d_6) δ_H 7.89 (2H, d, J = 7.5), 7.58 (1H, dd, J = 7.7, 6.7), 7.49 (2H, dd, J = 8.0, 7.7), 4.48 (1H, m), 4.17 (1H, m), 3.81 (1H, dd, J = 11.0, 4.9 Hz), 3.69 (1H, dd, J = 11.0, 6.7 Hz), 1.48 (3H, d, J = 6.7 Hz); ^{13}C NMR (126 MHz, acetone-d_6) δ_C 169.7, 132.5, 131.3, 128.7, 128.5, 84.9, 61.7, 46.8, 21.7; HRESIMS m/z 208.0786 $[M + H]^+$ (calcd for $C_{13}H_{14}N_2OS$, 208.0791).

*Compound **18**:* (mixture of *cis* and *trans* isomers, inseparable by C_{18} HPLC) ^{1}H NMR (500 MHz, acetone-d_6) δ_H (*trans*-**18**) 7.42 (1H, dd, J = 7.8, 1.4 Hz), 7.32 (1H, dd, J = 7.7, 7.4 Hz), 6.72 (1H, d, J = 8.4), 6.61 (H, m), 4.47 (1H, m), 3.74 (1H, dd, J = 11.0, 4.8 Hz), 3.57 (1H, dd, J = 11.0, 7.0 Hz), 3.91 (1H, m), 1.42 (3H, d, J = 6.7 Hz); (*cis*-**18**) δ_H 7.48 (1H, dd, J = 7.8, 1.4 Hz), 7.32 (1H, dd, J = 7.7, 7.4 Hz), 6.72 (1H, d, J = 8.4), 6.61 (H, m), 4.47 (1H, m), 4.20 (1H, dd, J = 11.0, 6.5 Hz), 3.93 (1H, m), 3.96 (1H, m), 1.27 (3H, d, J = 6.7 Hz); ^{13}C NMR (126 MHz, acetone-d_6) δ_C (*trans*-**18**) 168.2, 149.3, 132.1, 114.1, 110.0, 86.5, 62.3, 44.3, 21.7; (*cis*-**18**) 168.7, 149.3, 132.4, 114.1, 110.0, 81.5,

DOI: 10.1021/acs.jnatprod.7b00317
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60.8, 44.8, 16.6; HRESIMS m/z 237.1050 $[M + H]^+$ (calcd for $C_{12}H_{16}N_2OS$, 237.1062).

Compound ***19:*** ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 7.95 (1H, s), 7.74 (1H, dd, J = 7.7, 6.7 Hz), 7.68 (1H, d, J = 8.0 Hz), 7.47 (1H, dd, J = 7.7, 6.7 Hz), 7.31 (1H, s), 7.29 (1H, d, J = 8.0 Hz), 4.50 (1H, m), 4.05 (1H, m), 3.92 (1H, dd, J = 12.0, 5.2 Hz), 3.84 (1H, dd, J = 2.0, 4.4 Hz), 1.54 (3H, d, J = 6.7 Hz); ^{13}C NMR (126 MHz, $CDCl_3$) δ 175.3, 158.0, 114.1, 138.9, 128.8, 131.0, 126.1, 131.3, 130.0, 135.5, 121.1, 87.5, 65.7, 48.3, 24.4; HRESIMS m/z 274.1004 $[M + H]^+$ (calcd for $C_{15}H_{15}NO_2S$, 274.1014).

Compound ***20:*** ^{1}H NMR (500 MHz, CD_3CN) δ_H 11.04 (1H, brs, NH), 8.61 (1H, brs), 8.00 (1H, m), 7.66 (1H, m), 7.39 (2H, m), 4.35 (1H, dd, J = 10.4, 5.2 Hz), 4.23 (1H, dq, J = 7.4, 6.6 Hz), 3.94 (1H, dd, J = 12.6, 3.9 Hz), 3.79 (1H, dd, J = 12.6, 5.0 Hz), 1.54 (3H, d, J = 6.7 Hz); ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 9.00 (1H, brs, NH), 8.26 (1H, d, J = 7.4 Hz), 7.68 (1H, s), 7.40 (1H, d, J = 7.4 Hz), 7.27 (2H, m), 4.40 (1H, dd, J = 11.2, 5.8 Hz), 3.90 (2H, m), 3.76 (1H, dd, J = 11.2, 5.7 Hz), 1.50 (3H, d, J = 6.6 Hz); HSQC (500 MHz, CDCl) δ 128.9 (CH), 123.3 (CH), 121.8 (CH), 121.6 (CH), 111.4 (CH), 85.2 (CH), 63.7 (CH_2), 46.4 (CH), 21.2 (CH_3); HRESIMS m/z 247.0895 $[M + H]^+$ (calcd for $C_{13}H_{14}N_2OS$, 247.0900).

Compound ***21:*** ^{1}H NMR (500 MHz, CD_3CN) δ_H 7.88 (1H, s), 7.54 (1H, s), 6.77 (1H, brs), 4.50 (1H, brs), 4.23 (1H, brs), 3.97 (2H, brs), 1.55 (3H, d, J = 6.9 Hz); HRESIMS m/z 198.0579 $[M + H]^+$ (calcd for $C_9H_{11}NO_2S$, 198.0583).

In the structure identification of compound **21**, we observed that the ^{1}H NMR spectrum of **21** gave very broad proton signals, even though we tried four different NMR solvents ($CDCl_3$, CD_3OD, DMSO-d_6, and CD_3CN). This suggested that the **21** presented as slowly rotating isomers through the single bond between the furan ring and the thiazoline ring. In order to stabilize the rotamers of **21**, we synthesized the *p*-bromobenzoyl ester of compound **21**. Indeed, the ^{1}H NMR spectrum of the *p*-bromobenzoyl ester of compound **21** gave a single well-resolved set of peaks. Compound **21** (2 mg) was dissolved in anhydrous pyridine (1 mL), one equivalent of *p*-bromobenzoyl chloride was add to the solution, and reaction was quenched by adding H_2O (1 mL) after 4 h. The mixture was extracted with EtOAc (2 mL) and purified by C_{18} HPLC (55% MeCN in water) to yield the *p*-bromobenzoyl ester of compound **21** (1.8 mg).

p-Bromobenzoyl ester of compound ***21:*** ^{1}H NMR (500 MHz, CD_3CN) δ_H 7.91 (2H, d, J = 8.1), 7.59 (2H, d, J = 8.1), 7.56 (1H, s), 6.94 (1H, s), 6.52 (1H, brs), 4.66 (1H, m), 4.58 (1H, m), 4.43 (1H, m), 3.98 (1H, m), 1.51 (3H, d, J = 6.8 Hz); ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 7.93 (2H, d, J = 7.8), 7.60 (2H, d, J = 7.8), 7.57 (1H, s), 6.94 (1H, d, J = 3.5 Hz), 6.53 (1H, brs), 4.68 (1H, m), 4.59 (1H, dd, J = 11.7, 4.7 Hz), 4.44 (1H, dd, J = 11.7, 6.5 Hz), 3.99 (1H, m), 1.52 (3H, d, J = 6.8 Hz); HSQC (500 MHz, $CDCl_3$) δ_C 145.1 (CH), 131.9 × 2 (CH), 131.2 × 2 (CH), 114.5 (CH), 111.9 (CH), 82.3 (CH), 65.2 (CH_2), 47.8 (CH), 22.4 (CH_3); ESIMS m/z 380 $[M + H]^+$.

Compound ***22:*** ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 7.76 (1H, d, J = 2.8 Hz), 7.43 (1H, d, J = 5.3 Hz), 7.28 (1H, dd, J = 5.3, 2.8 Hz), 4.28 (1H, m), 3.98 (1H, m), 3.93 (1H, dd, J = 11.5, 4.8 Hz), 3.69 (1H, dd, J = 11.5, 5.1 Hz), 1.44 (3H, d, J = 7.1 Hz); ^{13}C NMR (126 MHz, $CDCl_3$) δ_C 162.5, 136.6, 131.1, 130.1, 127.6, 85.2, 62.7, 47.7, 21.3; HRESIMS m/z 214.0350 $[M + H]^+$ (calcd for $C_9H_{11}NOS_2$, 214.0355).

Compound ***23:*** ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 7.76 (1H, d, J = 2.8 Hz), 7.43 (1H, d, J = 5.3 Hz), 7.28 (1H, dd, J = 5.3, 2.8 Hz), 4.28 (1H, m), 3.98 (1H, m), 3.93 (1H, dd, J = 11.5, 4.8 Hz), 3.69 (1H, dd, J = 11.5, 5.1 Hz), 1.44 (3H, d, J = 7.1 Hz); ^{13}C NMR (126 MHz, $CDCl_3$) δ_C 164.8, 135.1, 129.3, 126.8, 126.4, 84.8, 62.7, 46.9, 21.2; HRESIMS m/z 214.0350 $[M + H]^+$ (calcd for $C_9H_{11}NOS_2$, 214.0355).

Compound ***24:*** ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 7.69 (2H, d, J = 8.6 Hz), 6.84 (2H, d, J = 8.6 Hz), 4.30 (1H, ddd, J = 5.9, 5.8, 5.2 Hz), 3.97 (2H, t, J = 6.9 Hz), 3.89 (1H, dq, J = 6.6, 5.6 Hz), 3.83 (1H, m), 3.68 (1H, m), 1.76 (2H, m), 1.48 (2H, m), 1.45 (3H, d, J = 6.7 Hz), 0.96 (3H, t, J = 7.5 Hz); HSQC (500 MHz, $CDCl_3$) δ_C 129.9 (CH), 114.2 (CH), 85.9 (CH), 67.9 (CH_2), 63.5 (CH_2), 46.8 (CH), 31.1 (CH_2), 21.4 (CH_3), 19.3, (CH_2), 13.8 (CH_3); ^{13}C NMR (126 MHz, $CDCl_3$) δ_C 168.3, 161.7, 129.9 × 2, 125.7, 114.3 × 2, 85.8, 67.9, 63.5, 46.8, 31.2, 21.4, 19.2, 13.8; HRESIMS m/z 280.1360 $[M + H]^+$ (calcd for $C_{15}H_{21}NO_2S$, 280.1366).

Compound ***25:*** ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 7.68 (2H, d, J = 8.6 Hz), 6.84 (2H, d, J = 8.6 Hz), 4.29 (1H, m), 3.95 (2H, t, J = 6.9 Hz), 3.88 (1H, m), 3.79 (1H, m), 3.67 (1H, m), 1.76 (2H, m), 1.41 (3H, d, J = 6.7 Hz), 1.45−1.25 (6H, m), 0.89 (3H, t, J = 7.5 Hz); HSQC (500 MHz, $CDCl_3$) δ_C 129.9 (CH), 113.9 (CH), 86.1 (CH), 68.7 (CH_2), 65.1 (CH_2), 46.4 (CH), 28.6 (CH_2), 27.5 (CH_2), 26.7 (CH_2), 21.2 (CH_3), 14.9, (CH_3); ^{13}C NMR (126 MHz, $CDCl_3$) δ_C 169.2, 162.6, 130.8, 126.1, 115.1, 86.6, 69.1, 64.2, 47.6, 32.4, 30.0, 26.5, 23.5, 22.2, 14.9; HRESIMS m/z 308.1671 $[M + H]^+$ (calcd for $C_{17}H_{25}NO_2S$, 308.1679).

Compound ***26:*** ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 7.70 (2H, d, J = 8.6 Hz), 6.85 (2H, d, J = 8.6 Hz), 4.31 (1H, m), 3.97 (2H, t, J = 6.9 Hz), 3.84 (1H, m), 3.69 (1H, m), 3.67 (1H, m), 1.78 (2H, m), 1.46 (2H, m), 1.44 (3H, d, J = 6.7 Hz), 1.38−1.23 (p8H, m), 0.89 (3H, t, J = 7.5 Hz); HSQC (500 MHz, $CDCl_3$) δ_C 130.0 (CH), 114.1 (CH), 86.3 (CH), 68.3 (CH_2); 63.8 (CH_2), 46.6 (CH), 28.1 (CH_2), 29.7 (CH_2), 26.7 (CH_2), 20.1 (CH_3), 15.9 (CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ_C 168.2, 161.7, 129.9, 125.8, 114.3, 85.9, 76.8, 68.2, 63.5, 46.8, 31.8, 29.3, 29.2, 29.1, 26.0, 22.6, 21.4, 14.0; HRESIMS m/z 336.1983 $[M + H]^+$ (calcd for $C_{19}H_{29}NO_2S$, 336.1992).

Compound ***27:*** ^{1}H NMR (600 MHz, acetone-d_6) δ_H 9.00 (1H, brs), 7.89 (2H, m), 7.67 (1H brs), 7.60 (1H, m), 7.52 (2H, m), 4.75 (1H, brs), 4.04 (1H, brs), 3.35 (1H, m), 3.21 (1H, m), 1.52 (3H, brs); key HSQC (600 MHz, acetone-d_6) δ_C 83.1 (CH), 51.3 (CH), 29.7, (CH_2), 22.3 (CH_3); HRESIMS m/z 258.1052 $[M + H]^+$ (calcd for $C_{14}H_{15}N_3OS$, 258.1059).

Compound ***28:*** ^{1}H NMR (600 MHz, acetone-d_6) δ_H 8.97 (1H, brs), 7.89 (2H, m), 7.69 (1H brs), 7.57 (1H, m), 7.51 (2H, m), 4.75 (1H, brs), 4.04 (1H, brs), 3.35 (1H, m), 3.21 (1H, m), 1.52 (3H, brs); key HSQC (600 MHz, acetone-d_6) δ_C 79.1 (CH), 49.8 (CH), 26.9 (CH_2), 17.9 (CH_3); HRESIMS m/z 258.1052 $[M + H]^+$ (calcd for $C_{14}H_{15}N_3OS$, 258.1059).

Compound ***29:*** ^{1}H NMR (600 MHz, DMSO-d_6) δ_H 9.05 (1H, brs), 7.88 (2H, d, J = 8.7 Hz), 7.82 (2H, d, J = 8.7 Hz), 7.76 (2H, d, J = 7.7 Hz), 7.59 (1H, brs), 7.54 (2H, t, J = 7.7 Hz), 7.46 (1H, t, J = 7.7 Hz), 4.65 (1H, m), 3.92 (1H, m), 3.07 (1H, dd, J = 15.0, 6.0 Hz), 2.98 (1H, dd, J = 15.0, 8.3 Hz), 1.41 (3H, d, J = 6.8 Hz); ^{13}C NMR (126 MHz, acetone-d_6) δ_C 166.5, 143.9, 139.8, 133.6, 132.2, 131.2, 129.0 (2 × C), 128.9 (2 × C), 128.0 (2 × C), 127.0 (4 × C), 117.1, 82.6, 49.9, 28.6, 21.0; HRESIMS m/z 334.1363 $[M + H]^+$ (calcd for $C_{20}H_{19}N_3S$, 334.1378).

Compound ***30:*** ^{1}H NMR (500 MHz, acetone-d_6) δ_H 8.97 (1H, brs), 7.99 (2H, d, J = 8.7 Hz), 7.78 (2H, d, J = 8.7 Hz), 7.74 (2H, d, J = 7.7 Hz), 7.66 (1H, brs), 7.52 (2H, t, J = 7.7 Hz), 7.44 (1H, t, J = 7.7 Hz), 4.72 (1H, m), 4.25 (1H, m), 3.44 (1H, m), 3.38 (1H, m), 1.37 (3H, d, J = 6.8 Hz); ^{13}C NMR (126 MHz, acetone-d_6) δ_C 166.9, 143.9, 139.8, 133.6, 132.4, 129.0, 128.8, 128.0, 127.0, 116.8, 77.8, 48.4, 25.3, 16.2; HRESIMS m/z 334.1363 $[M + H]^+$ (calcd for $C_{20}H_{19}N_3S$, 334.1378).

PDSP Screening. Percentage inhibition studies, K_i determinations, and agonist and/or antagonist functional data were performed by the National Institute of Mental Health's Psychoactive Drug Screening Program (PDSP). For experimental details refer to the PDSP Web site: http://pdsp.med.unc.edu/.

***In Vivo* Assays.** *Animal Protocol.* This work was performed under IACUC protocol number 15-10007. NIH guidelines for animal research were followed.

Synthetic **1** was suspended in 30% polyethylene glycol 400 with a few drops of DMSO. Compound **1** was administered to mice in a volume of 0.01 mL/g of body weight via the i.p. route.

Maximal Electroshock Test. For the MES, a drop of anesthetic and electrolyte solution (0.5% tetracaine hydrochloride in 0.9% saline) was applied to the eyes of each animal prior to corneal stimulation. The electrical stimulus in the MES test (50 mA, 60 Hz, 0.2 s) was delivered via corneal electrodes by an apparatus similar to that originally described by Woodbury and Davenport.[19] Abolition of the hind leg tonic extensor component is taken as the end point for this test. Efficacy on four mice was compared by evaluating the following i.p.

DOI: 10.1021/acs.jnatprod.7b00317
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doses of **1**: 100, 30, and 10 mg/kg at various time points (0.25, 0.5, 1, 2, and 4 h).

6 Hz "Psychomotor" Seizure Test. The drug administration approach employed in the 6 Hz test was identical to that described above for the MES test. Mice were pretreated with **1** at the doses listed above. At varying times (1/4, 1/2, 1, 2, and 4 h after treatment) individual mice (four at each time point) were challenged with sufficient current, 32 and 44 mA for 3 s, delivered through corneal electrodes to elicit a partial psychomotor seizure.[35] Data were expressed as the number of mice protected out of the number of animals tested over time.

Tail Flick Assay. Thermal pain sensitivity was measured using the IITC Series 8 (model 336TG) automated plantar/tail analgesic meter (IITC Life Science). Mice were placed in a restraint tube, and a light beam (peak intensity 75 mJ, 50% maximal intensity) was focused on the distal tail, 2−5 mm from the tip. Control mice received an equivalent volume of vehicle at the same time of peak effect (TPE) for each test. The mean tail flick latency ± SEM for each group was compared using one-way ANOVA with Dunnett *post hoc* comparison to vehicle-treated mice (GraphPad Prism 5.0).

Hot Plate Test. Mice were allowed to acclimatize for 10 min prior to test. Pain reflexes in response to a thermal stimulus were measured using a hot plate analgesia meter from IITC. The surface of the hot plate was heated to a constant temperature of 55 °C, as measured by a built-in digital thermometer with an accuracy of 0.1 °C. Mice were placed on the hot plate (25.4 cm × 25.4 cm), surrounded by a clear acrylic cage (19 cm tall, open top). The latency to the response of choice (i.e., hindpaw lick, hindpaw flick, or jump) was measured to the nearest 0.1 s by manually stopping a timer when the response was observed. The mouse was immediately removed from the hot plate and returned to its home cage. If a mouse did not respond within 30 s, the test was terminated and the mouse was removed from the hot plate to avoid injury to the animal.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.7b00317.

Additional information (PDF)

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Notes

Note: Some of the compounds described here were the subject of a patent application.

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was supported by NIH R01GM107557 and Fogarty International Center of the National Institutes of Health under Award No. U19TW008163. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. Chemical resources reported in this work were created at the University of Utah. Serotonin receptor screening and functional analysis were generously provided by the National Institute of Mental Health's Psychoactive Drug Screening Program, Contract No. HHSN-271-2013-00017-C (NIMH PDSP). The NIMH PDSP is directed by B. L. Roth M.D., Ph.D., at the University of North Carolina at Chapel Hill, NC, and Project Officer J. Driscoll at NIMH, Bethesda, MD, USA. We thank the NIH/NINDS Epilepsy Therapy Screening Program (ETSP) for the epilepsy model testing performed.

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DOI: 10.1021/acs.jnatprod.7b00317
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DOI: 10.1021/acs.jnatprod.7b00317
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