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A Strategy to Minimize Reactive Metabolite Formation: Discovery of (*S*)-4-(1-Cyclopropyl-2-methoxyethyl)-6-[6-(difluoromethoxy)-2,5-dimethylpyridin-3-ylamino]-5-oxo-4,5-dihydropyrazine-2-carbonitrile as a Potent, Orally Bioavailable Corticotropin-Releasing Factor-1 Receptor Antagonist[†]

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Detailed metabolic characterization of **8**, an earlier lead pyrazinone-based corticotropin-releasing factor-1 (CRF_1) receptor antagonist, revealed that this compound formed significant levels of reactive metabolites, as measured by in vivo and in vitro biotransformation studies. This was of particular concern due to the body of evidence suggesting that reactive metabolites may be involved in idiosyncratic drug reactions. Further optimization of the structure−activity relationships and in vivo properties of pyrazinone-based CRF_1 receptor antagonists and studies to assess the formation of reactive metabolites led to the discovery of **19e**, a high affinity CRF_1 receptor antagonist (IC_{50} = 0.86 nM) wherein GSH adducts were estimated to be only 0.1% of the total amount of drug-related material excreted through bile and urine, indicating low levels of reactive metabolite formation in vivo. A novel 6-(difluoromethoxy)-2,5-dimethylpyridin-3-amine group in **19e** contributed to the potency and improved in vivo properties of this compound and related analogues. **19e** had excellent pharmacokinetic properties in rats and dogs and showed efficacy in the defensive withdrawal model of anxiety in rats. The lowest efficacious dose was 1.8 mg/kg. The results of a two-week rat safety study with **19e** indicated that this compound was well-tolerated.

Introduction

Stress-related diseases, such as anxiety and depression, affect a significant number of people. Although numerous currently marketed drugs exist to treat these conditions, shortcomings such as delayed onset of action, lack of efficacy in a subset of patients, and unwanted side effects highlight the need for new pharmacological approaches. The discovery of corticotropin releasing factor-1 (CRF_1) receptor antagonists, which may potentially be used to treat these conditions, has been the focus of research groups for some time.[1−7] CRF,[a] a 41 amino acid neuropeptide first isolated by Vale and co-workers,[8] is the primary regulator of the hypothalamic−pituitary−adrenal (HPA) axis and is primarily produced in the paraventricular region of the hypothalamus. Upon its secretion, CRF acts as the principal agent controlling the release of adrenocorticotropin hormone (ACTH) and other proopiomelanocortin (POMC)-derived peptides from the anterior pituitary gland. Numerous studies have documented the critical role of CRF in the regulation of the endocrine, behavioral, and autonomic responses to stress.[6,9,10] Compelling evidence exists to support the hypothesis that excess levels of CRF contribute to stress-related disorders such as anxiety and depression and that antagonists of CRF_1 receptors may be able to successfully treat these conditions.[9,11−14] Patients suffering from depression have been found to have elevated levels of CRF in cerebrospinal fluid.[15−17] In addition, a blunted ACTH release in response to CRF infusion has been observed,[18] as well as an elevated cortisol response in the dexamethasone/CRF test.[19,20] Moreover, a variety of small molecule CRF_1 receptor antagonists, such as **1** (DMP696),[21] were shown to be efficacious in behavioral models for anxiety and depression in preclinical studies.[1,5]

Over the past several years, a limited number of CRF_1 receptor antagonists have reached clinical development.[1] In a small open-label clinical trial with **2** (R121919, also known as NBI30775)[22] for depression, it was found that depressed patients showed reductions in depression symptoms, as rated by both patients and clinicians.[23,24] A placebo-controlled clinical study, designed to evaluate whether subchronic treatment with **3** (NBI-34041)[25] would decrease the stress hormone response following a psychological stressor, indicated that this drug candidate may improve resistance to psychological stress by reducing stress hormone secretion.[24,26] In a double-blind, placebo-controlled clinical trial to evaluate **4** (CP-316311)[27] for the treatment of major depressive disorder, it was found

[†]We acknowledge the 100th anniversary of the Division of Medicinal Chemistry.

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[a]Abbreviations: CRF, corticotropin-releasing factor; HPA, hypothalamic−pituitary−adrenal; ACTH, adrenocorticotropin hormone; POMC, proopiomelanocortin; SAR, structure−activity relationships; BDC, bile-duct cannulated; GSH, glutathione; cAMP, cyclic adenosine monophosphate.

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that this drug candidate lacked efficacy.[28] A small number of additional compounds, including **5** (CP-376395),[29] **6** (BMS-561388),[30] and **7** (BMS-562086)[31] (Figure 1), were reported to have entered clinical trials. The results from these clinical trials may provide further insights into the clinical utility of CRF_1 receptor antagonists. Although the outcome of the limited number of clinical trials reported to date has been mixed, the prospect that antagonism of CRF_1 receptors may offer therapeutic potential for the treatment of diseases resulting from elevated levels of CRF continues to provide motivation to discover novel CRF_1 receptor antagonists.

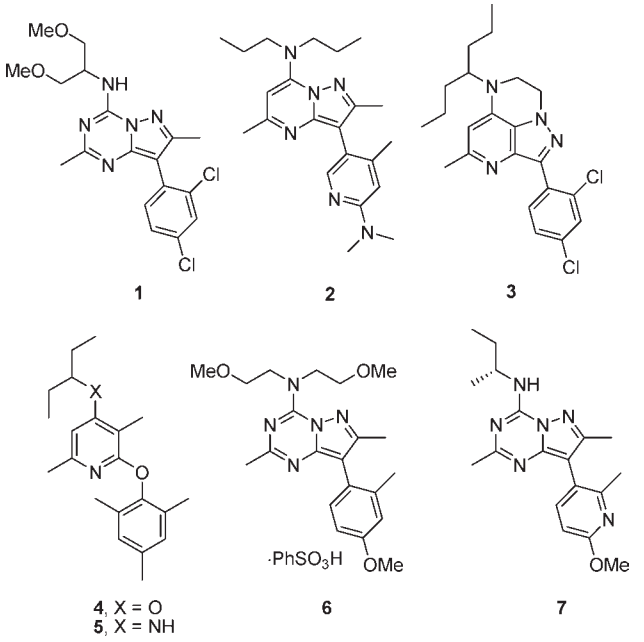


Figure 1. CRF_1 receptor antagonists.

During our investigation into the structure−activity relationships (SAR) of pyrazinone-based CRF_1 receptor antagonists,[32−34] compound **8** (Figure 2) was synthesized and was found to have good pharmacokinetic properties in rats as well as efficacy in the defensive withdrawal model of anxiety in rats.[34] In addition, we described how incorporation of the 1-cyclopropylethyl and difluoromethoxy groups, which were not readily metabolized, in strategic locations of **8** contributed to the improved pharmacokinetic properties of this compound compared to others within this chemotype.[34] However, subsequent in vivo metabolism and disposition studies in bile duct-cannulated (BDC) rats revealed that **8** was extensively metabolized in vivo. Approximately 60% of the total drug-related materials collected in bile (0−7 h) was identified as pyrazinone oxidation metabolites (17%, e.g. **9** and **10**, Figure 2), glutathione (GSH)-related adducts on the pyrazinone ring via epoxidation of the C5−C6 double bond (18%, e.g. **11**) and GSH-related adducts on the phenyl group (25%, e.g. **12**). A detailed account of these metabolism studies is described separately.[35] The formation of excessive levels of GSH adducts, an indication of reactive metabolite formation, was of particular concern due to the body of circumstantial evidence supporting the hypothesis that reactive metabolites may be involved in idiosyncratic drug reactions.[36−38] As a result, an investigation was undertaken as part of the medicinal chemistry optimization process to minimize reactive metabolite formation, to the extent possible, by making appropriate structural modifications.

A three-pronged optimization strategy to reduce the formation of reactive metabolites found with **8** was developed, as illustrated in Figure 3. First, to reduce the bioactivation level of the phenyl group, we directed our efforts toward incorporation of a pyridyl group as an isosteric replacement for the phenyl group. In an in vitro biotransformation study comparing a small number of pyridyl and phenyl analogues, it was found that pyridyl groups showed substantially reduced levels of metabolic activation relative to phenyl groups.[36]

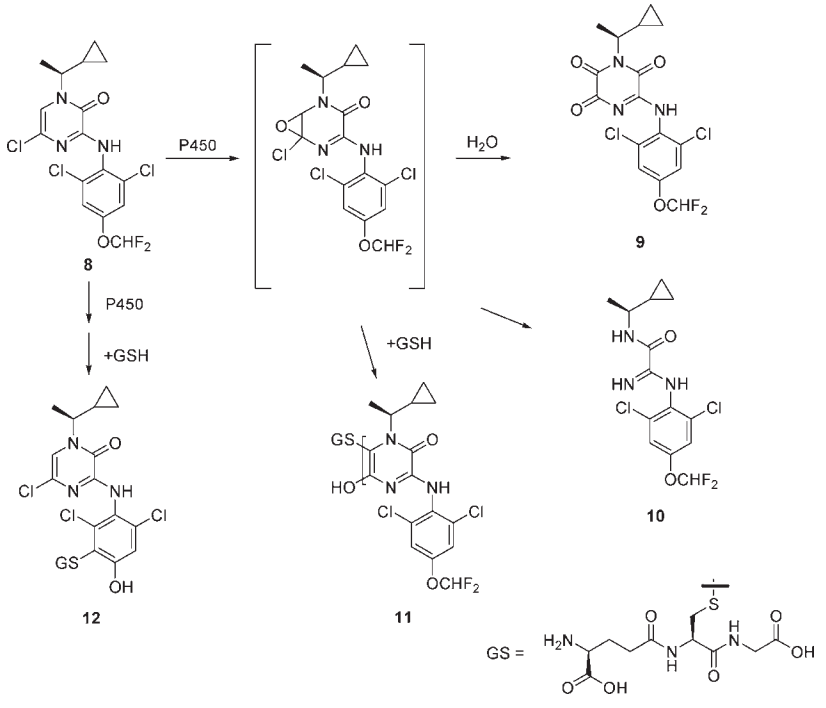


Figure 2. Formation of pyrazinone oxidation metabolites and related glutathione conjugates of compound **8**.

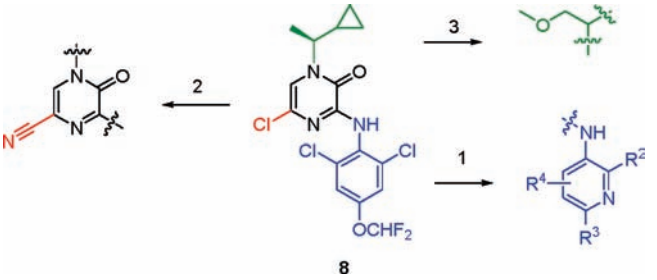


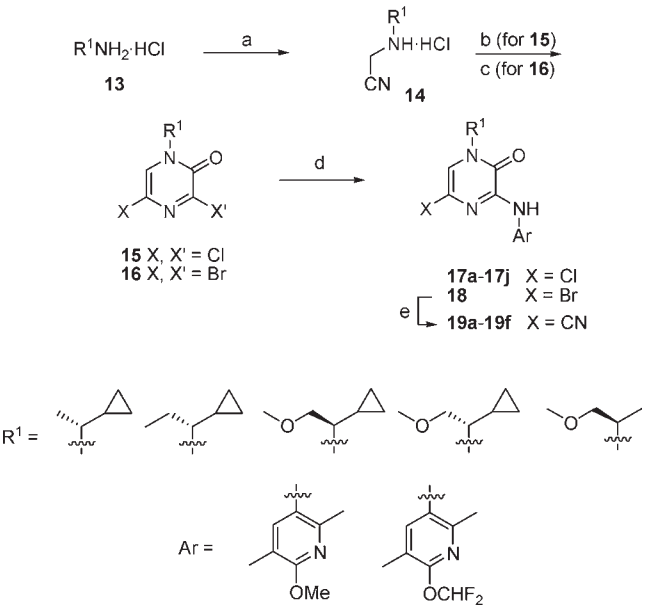
Figure 3. Illustration of a three-pronged approach to reduce the level of reactive metabolite formation on the pyrazinone and phenyl groups of **8**: (1) replacement of the phenyl group with a pyridyl group, (2) replacement of the 5-chloro substituent with a 5-cyano group, as the SAR allowed, and (3) inclusion of a benign metabolic "soft spot" (methoxy group) in the molecule.

A second area of focus was to reduce the level of pyrazinone bioactivation, measured as the sum of pyrazinone oxidation and GSH adduct formation on the pyrazinone ring, by decreasing the propensity of the pyrazinone ring to undergo epoxidation. A series of thrombin inhibitors containing a 6-methylpyrazinone core had been found to generate reactive metabolites.[39] It was discovered that replacement of the C6 methyl group with a chloro group rendered the pyrazinone less susceptible to epoxidation and led to an analogue with diminished potential for reactive metabolite formation.[36] Although 5-chloropyrazinones may be sufficiently electron deficient so as to mitigate reactive metabolite formation in some cases, we nevertheless sought to replace the 5-chloro substituent with the more strongly electron-withdrawing 5-cyano group, as the SAR allowed, to further reduce the potential for oxidation of the pyrazinone ring. Encouragingly, we had observed in an in vitro experiment that a C5-cyano-pyrazinone showed decreased levels of reactive metabolite formation relative to a C5-chloropyrazinone.[35] Third, we hypothesized that including a benign metabolic "soft spot" in the molecule would aid in diverting metabolism away from the pyrazinone core. It was anticipated that metabolism at this "soft spot" would result in the formation of innocuous metabolites and further minimize the formation of undesired reactive metabolites. We previously observed that including a methoxy group in R^1 resulted in an increased rate of clearance in some analogues as a result of *O*-demethylation, followed to some extent by oxidation of the resultant alcohol to a carboxylic acid (data not presented). Incorporating a methoxy group within R^1 may involve making a trade-off between low clearance and minimization of GSH adduct formation.

Results and Discussion

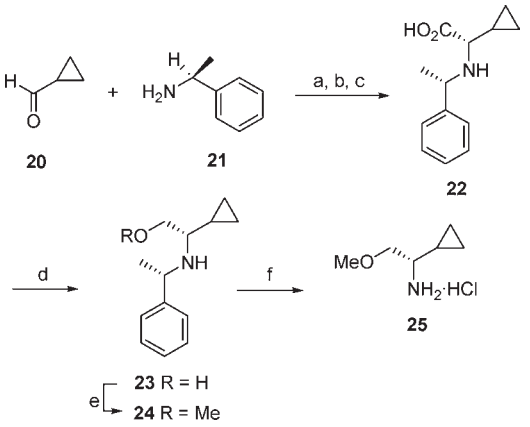
Chemistry. The synthetic route to prepare 1-alkyl-3-*N*-arylpyrazin-2-ones is summarized in Scheme 1, as previously described.[33] Briefly, treatment of alkylamine hydrochlorides **13** with chloroacetonitrile in the presence of potassium iodide and potassium carbonate in acetonitrile furnished the aminoacetonitrile intermediate **14** in high yield. The aminoacetonitrile intermediate was then condensed with oxalyl chloride or oxalyl bromide to form **15** or **16**, respectively. Coupling of **15** or **16** with a variety of aryl amines in the presence of NaHMDS then furnished the desired pyrazinone products **17** or **18**, respectively. Synthesis of 5-cyanopyrazinone analogues (**17**) was generally completed by treatment of bromopyrazinones **18** with zinc cyanide in the presence of a palladium catalyst.[40] Synthesis of

Scheme 1[a]



[a] Reagents and conditions: (a) chloroacetonitrile, KI, K_2CO_3, CH_3CN, 50 °C (84−96%); (b) $(COCl)_2$, toluene, 55 °C (43−71%) or $(COCl)_2$, dioxane/CH_2Cl_2, 55 °C (69−74%); (c) $(COBr)_2$, CH_2Cl_2, 45 °C (56−68%); (d) NaHMDS, $ArNH_2$, THF; (e) $Zn(CN)_2$, $Pd_2(dba)_3$, dppf, DMF, H_2O.

Scheme 2[a]

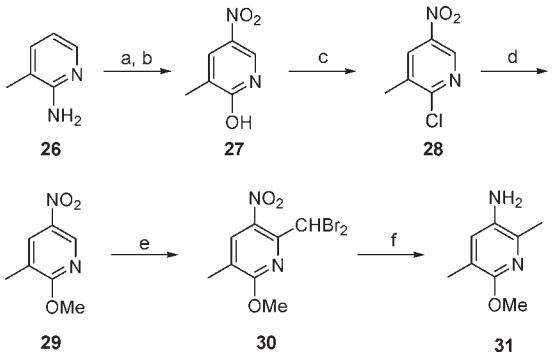


[a] Reagents and conditions: (a) MeOH, 75 °C; (b) KCN, MeOH; (c) conc HCl, 95 °C (52%, 3 steps); (d) $BH_3 \cdot SMe_2$, THF (44%); (e) NaH, MeI, THF (85%); (f) H_2, $Pd(OH)_2/C$, EtOH (99%).

5-cyanopyrazinone analogues (**19**) could also be carried out from chloropyrazinones **17** with higher catalyst loading.

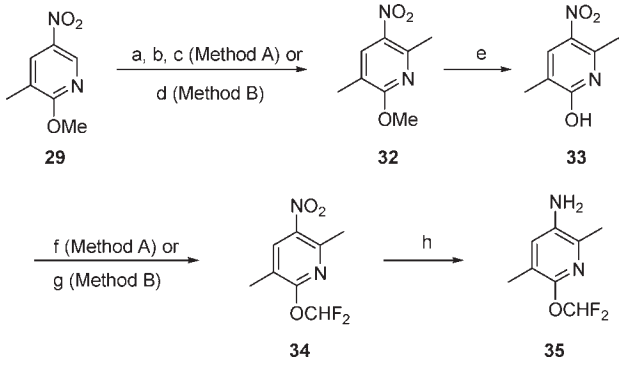
The amine groups that comprise R^1 were synthesized as previously described.[33] In addition, an alternate route was developed for the asymmetric synthesis of (*S*)-2-methoxy-1-cyclopropylethylamine (**25**) (Scheme 2). The synthesis of **22** was completed using the method of Bayston et al.,[41] whereby condensation of commercially available cyclopropane carboxaldehyde (**20**) and (*S*)-1-phenethyl amine (**21**) resulted in the formation of a chiral imine, which was treated in situ with potassium cyanide. Hydrolysis of the resultant α-amino nitrile intermediate under acidic conditions provided **22**. Borane reduction of **22** afforded **23**, which was then treated with sodium hydride and methyl iodide to provide **24** in 85% yield. Reductive cleavage of the benzyl group furnished **25** in high yield.

Scheme 3[a]



[a] Reagents and conditions: (a) HNO_3, H_2SO_4; (b) HNO_3, H_2O (75%, 2 steps); (c) $POCl_3$, 120 °C (92%); (d) NaOMe, MeOH (97%); (e) $CHBr_3$, KO*t*-Bu, THF, −78 °C (94%); (f) H_2, Pd/C, EtOH (73%).

Scheme 4[a]



[a] Reagents and conditions: (a) *t*-butylchloroacetate, KO*t*-Bu, THF; (b) TFA, reflux; (c) K_2CO_3, DMF (65%, 3 steps); (d) NaH, $Me_3SO^+I^-$, DMSO (41%); (e) conc HCl, 110 °C (94%); (f) $FSO_2CF_2CO_2SiMe_3$, NaH, CsF, MeCN (92%); (g) $FSO_2CF_2CO_2H$, NaH, MeCN (96%); (h) H_2, Pd/C, EtOH (89%).

Schemes 3 and 4 describe the synthesis of two pyridyl groups that were synthesized as isosteric replacements for the phenyl groups used in previously described analogues.[33,34] Synthesis of **31** began with commercially available 3-methylpyridin-2-amine (**26**) (Scheme 3). A modification of the two-step, one-pot procedure of Hawkins and Roe[42] afforded **27** in 75% overall yield. Subsequent treatment of **27** with phosphorus oxychloride followed by addition of sodium methoxide afforded **29** in high yield. Completion of the synthesis of **31** involved incorporation of a methyl group at the 6-position of **29** using a vicarious nucleophilic substitution reaction. Several conditions were explored, and it was found that treatment of **29** with bromoform and potassium *t*-butoxide resulted in the formation of **30** in high yield.[43] Simultaneous reduction of the nitro group and debromination by treatment with hydrogen in the presence of palladium on carbon furnished **31** in 73% yield.

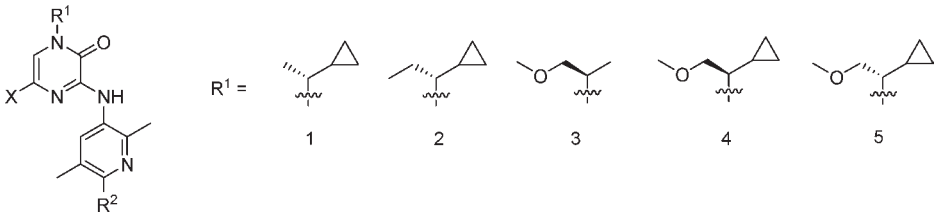
Synthesis of 6-(difluoromethoxy)-2,5-dimethylpyridin-3-amine (**35**) (Scheme 4) commenced with compound **29**, prepared as described in Scheme 3. We chose to investigate alternative nucleophiles for the vicarious nucleophilic substitution reaction. Two viable reaction conditions were identified. Method A involved treatment of **29** with *t*-butyl chloroacetate in the presence of potassium *t*-butoxide, followed by hydrolysis of the resultant ester to give the corresponding carboxylic acid. Decarboxylation was achieved by heating under basic conditions in DMF to provide **32**. Alternatively, in method B, the 6-methyl group was installed directly by treatment of **29** with trimethylsulfoxonium iodide in the presence of sodium hydride and DMSO. While method B is only a single step, method A proceeded in a higher overall yield and proved more convenient for larger scale preparations of **32**. Completion of the synthesis of **35** began by heating **32** under acidic conditions for 1−2 h to furnish **33** in excellent yield. The difluoromethoxy intermediate (**34**) was prepared by selective *O*-alkylation of **33**. Initial preparations involved use of sodium chlorodifluoroacetate;[44] however, yields were generally low and variable. The preferred methods involved the use of either trimethylsilyl 2,2-difluoro-2-(fluorosulfonyl)acetate in combination with cesium fluoride[45,46] (method A) or 2,2-difluoro-2-(fluorosulfonyl)-acetic acid[47] (method B). Both conditions provided **34** in high yield. Conversion of **34** to **35** was accomplished by reduction of the nitro group with palladium on carbon under a hydrogen atmosphere.

Biology. Compounds were tested in a CRF_1 receptor binding titration assay using rat frontal cortex homogenate. Inhibition of specific binding of [^{125}I]Tyr-*o*-CRF by test compounds was measured to determine the receptor binding affinity.[33] Potent compounds were subsequently incubated with rat and human liver microsomes to evaluate their metabolic stability. Selected compounds were examined for their functional activity in Y79 cells to assess antagonist properties and were screened for binding affinity at CRF_2 receptors. The CRF_2 receptor binding titration assay employed porcine choroid plexus homogenate and [^{125}I]-sauvagine binding inhibition to determine binding affinities. Compounds with appropriate profiles were assessed in pharmacokinetic studies, followed by evaluation in the defensive withdrawal model of anxiety in rats.[21,48] Along with the behavioral studies, the compounds were examined in an ex vivo binding assay to determine CRF_1 receptor occupancy. In vivo plasma levels after oral dosing were also measured. Key compounds were also assessed for the level of reactive metabolite formation in vitro, by incubation with NADPH-fortified rat and human liver microsomes in the presence of GSH, and in vivo, in BDC rats.[49]

On the basis of the SAR previously developed for N^3-phenyl pyrazinones,[33] we focused on a group of analogues wherein the 4-methoxy-2,5-dimethylaniline moiety was replaced with a similarly substituted pyridyl substituent (Table 1). Compounds **17a**−**17d** were very potent ($IC_{50} \leq$ 1.5 nM); however, replacement of the cyclopropyl group in R^1 with a methyl group resulted in a 12-fold decrease in potency (compare **17e** vs **17b**). Replacement of the chloro substituent (X) of **17a** with a cyano group (**19a**) resulted in an 8-fold decrease in potency. The loss in potency could be regained by replacement of the (*S*)-1-cyclopropyl-2-methoxyethyl substituent ($R^1 = 5$) with the (*R*)-1-cyclopropylpropyl group ($R^1 = 2$) (**19b**). The metabolic stability data (Table 1) for compounds where R^2 = OMe suggested a moderate rate of metabolism in human liver microsomes and, in general, a higher rate of clearance in rats. An improved metabolic stability profile would be desirable.

On the basis of our previous experience with this chemotype, we recognized that replacement of the methoxy substituent on the pyridyl moiety (R^2 = OMe) with a difluoromethoxy substituent should improve the metabolic stability of these compounds. A group of analogues containing the difluoromethoxy group at R^2 was synthesized, and indeed, incubation of **17f**−**17j** and **19d**−**19f** (Table 1) with

Table 1. CRF_1 Receptor Binding Affinities and Metabolic Stability Data for **17a**−**17j** and **19a**−**19f**



compd	R^1	R^2	X	IC_{50} (nM)[a]	metabolic stability (HLM, % remaining)[b,c]	metabolic stability (RLM, % remaining)[c,d]
17a	5	OMe	Cl	1.5 ± 0.6	48	24
17b	4	OMe	Cl	1.1 ± 0.2	40	13
17c	2	OMe	Cl	0.28 ± 0.05	51	5
17d	1	OMe	Cl	1.5 ± 0.5	77	55
17e	3	OMe	Cl	14 ± 4	39	8
19a	5	OMe	CN	12 ± 3	60	51
19b	2	OMe	CN	1.7 ± 0.5	81	38
19c	1	OMe	CN	10 ± 3	99	72
17f	1	$OCHF_2$	Cl	0.30 ± 0.03	100	86
17g	2	$OCHF_2$	Cl	0.21 ± 0.03	100	56
17h	3	$OCHF_2$	Cl	1.6 ± 0.3	87	66
17i	4	$OCHF_2$	Cl	0.50 ± 0.09	84	46
17j	5	$OCHF_2$	Cl	0.24 ± 0.10	93	69
19d	1	$OCHF_2$	CN	0.82 ± 0.09	100	89
19e	5	$OCHF_2$	CN	0.86 ± 0.12	74	70
19f	4	$OCHF_2$	CN	1.4 ± 0.3	78	64

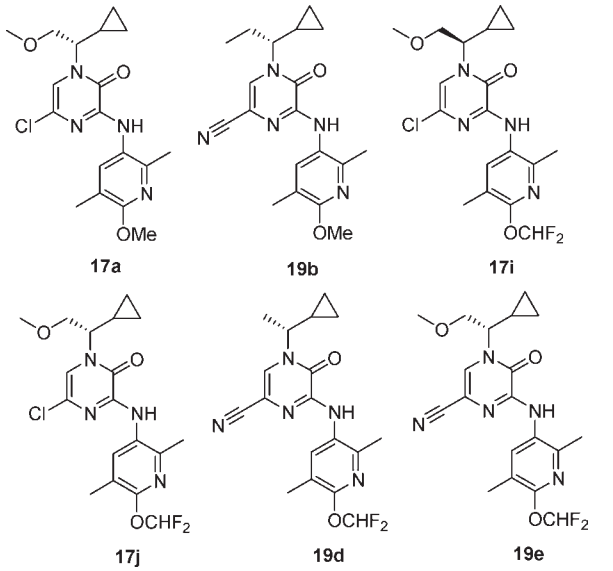
[a] All values are the average of at least $n = 3 \pm$ standard deviation. The IC_{50} of *o*-CRF = 2.9 ± 1.0 nM and the IC_{50} of **1** = 1.2 ± 0.2 nM in this assay. [b] HLM = human liver microsomes. [c] Percent remaining after 10 min. [d] RLM = rat liver microsomes.

human and rat liver microsomes revealed that compounds with the difluoromethoxy group at R^2 had improved metabolic stability in both human and rat liver microsomes compared to the corresponding methoxypyridyl analogues. In addition, analogues containing the difluoromethoxy group at R^2 were more potent than the corresponding analogues containing a methoxy group at R^2. In many cases, these compounds had subnanomolar IC_{50}'s (Table 1). As before, it was observed that replacement of the chloro group at X with a cyano group resulted in a slight decrease in potency (compare **17f** vs **19d** and **17j** vs **19e**); nevertheless, the binding affinities of **19d** and **19e** were subnanomolar.

Shown in Table 2 are the pharmacokinetic profiles of **17a**, **19b**, **17i**, **17j**, **19d**, and **19e** after iv (2 mg/kg) and oral dosing (10 mg/kg) in Sprague−Dawley rats. Compounds **17a** and **19b** were of interest as a result of their potency and desirable structural features. Both compounds contained a pyridyl group in place of a phenyl group. In addition, **17a** contained a methoxy group within R^1 and **19b** contained a cyano group on the pyrazinone ring. Compound **17a** had a moderately high rate of clearance in rats (38 mL/min/kg) and **19b** had a high rate of clearance (53 mL/min/kg), most likely due to oxidation of the top-chain alkyl group (R^1). Both **17a** and **19b** had low oral bioavailability when dosed as an aqueous suspension (1% polysorbate 80 in 0.5% methylcellulose). The low aqueous solubility of **17a** and **19b** likely contributed to their low oral bioavailability. The aqueous solubility of **17a** (LogD = 3.9[50]) was 6 μg/mL at pH 6.5 and 116 μg/mL at pH 1, and the aqueous solubility of **19b** (LogD = 4.1) was 1 μg/mL at pH = 6.5 and 23 μg/mL at pH = 1. The improved solubility of **17a** at low pH is likely due to its higher pK_a value compared to **19b** (3.1 for **17a** vs < 2 for **19b**).[51] Because of the low aqueous solubility of these compounds, they were also dosed orally as a solution in an oleoyl macrogolglycerides-based vehicle[52] (Table 2). An improvement in the oral bioavailability was observed (F = 26% and 33%, respectively), although it was still modest.

On the basis of the metabolic stability results shown in Table 1, it appeared that compounds **17i**, **17j**, **19d**, and **19e** may have improved rat pharmacokinetic profiles relative to **17a** and **19b**. When dosed iv, the rate of clearance of these four compounds was comparable to or only somewhat lower than that of **17a** (Table 2) despite their substantially greater in vitro metabolic stability in rats (Table 1). The low correlation between the in vitro metabolic stability results obtained by incubation with rat liver microsomes and the in vivo clearance in rats may be due to the lipophilic nature of these compounds, resulting in a low unbound fraction[53] and relatively large volume of distribution. When compounds **17i**, **17j**, **19d**, and **19e** ($R^2 = OCHF_2$) were dosed orally, a substantial increase in AUC, C_{max}, and oral bioavailability was observed relative to **17a** and **19b** (R^2 = OMe). In addition, it was discovered that the *S* enantiomer (**17j**) had a nearly 2-fold higher exposure, as indicated by the AUC value, and a corresponding increase in oral bioavailability (F = 86%) compared to the *R* enantiomer (**17i**) (F = 48%). After oral administration, the C_{max} in plasma for **17i**, **17j**, **19d**, and **19e** occurred 2−4 h after dosing. As shown in Table 2, **17j**, **19d**, and **19e** each had an improved oral bioavailability when dosed as a solution compared to dosing as an aqueous suspension, likely due to their low aqueous solubility. For example, the aqueous solubility of **19e** (LogD = 3.9) was 4 μg/mL at pH 6.5 and 3.0 μg/mL at pH 1. Unlike compounds **17a** and **19b**, there was no improvement in solubility at low pH, likely due to the reduced basicity of the pyridyl nitrogen (pK_a < 2 for **19e**). It was found in additional studies that **19e** had somewhat greater

Table 2. Discrete Pharmacokinetic Properties of Compounds **17a**, **19b**, **17i**, **17j**, **19d**, and **19e** in Rats



PK parameters	17a	19b	17i	17j	19d	19e
iv dose (2 mg/kg)[a]						
Cl (mL/min/kg)	38	53	31	36	36	29
V_{ss} (L/kg)	19	11	42	15	11	11
$t_{1/2}$ (h)	19	9.0	16	9.2	7.2	9.3
po dose (10 mg/kg)[b]						
AUC (nM·h)	1300	240	ND[d]	7000	4600	4100
C_{max} (nM)	180	50	ND	770	560	860
F%	11	3	ND	66	38	31
po dose (10 mg/kg)[c]						
AUC (nM·h)	3000	3000	4700	9000	9900	8800
C_{max} (nM)	510	590	440	1300	1400	1600
F%	26	33	48	86	82	66

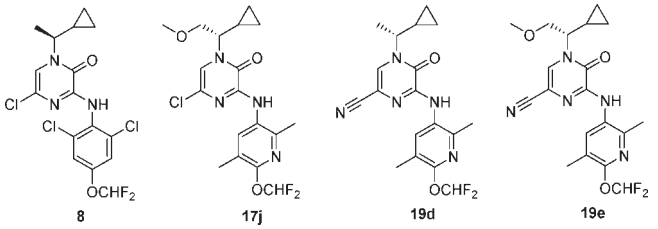
[a] Vehicle: PEG/ethanol, 90:10 (v/v); $n = 3$ rats. [b] Vehicle: 1% polysorbate 80 in 0.5% methylcellulose; $n = 3$ rats. [c] Vehicle: oleoyl macrogolglycerides[52]/DMAC/polysorbate 80, 85:10:5; $n = 3$ rats. [d] ND = not determined.

solubility in simulated gastric fluid (15 μg/mL) and in simulated intestinal fluid (fed) (7.4 μg/mL).

Compounds **17j**, **19d**, and **19e** were chosen for evaluation of potential pyrazinone bioactivation. These compounds incorporated structural features that were meant to minimize the formation of reactive metabolites (vide supra). Cytochrome P450 (CYP)-catalyzed pyrazinone bioactivation of **17j**, **19d**, and **19e** was assessed in vitro by incubation with rat and human liver microsomes for 30 min at a concentration of 10 μM in the presence of NADPH and GSH to trap potential reactive intermediates (Table 3). The bioactivation potential was measured as the sum of pyrazinone oxidation metabolite formation and GSH adduct formation via the proposed pyrazinone oxidation pathway shown in Figure 2. These three analogues showed a substantially diminished level of pyrazinone oxidation relative to **8**, while their in vitro metabolism turnovers were comparable to or higher than that of **8** in human liver microsomal and rat liver microsomal incubations. Among them, **19e** produced the lowest level of pyrazinone oxidation metabolites and related GSH adducts, with the combined amount accounting for $< 2\%$ and $< 4\%$ of the total drug related materials in human and rat liver microsomal incubations, respectively.[54] The major in vitro metabolic pathway with **19e** was identified as *O*-demethylation.

Compound **19e** was subsequently evaluated in metabolism and disposition studies in vivo in BDC rats (20 mg/kg, oral). Plasma, bile, and urine samples were collected and analyzed by HPLC/UV/MS. Compound **19e** was the predominant drug-related species in plasma, and the main circulating metabolite was the alcohol derived from *O*-demethylation. Compound **19e** was extensively metabolized in rat liver with only 3% of the total drug-related materials being excreted as the unchanged parent drug in bile samples. Metabolic analysis indicated that the major metabolic clearance pathways in liver were *O*-demethylation, followed by oxidation of the resultant primary alcohol to the carboxylic acid, which to some extent underwent subsequent glucuronidation. LC/MS analysis did not reveal any sign of acyl migration of the glucoronide product, a potential bioactivation mechanism. In rat urine, the major metabolites detected were formed through either *O*-demethylation or oxidation of the methyl

Table 3. Percentage of Parent Compound Remaining and Pyrazinone Oxidation Metabolites and GSH Adducts Formed via Pyrazinone Oxidation in NADPH- and GSH-Fortified Human and Rat Liver Microsomal Incubations (30 min) with **8**, **17j**, **19d**, and **19e** at 10 μM Substrate Concentrations



	human liver microsomes[a]			rat liver microsomes[a]		
compd	% parent compd remaining	% pyrazinone oxidation metabolites	% GSH adducts	% parent compd remaining	% pyrazinone oxidation metabolites	% GSH adducts
8	85	12	2	74	20	5
17j	75	1	5	42	1	3
19d	88	< 1	7	80	< 1	8
19e	56	< 1	1	82	< 1	3

[a] The results were obtained after incubation of each compound with human and rat liver microsomes for 30 min.

groups on the pyridyl substituent. In bile and urine (0−24 h), the amount of GSH adducts, identical to those detected in vitro, was estimated to be only 0.1% of the total drug-related materials.[55] No pyrazinone oxidation metabolites were detected in bile, urine, or plasma. It was previously noted in studies with **8** (vide supra) that approximately 60% of the total drug-related materials collected in bile (0−7 h) was identified as pyrazinone oxidation metabolites (17%, e.g. **9** and **10**, Figure 2), GSH-related adducts on the pyrazinone ring via epoxidation of the C5−C6 double bond (18%, e.g. **11**, Figure 2), and GSH-related adducts on the phenyl group (25%, e.g. **12**). These results indicate that the excessive levels of reactive metabolite formation arising from metabolism of **8** in vivo, detected as GSH adducts and pyrazinone oxidation metabolites, were lowered through structural modifications in key regions of the molecule to provide **19e**, in which the level of metabolic activation was minimal.

Additional profiling of **19e** was also conducted. The antagonist properties of **19e** were assessed by measuring its inhibition of CRF-stimulated cyclic adenosine monophosphate (cAMP) production in human Y-79 retinoblastoma cells. Compound **19e** produced a concentration-dependent inhibition of CRF (1nM)-induced cAMP production with an IC_{50} value of 1.9 ± 0.1 nM and completely suppressed CRF-stimulated cAMP production at higher concentrations, indicating that this compound behaves as an antagonist. In addition, **19e** was not active in a CRF_2 receptor binding assay (CRF_2 IC_{50} > 10 μM).[56] Compound **19e** was also profiled in a broad array of receptor binding assays.[57] Compound **19e** (10 μM) produced < 50% inhibition of binding to 43 different receptors, transporters, and ion channels.

Compound **19e** was tested in rats in the defensive withdrawal model of anxiety to determine behavioral efficacy.[21,48] CRF_1 receptor occupancy and plasma concentrations at each dose were also measured. In this experiment, the time required for a rat to emerge from a darkened chamber placed within an open, illuminated field was measured. A compound was considered to have an anxiolytic effect if the latency time period for the rat to emerge from the chamber was significantly reduced relative to vehicle-treated animals. Figure 4 and Table 4 summarize the results of the behavioral studies for **19e** following oral dosing at 0.56, 1, 1.8, and 3 mg/kg. Figure 4a shows exit latencies for each dose at 60 min after oral administration of either **19e** or the positive control compound **1** (dosed at 10 mg/kg).[58] The results show that **19e** was effective at reducing exit latency at doses of 1.8 and 3 mg/kg (n = 8) (Table 4). At the lowest efficacious dose of 1.8 mg/kg, **19e** reduced exit latency by 60% relative to vehicle-treated controls. CRF_1 receptor occupancy in the parietal cortex was determined by ex vivo autoradiography.[58] Compound **19e** occupied CRF_1 receptors in a dose-dependent manner (Figure 4b). CRF_1 receptor occupancy greater than 50% was necessary to achieve a statistically significant anxiolytic effect.

Shown in Table 5 is the pharmacokinetic profile of **19e** in beagle dogs (fasted) after iv (2 mg/kg) and oral dosing. Compound **19e** had a low rate of clearance (Cl = 7.3 mL/min/kg), a long iv half-life ($t_{1/2}$ = 30 h), and a volume of distribution of 13 L/kg. Compound **19e** had high plasma exposures, as measured by the AUC values, and had excellent oral bioavailability when dosed as either an aqueous suspension (2 mg/kg) or as a solution (3 mg/kg) (F = 53% and 70%, respectively).

Compound **19e** was evaluated in a two-week rat safety study at doses of 20, 60, and 175 mg/kg. No significant toxicity findings, gross observations, or histopathological findings were observed at all dose levels. The results of this study indicated that this compound was well-tolerated.

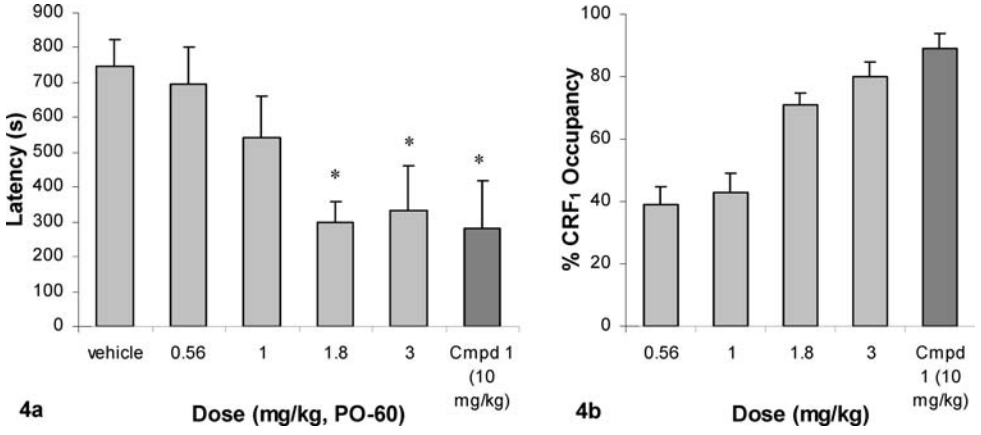


Figure 4. (a) Anxiolytic-like effects of **19e** in the defensive withdrawal test in rats at 0.56, 1, 1.8, and 3 mg/kg with **1** as a positive control (* p < 0.05 vs vehicle) and (b) CRF_1 receptor occupancy of **19e** in rats at 0.56, 1, 1.8, and 3 mg/kg with **1** as a positive control.

Table 4. Mean Total and Plasma Free Concentrations and CRF_1 Receptor Occupancies in the Defensive Withdrawal Test in Rats Following Oral Doses of Compound **19e**

oral dose (mg/kg)	mean total plasma conc (nM)[a,b]	mean plasma free conc (nM)[a,c]	CRF_1 receptor occupancy (%)[a,d,e]	% decrease in exit latency[b]
0.56	24 ± 4	0.84 ± 0.14	39 ± 6	7
1.0	45 ± 5	1.6 ± 0.2	43 ± 6	28
1.8	83 ± 7	2.9 ± 0.3	71 ± 4	60[f]
3.0	140 ± 10	5.0 ± 0.4	80 ± 5	56[f]

[a] ±SEM. [b] n = 8. [c] Based on an unbound fraction of 3.5% in plasma determined by equilibrium dialysis. [d] n = 4. [e] Receptor occupancy of **1** at 10 mg/kg = 89 ± 5. [f] p < 0.05 vs vehicle.

Table 5. Discrete Pharmacokinetic Properties of Compound **19e** in Beagle Dogs

PK parameters	**19e** in dogs
iv dose (2 mg/kg)[a]	
Cl (mL/min/kg)	7.3
V_{ss} (L/kg)	13
$t_{1/2}$ (h)	30
po dose (2 mg/kg)[b]	
AUC (nM·h)	7600
C_{max} (nM)	880
F%	53
po dose (3 mg/kg)[c]	
AUC (nM·h)	17000
C_{max} (nM)	1400
F%	70

[a] Vehicle: PEG/ethanol, 90:10 (v/v); $n = 4$. [b] Vehicle: 0.2% polysorbate 80 in 0.5% methylcellulose; $n = 4$. [c] Vehicle: oleoyl macrogolglycerides[52]/DMAC/polysorbate 80, 85:10:5; $n = 2$.

In addition, **19e** did not alter motor coordination and balance in rats at doses of up to 60 mg/kg in a rotarod study.

Conclusion

In conclusion, studies to minimize the formation of reactive metabolites in the pyrazinone chemotype led to the discovery of (*S*)-4-(1-cyclopropyl-2-methoxyethyl)-6-[6-(difluoromethoxy)-2,5-dimethylpyridin-3-ylamino]-5-oxo-4,5-dihydropyrazine-2-carbonitrile (**19e**),[59] a high affinity CRF_1 receptor antagonist ($IC_{50} = 0.86$ nM) and a potent inhibitor of CRF-stimulated cAMP production in human Y-79 retinoblastoma cells ($IC_{50} = 1.9$ nM). We have shown that excessive levels of reactive metabolite formation arising from metabolism of **8** could be lowered to minimal levels by: (1) replacement of the phenyl group with a pyridyl group, (2) replacement of the chloro group on the pyrazinone with the more strongly electron withdrawing cyano group, and (3) incorporation of a methoxy group within R^1 to act as a metabolic "soft spot" and aid in directing metabolism away from the pyrazinone ring. In addition, a novel 6-(difluoromethoxy)-2,5-dimethylpyridin-3-amine group, which contributed to the potency and improved in vivo properties of **19e** and related analogues, was discovered. Compound **19e** had excellent pharmacokinetic properties in rats and dogs and was efficacious in the defensive withdrawal model of anxiety in rats. The lowest efficacious dose was 1.8 mg/kg. The results of a two-week rat safety study with **19e** indicated that this compound was well-tolerated. On the basis of its overall profile, **19e** was selected as a candidate for further development.

Experimental Section

Chemistry. All procedures were carried out under a nitrogen atmosphere unless otherwise indicated using anhydrous solvents purchased from commercial sources without further purification. Reactions requiring anhydrous conditions were performed in glassware, which was flame-dried or oven-dried and placed under a nitrogen atmosphere. Column chromatography was performed on silica gel using the solvent systems indicated. Solvent systems are reported as v/v percent ratios. All reactions were monitored by TLC using EM Science, 0.25 mm, precoated silica gel plates or by LC/MS. Yields refer to chromatographically and spectroscopically pure compounds, except as otherwise indicated. Melting points were obtained on a Laboratory Devices, Inc. Mel Temp 3.0 melting point apparatus and are uncorrected. Proton NMR spectra were recorded on either a Bruker 400 or 500 MHz NMR spectrometer. Chemical shifts are reported in δ values relative to tetramethylsilane. Atmosphere pressure chemical ionization (APCI) low-resolution mass spectra were obtained on a Finnigan Navigator LC/MS single quadrupole mass spectrometer. Electrospray ionization (ESI) high-resolution mass spectra were obtained on a Finnigan MAT95S or Thermo Scientific MAT900 mass spectrometer. All final products had a purity of ≥95%. The purity of final products was determined by either combustion analysis or HPLC. Combustion analyses were performed by Quantitative Technologies, Inc., Whitehouse, NJ. HPLC purity was measured using two methods for each compound. Method A: Phenominex analytical C18 column (4.6 mm × 50 mm, 5 μm); mobile phase: $A = H_2O$ with 0.1% TFA, B = acetonitrile with 0.1% TFA, 0−1 min, 20% B; 1−7 min, 20% B → 95% B; 7−8 min, 95% B; flow rate = 3 mL/min; $\lambda = 254$ nm; run time = 8 min. Method B: Phenominex analytical Synergi polar RP(phenoxy) column (4.6 mm × 50 mm, 4 μm); mobile phase: $A = 90\%$ H_2O/10% methanol with 0.1% TFA, $B = 90\%$ methanol/10% H_2O with 0.1% TFA, 0−4 min, 40% B → 100% B; 4−6 min, 100% B; flow rate = 4 mL/min; $\lambda = 254$ nm; run time = 6 min.

General procedures for the preparation of compounds **14**−**16** were previously described.[33]

(*S*)-5-Chloro-1-(1-cyclopropyl-2-methoxyethyl)-3-(6-methoxy-2,5-dimethylpyridin-3-ylamino)pyrazin-2(1*H*)-one (17a). To a solution of (*S*)-3,5-dichloro-1-(1-cyclopropyl-2-methoxyethyl)pyrazin-2(1*H*)-one (**15**) (4.00 g, 15.2 mmol) and **31** (2.31 g, 15.2 mmol) in THF (76 mL) at 0 °C was added NaHMDS (31.9 mL, 31.9 mmol, 1 M in THF) dropwise. The reaction mixture was stirred at 0 °C for 2 h. The mixture was transferred to a separatory funnel containing saturated aqueous $NaHCO_3$ solution (50 mL), and the aqueous layer was extracted with ethyl acetate (3 × 100 mL). The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, and concentrated. The residue was purified by column chromatography on silica gel (10% → 40% ethyl acetate in hexanes) to afford **17a** (4.94 g, 86% yield) as a light-brown amorphous solid. The product was recrystallized from hexanes/ethyl acetate (1:1, 10 mL) to afford **17a** as a light-brown crystalline solid: mp 99−100.5 °C; $[\alpha]^{25}_D$ −52.3 (*c* 0.636, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.05 (s, 1H), 7.88 (s, 1H), 6.90 (s, 1H), 4.19−4.14 (m, 1H), 3.92 (s, 3H), 3.74 (dd, J_{AB}=10.3, J_{AX}=6.2 Hz, 1H), 3.67 (dd, J_{BA}=10.3, J_{BX}= 3.5 Hz, 1H), 3.34 (s, 3H), 2.41 (s, 3H), 2.17 (s, 3H), 1.31−1.24 (m, 1H), 0.80−0.73 (m, 1H), 0.62−0.56 (m, 1H), 0.53−0.47 (m, 1H), 0.36−0.30 (m, 1H). HRMS (ESI) *m*/*e* 379.1537 [(M + H)$^+$, calcd for $C_{18}H_{24}N_4O_3Cl$ 379.1537]. Anal. ($C_{18}H_{23}N_4O_3Cl$) C, H, N.

(*R*)-5-Chloro-1-(1-cyclopropyl-2-methoxyethyl)-3-(6-methoxy-2,5-dimethylpyridin-3-ylamino)pyrazin-2(1*H*)-one (17b). Compound **17b** was prepared according to the procedure described for the synthesis of **17a** using (*R*)-3,5-dichloro-1-(1-cyclopropyl-2-methoxyethyl)pyrazin-2(1*H*)-one (**15**) (300 mg, 1.14 mmol) and **31** (174 mg, 1.14 mmol). The product was purified by column chromatography to afford **17b** (310 mg, 72% yield) as a colorless solid. The product was subsequently recrystallized from hexanes/ethyl acetate to afford **17b** as a pale-yellow crystalline solid: mp 99.0−99.5 °C; $[\alpha]^{25}_D$ +48.2 (*c* 0.247, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.07 (s, 1H), 7.89 (s, 1H), 6.91 (s, 1H), 4.20−4.15 (m, 1H), 3.93 (s, 3H), 3.75 (dd, J_{AB}=10.5, J_{AX}=6.1 Hz, 1H), 3.67 (dd, J_{BA}=10.3, J_{BX}=3.4 Hz, 1H), 3.35 (s, 3H), 2.43 (s, 3H), 2.18 (s, 3H), 1.32−1.24 (m, 1H), 0.81−0.74 (m, 1H), 0.63−0.57 (m, 1H), 0.54−0.48 (m, 1H), 0.37−0.31 (m, 1H). HRMS (ESI) *m*/*e* 379.1538 [(M + H)$^+$, calcd for $C_{18}H_{24}N_4O_3Cl$ 379.1537]. Anal. ($C_{18}H_{23}N_4O_3Cl$) C, H, N.

(*R*)-5-Chloro-1-(1-cyclopropylpropyl)-3-(6-methoxy-2,5-dimethylpyridin-3-ylamino)pyrazin-2(1*H*)-one (17c). Compound **17c** was prepared according to the procedure described for the synthesis of **17a** using (*R*)-3,5-dichloro-1-(1-cyclopropylpropyl)pyrazin-2(1*H*)-one (**15**) (100 mg, 0.390 mmol) and **31** (60 mg, 0.39 mmol). The product was purified by column chromatography

to afford **17c** (30 mg, 21% yield) as a dark-yellow solid: $[\alpha]^{25}$ −7.5 (*c* 0.265, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.11 (s, 1H), 7.93 (s, 1H), 6.75 (s, 1H), 4.06−3.99 (m, 1H), 3.95 (s, 3H), 2.45 (s, 3H), 2.19 (s, 3H), 1.95−1.77 (m, 2H), 1.09−1.01 (m, 1H), 0.93 (t, *J* = 7.6 Hz, 3H), 0.82−0.75 (m, 1H), 0.55−0.47 (m, 2H), 0.32−0.27 (m, 1H). HRMS (ESI) *m*/*e* 363.1591 [(M + H)$^+$, calcd for $C_{18}H_{24}N_4O_2Cl$ 363.1588]. Anal. ($C_{18}H_{23}N_4O_2Cl$) C, H, N.

(*R*)-5-Chloro-1-(1-cyclopropylethyl)-3-(6-methoxy-2,5-dimethylpyridin-3-ylamino)pyrazin-2(1*H*)-one (17d). Compound **17d** was prepared according to the procedure described for the synthesis of **17a** using (*R*)-3,5-dichloro-1-(1-cyclopropylethyl)pyrazin-2(1*H*)-one (**15**) (150 mg, 0.650 mmol) and **31** (100 mg, 0.650 mmol). The product was purified by column chromatography to afford **17d** (150 mg, 66% yield) as a pale-yellow crystalline solid. The product was subsequently recrystallized from ethyl acetate/hexanes to afford **17d** a pale-yellow crystalline solid: mp 135−136 °C; $[\alpha]^{25}_D$ −19.1 (*c* 0.347, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.06 (s, 1H), 7.91 (s, 1H), 6.83 (s, 1H), 4.27−4.20 (m, 1H), 3.95 (s, 3H), 2.45 (s, 3H), 2.19 (s, 3H), 1.43 (d, *J* = 6.9 Hz, 3H), 1.12−1.05 (m, 1H), 0.79−0.72 (m, 1H), 0.59−0.53 (m, 1H), 0.50−0.44 (m, 1H), 0.38−0.31 (m, 1H). HRMS (ESI) *m*/*e* 349.1421 [(M + H)$^+$, calcd for $C_{17}H_{22}N_4O_2Cl$ 349.1431]. Anal. ($C_{17}H_{21}N_4O_2Cl$) C, H, N.

(*R*)-5-Chloro-3-(6-methoxy-2,5-dimethylpyridin-3-ylamino)-1-(1-methoxypropan-2-yl)pyrazin-2(1*H*)-one (17e). Compound **17e** was prepared according to the procedure described for the synthesis of **17a** using (*R*)-3,5-dichloro-1-(1-methoxypropan-2-yl)pyrazin-2(1*H*)-one (**15**) (100 mg, 0.422 mmol) and **31** (64 mg, 0.422 mmol). The product was purified by column chromatography to afford **17e** (65 mg, 44% yield) as a tan solid: $[\alpha]^{25}_D$ +40.2 (*c* 0.416, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.01 (s, 1H), 7.91 (s, 1H), 6.76 (s, 1H), 5.14 (td, *J* = 6.74, 4.15 Hz, 1H), 3.91 (s, 3H), 3.66−3.50 (m, 2H), 3.34 (s, 3H), 2.41 (s, 3H), 2.17 (s, 3H), 1.39 (d, *J* = 7.05 Hz, 3H). HRMS (ESI) *m*/*e* 353.1381 [(M + H)$^+$, calcd for $C_{16}H_{22}N_4O_3Cl$ 353.1380]. HPLC method A: t_R = 4.74 min, >99%; method B: t_R = 3.30 min, >99%.

(*R*)-5-Chloro-1-(1-cyclopropylethyl)-3-[6-(difluoromethoxy)-2,5-dimethylpyridin-3-ylamino]pyrazin-2(1*H*)-one (17f). Compound **17f** was prepared according to the procedure described for the synthesis of **17a** using (*R*)-3,5-dichloro-1-(1-cyclopropylethyl)pyrazin-2(1*H*)-one (**15**) (50 mg, 0.210 mmol) and **35** (48 mg, 0.210 mmol). The product was purified by column chromatography to afford **17f** (32 mg, 39% yield) as a colorless solid: $[\alpha]^{25}_D$ −8.1 (*c* 0.136, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.39 (s, 1H), 8.03 (s, 1H), 7.46 (t, *J* = 73.8 Hz, 1H), 6.88 (s, 1H), 4.24−4.20 (m, 1H), 2.44 (s, 3H), 2.26 (s, 3H), 1.43 (d, *J* = 6.8 Hz, 3H), 1.11−1.06 (m, 1H), 0.78−0.72 (m, 1H), 0.58−0.53 (m, 1H), 0.50−0.44 (m, 1H), 0.36−0.31 (m, 1H). HRMS (ESI) *m*/*e* 385.1232 [(M + H)$^+$, calcd for $C_{17}H_{20}N_4O_2ClF_2$ 385.1243]. HPLC method A: t_R = 6.18 min, 95.7%; method B: t_R = 3.82 min, 96.3%.

(*R*)-5-Chloro-1-(1-cyclopropylpropyl)-3-(6-(difluoromethoxy)-2,5-dimethylpyridin-3-ylamino)pyrazin-2(1*H*)-one (17g). Compound **17g** was prepared according to the procedure described for the synthesis of **17a** using (*R*)-3,5-dichloro-1-(1-cyclopropylpropyl)pyrazin-2(1*H*)-one (**15**) (30 mg, 0.12 mmol) and **35** (26 mg, 0.12 mmol). The product was purified by column chromatography to afford **17g** (23 mg, 48% yield) as a colorless solid: $[\alpha]^{25}_D$ −6.7 (*c* 0.173, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.43 (s, 1H), 8.06 (s, 1H), 7.46 (t, *J* = 73.8 Hz, 1H), 6.79 (s, 1H), 4.05−3.98 (m, 1H), 2.44 (s, 3H), 2.26 (s, 3H), 1.96−1.86 (m, 1H), 1.80 (dt, *J* = 14.9, 7.5 Hz, 1H), 1.10−1.01 (m, 1H), 0.92 (t, *J* = 7.4 Hz, 3H), 0.82−0.73 (m, 1H), 0.55−0.46 (m, 2H), 0.32−0.24 (m, 1H). HRMS (ESI) *m*/*e* 399.1382 [(M + H)$^+$, calcd for $C_{18}H_{22}N_4O_2ClF_2$ 399.1399]. HPLC method A: t_R = 6.36 min, >99%; method B: t_R = 4.61 min, >99%.

(*R*)-5-Chloro-3-[6-(difluoromethoxy)-2,5-dimethylpyridin-3-ylamino]-1-(2-methoxy-1-methylethyl)pyrazin-2(1*H*)-one (17h). Compound **17h** was prepared according to the procedure described for the synthesis of **17a** using (*R*)-3,5-dichloro-1-(1-methoxypropan-2-yl)pyrazin-2(1*H*)-one (**15**) (217 mg, 0.917 mmol) and **35** (200 mg, 0.917 mmol). The product was purified by column chromatography to afford **17h** (214 mg, 60% yield) as a pale-yellow solid. The product was recrystallized from hexanes/ethyl acetate to afford **17h** as a pale-yellow crystalline solid: mp 106.5−107.5 °C; $[\alpha]^{25}_D$ +47.5 (*c* 0.200, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.37 (s, 1H), 8.03 (s, 1H), 7.46 (t, *J* = 73.8 Hz, 1H), 6.82 (s, 1H), 5.16−5.12 (m, 1H), 3.62−3.53 (m, 2H), 3.34 (s, 3H), 2.44 (s, 3H), 2.26 (s, 3H), 1.40 (d, *J* = 7.0 Hz, 3H). HRMS (ESI) *m*/*e* 389.1210 [(M + H)$^+$, calcd for $C_{16}H_{20}N_4O_3ClF_2$ 389.1192]. Anal. ($C_{16}H_{19}N_4O_3ClF_2$) C, H, N.

(*R*)-5-Chloro-1-(1-cyclopropyl-2-methoxyethyl)-3-[6-(difluoromethoxy)-2,5-dimethylpyridin-3-ylamino]pyrazin-2(1*H*)-one (17i). Compound **17i** was prepared according to the procedure described for the synthesis of **17a** using (*R*)-3,5-dichloro-1-(1-cyclopropyl-2-methoxyethyl)pyrazin-2(1*H*)-one (**15**) (238 mg, 0.906 mmol) and **35** (198 mg, 0.906 mmol). The product was purified by column chromatography to afford compound **17i** (292 mg, 78% yield) as an off-white solid. The product was recrystallized from hexanes/ethyl acetate to afford **17i** as an off-white crystalline solid: mp 102.5−103.5 °C; $[\alpha]^{25}_D$ +36.4 (*c* 0.643, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.42 (s, 1H), 8.01 (s, 1H), 7.46 (t, *J* = 74.0 Hz, 1H), 6.96 (s, 1H), 4.19−4.14 (m, 1H), 3.75 (dd, J_{AB} = 10.3, J_{AX} = 6.3 Hz, 1H), 3.67 (dd, J_{BA} = 10.3, J_{BX} = 3.5 Hz, 1H), 3.34 (s, 3H), 2.44 (s, 3H), 2.26 (s, 3H), 1.32−1.25 (m, 1H), 0.81−0.74 (m, 1H), 0.63−0.56 (m, 1H), 0.54−0.47 (m, 1H), 0.36−0.31 (m, 1H). HRMS (ESI) *m*/*e* 415.1361 [(M + H)$^+$, calcd for $C_{18}H_{22}N_4O_3ClF_2$ 415.1349]. Anal. ($C_{18}H_{21}N_4O_3ClF_2$) C, H, N.

(*S*)-5-Chloro-1-(cyclopropyl-2-methoxyethyl)-3-[6-(difluoromethoxy)-2,5-dimethylpyridin-3-ylamino]pyrazin-2(1*H*)-one (17j). (*S*)-3,5-Dichloro-1-(1-cyclopropyl-2-methoxyethyl)pyrazin-2(1*H*)-one (**15**) (15.0 g, 57.01 mmol) and **35** (10.73 g, 57.01 mmol) were combined in a 2 L, three-neck round-bottom flask equipped with a thermometer and an addition funnel and placed under N_2. THF (570 mL) was added, and the mixture was cooled to 0 °C. NaHMDS (119.7 mL, 119.7 mmol, 1 M in THF) was added dropwise via the addition funnel over 20 min (the internal temperature was maintained below 5 °C). After the addition was complete, the reaction mixture was stirred at 0 °C for an additional 15 min. The reaction was quenched by the addition of saturated aqueous NH_4Cl solution (60 mL). The mixture was transferred to a separatory funnel containing water (400 mL), and the aqueous layer was extracted with ether (3 × 300 mL). The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, and concentrated. The product was purified by column chromatography on silica gel (30% ethyl acetate in hexanes) to afford **17j** (22.14 g, 94% yield) as a pale-yellow solid, which was subsequently recrystallized from heptane to furnish colorless needles: mp 103.4−104.4 °C; $[\alpha]^{25}_D$ −41.9 (*c* 0.807, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.42 (s, 1H), 8.02 (s, 1H), 7.46 (t, *J* = 74.0 Hz, 1H), 6.96 (s, 1H), 4.19−4.14 (m, 1H), 3.75 (dd, J_{AB} = 10.3, J_{AX} = 6.3 Hz, 1H), 3.67 (dd, J_{BA} = 10.3, J_{BX} = 3.5 Hz, 1H), 3.34 (s, 3H), 2.44 (s, 3H), 2.26 (s, 3H), 1.32−1.26 (m, 1H), 0.81−0.74 (m, 1H), 0.63−0.54 (m, 1H), 0.52−0.47 (m, 1H), 0.36−0.29 (m, 1H). HRMS (ESI) *m*/*e* 415.1360 [(M + H)$^+$, calcd for $C_{18}H_{22}N_4O_3ClF_2$ 415.1349]. Anal. ($C_{18}H_{21}N_4O_3ClF_2$) C, H, N.

(*S*)-4-(1-Cyclopropyl-2-methoxyethyl)-6-(6-methoxy-2,5-dimethylpyridin-3-ylamino)-5-oxo-4,5-dihydropyrazine-2-carbonitrile (19a). To a solution of **17a** (200 mg, 0.53 mmol) in DMF (3 mL) and H_2O (0.03 mL) was added zinc cyanide (62 mg, 0.53 mmol). Nitrogen was bubbled into the reaction mixture for 5 min. $Pd_2(dba)_3$ (146 mg, 0.16 mmol) and dppf (216 mg, 0.39 mmol) were added, and the reaction mixture was heated at 120 °C for 24 h. The mixture was cooled to room temperature and was transferred to a separatory funnel containing ethyl acetate. The organic layer was washed with saturated aqueous NH_4Cl solution (2×) and brine, dried over $MgSO_4$, filtered, and

concentrated. The product was purified by column chromatography on silica gel (30% ethyl acetate in hexanes) to afford **19a** (120 mg, 62% yield) as a tan solid: mp 120−121 °C; $[\alpha]^{25}$ −77.8 (*c* 0.573, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.03 (s, 1H), 7.86 (s, 1H), 7.48 (s, 1H), 4.14 (ddd, J = 10.1, 5.1, 3.1 Hz, 1H), 3.92 (s, 3H), 3.73 (dd, J = 10.3, 5.3 Hz, 1H), 3.65 (dd, J = 10.3, 3.0 Hz, 1H), 3.34 (s, 3H), 2.41 (s, 3H), 2.18 (s, 3H), 1.41−1.29 (m, 1H), 0.86−0.76 (m, 1H), 0.68−0.58 (m, 1H), 0.52 (dq, J = 9.6, 4.8 Hz, 1H), 0.35−0.26 (m, 1H). HRMS (ESI) *m/e* 370.1885 [$(M+H)^+$, calcd for $C_{19}H_{24}N_5O_3$ 370.1879]. Anal. ($C_{19}H_{23}N_5O_3$) C, H, N.

(*R*)-4-(1-Cyclopropylpropyl)-6-(6-methoxy-2,5-dimethylpyridin-3-ylamino)-5-oxo-4,5-dihydropyrazine-2-carbonitrile (19b). Part 1. To a solution of (*R*)-3,5-dibromo-1-(1-cyclopropylpropyl)pyrazin-2(1*H*)-one (**16**) (18.0 g, 54.0 mmol) and **31** (8.22 g, 54.0 mmol) in THF (200 mL) at 0 °C was added NaHMDS (113 mL, 113 mmol, 1 M in THF). The reaction mixture was stirred at 0 °C for 1 h. The mixture was transferred to a separatory funnel containing saturated aqueous $NaHCO_3$ solution (200 mL), and the aqueous layer was extracted with ethyl acetate (3 × 250 mL). The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, and concentrated. The residue was purified by column chromatography on silica gel (10% → 30% ethyl acetate in hexanes) to afford (*R*)-5-bromo-1-(1-cyclopropylpropyl)-3-(6-methoxy-2,5-dimethylpyridin-3-ylamino)pyrazin-2(1*H*)-one (**18**) (20.12 g, 94% yield) as a light-brown solid. An analytical sample was recrystallized from cyclohexane then tritrated with MeOH to furnish a colorless crystalline solid: mp 114.5−115.7 °C; $[\alpha]^{25}$ −9.5 (*c* 0.443, EtOH). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.10 (s, 1H), 7.92 (s, 1H), 6.82 (s, 1H), 4.00 (q, J = 8.8 Hz, 1H), 3.93 (s, 3H), 2.43 (s, 3H), 2.18 (s, 3H), 1.94−1.76 (m, 2H), 1.08−1.00 (m, 1H), 0.92 (t, J = 7.3 Hz, 3H), 0.81−0.73 (m, 1H), 0.54−0.45 (m, 2H), 0.31−0.24 (m, 1H). HRMS (ESI) *m/e* 407.1083 [$(M + H)^+$, calcd for $C_{18}H_{24}N_4O_2Br$ 407.1083]. Anal. ($C_{18}H_{23}N_4O_2Br$) C, H, N.

Part 2. To a solution of (*R*)-5-bromo-1-(1-cyclopropylpropyl)-3-(6-methoxy-2,5-dimethylpyridin-3-ylamino)pyrazin-2(1*H*)-one (**18**) (16.0 g, 39.3 mmol) from above in DMF (200 mL) and H_2O (2.0 mL) was added zinc cyanide (4.60 mg, 39.3 mmol). Nitrogen was bubbled into the reaction mixture for 10 min. $Pd_2(dba)_3$ (0.83 g, 2.00 mmol) and dppf (2.60 g, 4.70 mmol) were added, and the reaction mixture was heated at 120 °C for 7 h. The mixture was cooled to room temperature, filtered through celite, and the filtrate was transferred to a separatory funnel containing ethyl acetate. The organic layer was washed with saturated aqueous NH_4Cl solution (2×) and brine, dried over $MgSO_4$, filtered, and concentrated. The product was purified by column chromatography on silica gel (10% → 30% ethyl acetate in hexanes) to afford a pale-green solid, which was subsequently recrystallized from cyclohexane to furnish **19b** (5.50 g). Mixed fractions from the column chromatography, and the filtrate from the recrystallization were combined, repurified by column chromatography, and recrystallized from cyclohexane to give an additional 6.65 g of product. A total of 12.15 g (88% yield) of **19b** was isolated as a pale-yellow crystalline solid: mp 150.2−151.2 °C; $[\alpha]^{25}$ −23.0 (*c* 0.364, EtOH). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.05 (s, 1H), 7.91 (s, 1H), 7.28 (s, 1H), 4.04−3.98 (m, 1H), 3.93 (s, 3H), 2.42 (s, 3H), 2.18 (s, 3H), 2.02−1.77 (m, 2H), 1.09−1.01 (m, 1H), 0.93 (t, J = 7.5 Hz, 3H), 0.88−0.79 (m, 1H), 0.58−0.49 (m, 2H), 0.29−0.24 (m, 1H). HRMS (ESI) *m/e* 354.1925 [$(M + H)^+$, calcd for $C_{19}H_{24}N_5O_2$ 354.1930]. Anal. ($C_{19}H_{23}N_5O_2$) C, H, N.

(*R*)-4-(1-Cyclopropylethyl)-6-(6-methoxy-2,5-dimethylpyridin-3-ylamino)-5-oxo-4,5-dihydropyrazine-2-carbonitrile (19c). To a solution of (*R*)-5-bromo-1-(1-cyclopropylethyl)-3-(6-methoxy-2,5-dimethylpyridin-3-ylamino)pyrazin-2(1*H*)-one (**18**) (153 mg, 0.389 mmol), prepared according to the procedure described in Part 1 for the preparation of **19b** using appropriate starting materials in DMF (1 mL) was added zinc cyanide (46 mg, 0.389 mmol) and $Pd(PPh_3)_4$ (135 mg, 0.117 mmol) and the reaction mixture was heated under N_2 at 100 °C for 1.5 h. The mixture was then cooled to room temperature and transferred to a separatory funnel containing saturated aqueous $NaHCO_3$ solution (15 mL). The aqueous layer was extracted with ethyl acetate (3 × 15 mL). The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, and concentrated. The residue was purified by column chromatography on silica gel (20% ethyl acetate in hexanes) to furnish **19c** (105 mg, 80% yield) as a colorless solid: mp 138−140 °C; $[\alpha]^{25}_D$ −36.6 (*c* 0.477, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.00 (s, 1H), 7.88 (s, 1H), 7.37 (s, 1H), 4.24−4.20 (m, 1H), 3.92 (s, 3H), 2.41 (s, 3H), 2.18 (s, 3H), 1.44 (d, J = 6.8 Hz, 3H), 1.10−1.06 (m, 1H), 0.82−0.78 (m, 1H), 0.61−0.57 (m, 1H), 0.51−0.48 (m, 1H), 0.36−0.31 (m, 1H). HRMS (ESI) *m/e* 340.1786 [$(M + H)^+$, calcd for $C_{18}H_{22}N_5O_2$ 340.1774]. HPLC method A: t_R = 5.28 min, >99%; method B: t_R = 3.30 min, >99%.

(*R*)-4-(1-Cyclopropylethyl)-6-(6-(difluoromethoxy)-2,5-dimethyl-pyridin-3-ylamino)-5-oxo-4,5-dihydropyrazine-2-carbonitrile (19d). Compound **19d** was prepared according to the two-step procedure described for the synthesis of **19b** using (*R*)-3,5-dibromo-1-(1-cyclopropylethyl)pyrazin-2(1*H*)-one (**16**) (15.0 g, 46.9 mmol) and **35** (8.8 g, 46.9 mmol). Purification by column chromatography on silica gel (0 → 30% ethyl acetate in hexanes) afforded **19d** (12.4 g, 93% yield, 2 steps) as a pale-yellow solid. Subsequent recrystallization from heptane and a minimal amount of ethyl acetate afforded **19d** as white needles: mp 149−150 °C; $[\alpha]^{25}_D$ −24.47 (*c* 0.509, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.37 (s, 1H), 8.01 (s, 1H), 7.46 (t, J = 73.5 Hz, 1H), 7.43 (s, 1H), 4.26−4.18 (m, 1H), 2.44 (s, 3H), 2.27 (s, 3H), 1.45 (d, J = 6.8 Hz, 3H), 1.13−1.06 (m, 1H), 0.85−0.78 (m, 1H), 0.64−0.57 (m, 1H), 0.54−0.47 (m, 1H), 0.36−0.29 (m, 1H). HRMS (ESI) *m/e* 376.1595 [$(M + H)^+$, calcd for $C_{18}H_{20}N_5O_2F_2$ 376.1585]. Anal. ($C_{18}H_{19}N_5O_2F_2$) C, H, N.

(*S*)-4-(1-Cyclopropyl-2-methoxyethyl)-6-[6-(difluoromethoxy)-2,5-dimethylpyridin-3-ylamino]-5-oxo-4,5-dihydropyrazine-2-carbonitrile (19e). (*S*)-2-(1-Cyclopropyl-2-methoxyethylamino)acetonitrile hydrochloride (**14**) (15.0 g, 78.7 mmol) was suspended in anhydrous dichloromethane (300 mL) in a 1 L, three-neck round-bottom flask equipped with an addition funnel. The mixture was cooled to −60 °C and oxalyl bromide (41.3 mL, 440 mmol) was added dropwise over 15 min. After addition was complete, the cooling bath was removed and the reaction mixture was allowed to warm to room temperature and was then heated at 40 °C for 3 h. The mixture was cooled to room temperature and concentrated under vacuum. The residue was transferred directly onto a silica gel column in a fume hood (caution: this purification should be performed in a fume hood as some gas evolution occurred as residual oxalyl bromide decomposed) and was eluted (5% → 20% ethyl acetate in hexanes) to give (*S*)-3,5-dibromo-1-(1-cyclopropyl-2-methoxyethyl)pyrazin-2(1*H*)-one (**16**) (15.5 g, 56% yield) as an off-white solid: mp 98.5−100.5 °C; $[\alpha]^{25}_D$ −71.8 (*c* 1.19, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.61 (s, 1H), 4.08−4.04 (m, 1H), 3.72 (dd, J_{AB} = 10.5, J_{AX} = 4.5 Hz, 1H), 3.61 (dd, J_{BA} = 10.3, J_{BX} = 3.0 Hz, 1H), 3.32 (s, 3H), 1.41−1.36 (m, 1H), 0.82−0.76 (m, 1H), 0.65−0.59 (m, 1H), 0.54−0.48 (m, 1H), 0.32−0.27 (m, 1H). LRMS (APCI) *m/e* 351.1 [$(M + H)^+$, calcd for $C_{10}H_{13}N_2O_2Br_2$ 350.9].

(*S*)-3,5-Dibromo-1-(1-cyclopropyl-2-methoxyethyl)pyrazin-2(1*H*)-one (**16**) from the above procedure (18.1 g, 51.7 mmol) and **35** (9.70 mg, 51.7 mmol) were combined in a 2 L, three-necked round-bottom flask equipped with a thermometer and an addition funnel and placed under N_2. THF (360 mL) was added and the mixture was cooled to 0 °C. NaHMDS (109 mL, 109 mmol, 1 M in THF) was added dropwise via the addition funnel over 15 min (the internal temperature was maintained below 5 °C). After the addition was complete, the reaction mixture was stirred at 0 °C for an additional 30 min. The reaction was quenched by the addition of saturated aqueous NH_4Cl solution (120 mL). The mixture was transferred to a

separatory funnel containing water (100 mL), and the aqueous layer was extracted with ethyl acetate (3 × 250 mL). The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, and concentrated to afford (*S*)-5-bromo-1-(cyclopropyl-2-methoxyethyl)-3-[6-(difluoromethoxy)-2,5-dimethylpyridin-3-ylamino]pyrazin-2(1*H*)-one (**18**) (22.40 g, 95% yield) as a brown solid, which was used in the next step without further purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.40 (s, 1H), 7.98 (s, 1H), 7.53 (s, 1H), 7.46 (t, J = 73.7 Hz, 1H), 4.16−4.12 (m, 1H), 3.74 (dd, J_{AB} = 10.5, J_{AX} = 5.2 Hz, 1H), 3.66 (dd, J_{BA} = 10.3, J_{BX} = 3.3 Hz, 1H), 3.35 (s, 3H), 2.44 (s, 3H), 2.27 (s, 3H), 1.39−1.34 (m, 1H), 0.85−0.79 (m, 1H), 0.66−0.62 (m, 1H), 0.56−0.51 (m, 1H), 0.34−0.29 (m, 1H). HRMS (ESI) *m*/*e* 459.0864 [(M + H)$^+$, calcd for $C_{18}H_{22}N_4O_3BrF_2$ 459.0843].

(*S*)-5-Bromo-1-(cyclopropyl-2-methoxyethyl)-3-[6-(difluoromethoxy)-2,5-dimethylpyridin-3-ylamino]pyrazin-2(1*H*)-one (**18**) from the above procedure (22.40 g, 48.8 mmol) was dissolved in dimethylformamide (480 mL) and water (24 mL) at room temperature. Nitrogen was bubbled through the reaction mixture for 15 min. Zinc cyanide (6.00 g, 51.0 mmol), $Pd_2(dba)_3$ (2.23 g, 2.40 mmol), and dppf (3.24 g, 5.85 mmol) were added and the reaction mixture was heated at 120 °C for 3 h. The reaction mixture was cooled and filtered through a pad of celite. The filtrate was concentrated in vacuo, and the residue was transferred to a separatory funnel containing saturated aqueous NH_4Cl solution (200 mL). The aqueous layer was extracted with ethyl acetate (3 × 400 mL). The combined organic layers were washed with brine (300 mL), dried over $MgSO_4$, filtered, and concentrated. Purification by column chromatography on silica gel (70% → 100% CH_2Cl_2 in hexanes then 2% → 5% ethyl acetate in CH_2Cl_2) afforded **19e** (14.23 g, 72% yield) as a pale-yellow solid. Recrystallization from a mixture of ethanol:2-butanol (10:1) afforded **19e** as pale-yellow needles: mp 146.8−147.7 °C; $[\alpha]^{25}_D$ −63.7 (*c* 0.486, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.40 (s, 1H), 7.98 (s, 1H), 7.53 (s, 1H), 7.46 (t, J = 73.5 Hz, 1H), 4.16−4.12 (m, 1H), 3.74 (dd, J_{AB} = 10.3, J_{AX} = 5.0 Hz, 1H), 3.67 (dd, J_{BA} = 10.4, J_{BX} = 3.0 Hz, 1H), 3.35 (s, 3H), 2.44 (s, 3H), 2.27 (s, 3H), 1.39−1.33 (m, 1H), 0.85−0.79 (m, 1H), 0.67−0.61 (m, 1H), 0.56−0.50 (m, 1H), 0.34−0.29 (m, 1H). HRMS (ESI) *m*/*e* 406.1704 [(M+H)$^+$, calcd for $C_{19}H_{22}N_5O_3F_2$ 406.1691]. Anal. ($C_{19}H_{21}N_5O_3F_2$) C, H, N.

When the chiral amine intermediate (compound **25**) was prepared via Scheme 2, residual amounts of the undesired enantiomer of **19e** (~97% ee) were removed by super critical fluid chromatography (SFC) on chiral support: Chiralpak OD-H column (5 cm × 25 cm), mobile phase = 15% isopropanol/acetonitrile (1:1) in CO_2; flow rate = 200 mL/min, pressure = 100 bar, temperature = 35 °C, λ = 254 nm, 5 mL of 91 mg/mL in isopropanol/acetonitrile (1:1) per injection per 6 min. The purified material had an optical purity > 99% ee as determined by analytical SFC: Chiralcel OD-H column (4.6 mm × 250 mm, 5 μm); mobile phase = 8% ethanol in CO_2; flow rate = 2 mL/min @ 150 bar; λ = 215 nm; t_R = 6.5 min. Compound **19e** was then recrystallized from anhydrous 2-butanol.

(*R*)-4-(1-Cyclopropyl-2-methoxyethyl)-6-[6-(difluoromethoxy)-2,5-dimethylpyridin-3-ylamino]-5-oxo-4,5-dihydropyrazine-2-carbonitrile (19f). Nitrogen was bubbled into a mixture of **17i** (500 mg, 1.21 mmol), zinc cyanide (143 mg, 1.21 mmol), and zinc dust (39 mg, 0.604 mmol) in DMF (25 mL) and H_2O (0.3 mL) for 5 min. $Pd_2(dba)_3$ (332 mg, 0.362 mmol) and dppf (335 mg, 0.604 mmol) were added, and the reaction mixture was heated at 120 °C for 5 h. The mixture was cooled to room temperature and was transferred to a separatory funnel containing saturated aqueous NH_4Cl solution. The aqueous layer was extracted with ethyl acetate (3 × 25 mL). The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, and concentrated. The product was purified by column chromatography on silica gel (30% ethyl acetate in hexanes) to afford **19f** (400 mg, 82% yield) as an off-white solid, which was subsequently recrystallized from heptane to furnish a colorless solid: mp 131−132 °C; $[\alpha]^{25}_D$ +56.8 (*c* 0.264, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.40 (s, 1H), 7.99 (s, 1H), 7.54 (s, 1H), 7.46 (t, J = 73.7 Hz, 1H), 4.14 (ddd, J = 10.3, 5.2, 3.1 Hz, 1H), 3.73 (dd, J = 10.3, 5.3 Hz, 1H), 3.66 (dd, J = 10.3, 3.0 Hz, 1H), 3.34 (s, 3H), 2.43 (s, 3H), 2.27 (s, 3H), 1.41−1.31 (m, 1H), 0.87−0.78 (m, 1H), 0.68−0.59 (m, 1H), 0.57−0.49 (m, 1H), 0.35−0.27 (m, 1H). HRMS (ESI) *m*/*e* 406.1692 [(M + H)$^+$, calcd for $C_{19}H_{22}N_5O_3F_2$ 406.1691]. Anal. ($C_{19}H_{21}N_5O_3F_2$) C, H, N.

(*S*)-2-Cyclopropyl-2-[(*S*)-1-phenylethylamino]ethanol (23). To a solution of **22**[41] (400 g, 1.82 mol) in dry THF (5.2 L) at 0 °C was added borane dimethylsulfide (neat) (485 g, 6.39 mol) with vigorous stirring. The reaction mixture was stirred overnight at room temperature. Reaction progress was monitored by HPLC. Stirring was continued until the acid was consumed completely (18−20 h). Upon completion, the reaction mixture was cooled to 0 °C and methanol (6 L) was added dropwise. The mixture was concentrated under vacuum, and the residue was dissolved in chloroform (5 L). The organic layer was washed with 10% aqueous $NaHCO_3$ solution (2 × 1 L) followed by brine, dried over Na_2SO_4, filtered, and concentrated to afford a tan−yellow oil. The oil was distilled under reduced pressure to afford **23** (166 g, 44% yield) as a colorless oil: bp 175−184 °C, 0.1 mmHg; $[\alpha]^{25}_D$ −52.1 (*c* 1.0, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33−7.20 (m, 5H), 3.91−3.86 (m, 1H), 3.67 (dd, J_{AB} = 10.6, J_{AX} = 3.8 Hz, 1H), 3.39 (dd, J_{BA} = 10.6, J_{BX} = 4.3 Hz, 1H), 2.40 (s br, 2H), 1.70−1.65 (m, 1H), 1.35 (d, J = 6.6 Hz, 3H), 0.88−0.82 (m, 1H), 0.47−0.37 (m, 2H), 0.05− −0.06 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 146.8, 127.6, 126.2, 125.9, 63.0, 60.5, 54.4, 24.4, 13.3, 3.0, 1.5; LRMS (ESI) *m*/*e* 206.3 [(M + H)$^+$, calcd for $C_{13}H_{20}NO$ 206.2].

(*S*)-1-Cyclopropyl-2-methoxy-*N*-[(*S*)-1-phenylethyl]ethanamine (24). To a solution of **23** (29.2 g, 0.143 mol) in THF (700 mL) at 0 °C was added sodium hydride (6.29 g, 0.157 mol, 60% dispersion in mineral oil). The cooling bath was removed and the reaction mixture was allowed to warm to room temperature and was stirred at room temperature for 30 min. Methyl iodide (20.30 g, 0.143 mol) was then added dropwise via syringe. Some warming occurred soon after the addition was complete. The temperature of the reaction mixture was controlled at ~25 °C with a water bath containing a small amount of ice. The reaction mixture was stirred at room temperature for 4 h. The reaction mixture was then slowly quenched with saturated aqueous $NaHCO_3$ solution and was transferred to a separatory funnel containing saturated aqueous $NaHCO_3$ solution (400 mL). The aqueous layer was extracted with ethyl acetate (3 × 300 mL). The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, and concentrated. The crude product was purified by column chromatography on silica gel (5% MeOH in 1:1 ethyl acetate/hexanes) to afford **24** (26.52 g, 85% yield) as a light-brown oil: $[\alpha]^{25}_D$ −61.5 (*c* 0.72, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.30−7.25 (m, 4H), 7.20−7.16 (m, 1H), 3.98−3.93 (q, J = 6.8 Hz, 1H), 3.50 (dd, J_{AB} = 9.5, J_{AX} = 3.5 Hz, 1H), 3.35 (s, 3H), 3.34 (dd, J_{BA} = 9.5, J_{BX} = 5.8 Hz, 1H), 1.79−1.74 (m, 2H), 1.33 (d, J = 6.5 Hz, 3H), 0.73−0.67 (m, 1H), 0.36−0.34 (m, 2H), 0.03− −0.06 (m, 2H). GC/MS (ESI) *m*/*e* 220.2 [(M + H)$^+$, calcd for $C_{14}H_{22}NO$ 220.2].

(*S*)-1-Cyclopropyl-2-methoxyethanamine Hydrochloride (25). A mixture of **24** (100 g, 458 mmol), $Pd(OH)_2/C$ (50 g, 20% on carbon), and ethanol (1.2 L) in a Parr bottle was placed under a H_2 atmosphere (15 psi) and was shaken for 18 h. The reaction mixture was then filtered through a pad of celite into a flask containing 2 N HCl in Et_2O (360 mL) with stirring. The resulting filtrate was concentrated to a yellow solid, which was then coevaporated with Et_2O (500 mL). The resulting solid was dried overnight in vacuo to give **25** (69 g, 99% yield) as a white solid: mp 190−192 °C; $[\alpha]^{25}_D$ +16.3 (*c* 0.446, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.41 (s br, 3H), 3.68 (d, J = 5.6 Hz, 2H), 3.39 (s, 3H), 2.64−2.60 (m, 1H), 1.20−1.13 (m, 1H), 0.71−0.58 (m, 3H), 0.32−0.28 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$)

δ 72.1, 59.2, 57.5, 10.7, 4.2, 4.1. LRMS (ESI) *m*/*e* 231.2 [(2 M+ H)$^+$, calcd for $C_{12}H_{27}N_2O_2$ 231.2].

3-Methyl-5-nitropyridin-2-ol (27). (Caution: Careful temperature control must be maintained while performing the following nitration procedure to avoid the risk of an uncontrolled exothermic reaction. A detailed procedure follows.) A three-necked, 2 L, round-bottomed flask equipped with a mechanical stirrer, an addition funnel, and a thermometer was placed in an ice−water bath. Conc H_2SO_4 (150 mL) was added to the flask. 2-Amino-3-methylpyridine (**26**) (50.0 g, 0.463 mol, Lancaster, CAS 1603-40-3, mp 29 °C, prewarmed in a warm water bath to melt it) was weighed out in a 125 mL Erlenmeyer flask and was subsequently added in small portions. (The Erlenmeyer flask was kept in a warm water bath during the addition to prevent the starting material from solidifying.) The temperature rose to ca. 45 °C during the addition, and white smoke/fog formed within the flask. The Erlenmeyer flask was rinsed with conc H_2SO_4 (100 mL), which was then added to the reaction flask. The resulting mixture was a milky-white suspension. A solution of conc H_2SO_4 (35 mL) and 70% nitric acid (35 mL) was premixed with ice−water bath cooling and transferred into the addition funnel. After the internal temperature of the reaction mixture had cooled to 10−15 °C (but not below 10 °C), the premixed H_2SO_4/HNO_3 acid mixture was added dropwise at a rate such that the internal reaction temperature rose to 20−25 °C (5−10 min addition time). After the addition was complete, the ice−water bath was replaced with a tap water bath. The reaction temperature slowly increased to ca. 30 °C and then cooled down to room temperature. The reaction should be monitored during this time to ensure that the temperature does not rise too high. The reaction mixture was then stirred overnight at room temperature. 70% Nitric acid (35 mL) was then added dropwise via the addition funnel to the dark red−brown mixture at a rate of addition such that the temperature did not exceed 35 °C. During this time, the reaction flask was in a room temperature water bath. Water (500 mL) was then added to the reaction flask in portions via the addition funnel. The first ∼150 mL of water was added dropwise while allowing the internal temperature to climb slowly to 50−60 °C. The rate of stirring was increased in order to break up any foaming that occurred. Brown gas evolved during the addition of this initial ∼150 mL of water. The remaining ∼350 mL of water was added at a faster rate as the gas evolution had stopped and a temperature increase was no longer observed. The reaction turned from a dark cloudy brown to a clear-orange solution. Some yellow precipitate may form as the reaction cools to below 50 °C. The water bath was then removed and replaced with a heating mantle, and the addition funnel was replaced with a condenser. The reaction mixture (a light-orange or bright-yellow solution) was then heated at 115−118 °C for 1.75−2 h. Additional gas evolution occurred at ∼115 °C during this time. The reaction mixture was then cooled to room temperature with the aid of an ice−water bath and was then cooled further to 0 °C by adding ice directly into the reaction mixture. The solid that formed was collected on a Buchner funnel and was washed with cold water followed by a minimal amount of cold ethanol followed by a minimal amount of cold ether. The solid was then dried under vacuum to afford **27** (53.5 g, 75% yield) as a pale yellow solid: mp 228−229 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.55 (s, br, 1H), 8.54 (d, J = 3.0 Hz, 1H), 8.04 (d, J = 2.0 Hz, 1H), 2.04 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 162.2, 135.4, 130.0, 129.5, 128.1, 15.8. LRMS (ESI) *m*/*e* 152.96 [(M − H)$^-$, calcd for $C_6H_5N_2O_3$, 153.03]. Anal. ($C_6H_6N_2O_3$) C, H, N.

2-Chloro-3-methyl-5-nitropyridine (28). Compound **27** (134 g, 0.872 mol) was divided into three portions and placed in three 1 L round-bottom flasks. Phosphorus oxychloride (200 mL) was added to each flask, and the mixtures were heated at reflux for 2 h. The solutions were cooled, and the excess phosphorus oxychloride was removed in vacuo. The residues were poured into ice water (1 L) with stirring and the precipitate was collected by filtration and air-dried for 20 min. The combined products were recrystallized from 10% ethyl acetate in hexanes (300 mL) and air-dried to give **28** (139 g, 92% yield) as a white solid, which was used in the next step without further purification. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.04 (d, J = 2.5 Hz, 1H), 8.33 (d, J = 2.2 Hz, 1H), 2.50 (s, 3H).

2-Methoxy-3-methyl-5-nitropyridine (29). Compound **28** (139 g, 0.806 mol) from the above procedure was divided into two portions and placed in two 2 L round-bottom flasks with methanol (500 mL). The solutions were cooled in dry ice/isopropyl alcohol baths as solid sodium methoxide (26.5 g, 0.467 mol) was added portionwise to each flask such that the temperature remained below 20 °C. When the additions were complete, the resulting mixtures were heated to reflux for 1 h. The mixtures were cooled and diluted with ice−water (500 mL) to give white precipitates, which were collected by filtration. The combined filtrates were washed with water and air-dried to give **29** (127 g, 97% yield) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.91 (d, J = 2.0 Hz, 1H), 8.16 (s, 1H), 4.06 (s, 3H), 2.25 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.8, 141.9, 139.4, 132.9, 121.8, 54.8, 15.8. An analytical sample was recrystallized from hexane to give white needles, mp 95−96.5 °C. Anal. ($C_7H_8N_2O_3$) C, H, N.

2-(Dibromomethyl)-6-methoxy-5-methyl-3-nitropyridine (30). To a three-neck, 22 L round-bottom flask equipped with a mechanical stirrer and an addition funnel was added potassium *t*-butoxide (450 g, 4.00 mol) and THF (4 L). The mixture was stirred vigorously, and a solution of **29** (100 g, 0.595 mol) and bromoform (195.4 g, 0.773 mol) in 500 mL of dry THF was added dropwise at such a rate that the temperature did not rise above −74 °C (3 h addition time). The reaction mixture was stirred at −78 °C for an additional 15 min. The reaction mixture was then quenched by the dropwise addition of a mixture of methanol (400 mL) and conc HCl (600 mL) while maintaining the temperature below −68 °C. Water (1 L) was then added, and the aqueous layer was extracted with ethyl acetate (3 × 4 L). The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, and concentrated. The product was purified by column chromatography on silica gel (0% → 7% ethyl acetate in hexanes) to give **30** (190.0 g, 94% yield) as a red−brown solid: mp 58.7−61.2 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.04 (s, 1H), 7.60 (s, 1H), 4.15 (s, 3H), 2.26 (s, 1H). LRMS (ESI) *m*/*e* 339.0 [(M + H)$^+$, calcd for $C_8H_9N_2O_3Br_2$, 338.9].

6-Methoxy-2,5-dimethylpyridin-3-amine (31). To a solution of **30** (110 g, 0.323 mol) in EtOH (1.1 L) in a Parr bottle was added triethylamine (135 mL, 0.970 mol) followed by 10% Pd/C (11.0 g). The mixture placed on a Parr shaker under a hydrogen atmosphere at 45 psi for 18 h. The reaction mixture was filtered through celite, and the filtrate was concentrated. The residue was partitioned between water and ethyl acetate, and the organic layer was washed with water, brine, dried over $MgSO_4$, filtered, and concentrated. The residue was purified by column chromatography on silica gel (10% → 50% ethyl acetate in hexanes) to give **31** (35.7 g, 73% yield) as a light-brown solid: mp 39.8−41 °C. ^{1}H NMR (400 mHz, DMSO-d_6) δ 6.82 (s, 1H), 4.37 (s, 2H), 3.73 (s, 3H), 2.16 (s, 3H), 2.00 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 152.7, 135.8, 134.8, 126.3, 116.1, 52.1, 19.2, 14.7. LRMS (ESI) *m*/*e* 153.2 [(M + H)$^+$, calcd for $C_8H_{13}N_2O$, 153.1].

2-Methoxy-3,6-dimethyl-5-nitropyridine (32). Method A (three steps): A solution of **29** (68.8 g, 409 mmol) and *tert*-butyl 2-chloroacetate (77.0 g, 511 mmol) in THF (1 L) was stirred and cooled to −20 °C in a dry ice/isopropyl alcohol bath. Potassium *tert*-butoxide (115 g, 1.02 mol) was added at a rate such that the reaction temperature was less than −10 °C. The reaction mixture turned dark purple. When the addition was complete, the cooling bath was removed and the reaction was stirred for 30 min. The stirred reaction mixture was quenched with HCl (500 mL, 2.4 N). The purple solution turned pale yellow, and the mixture separated into two layers. The organic layer was separated, washed three times with brine, and concentrated in

vacuo. Hexane was added to the amber residue. The mixture was concentrated in vacuo and then dried under high vacuum to give *tert*-butyl 2-(6-methoxy-5-methyl-3-nitropyridin-2-yl)acetate (83.4 g, 72% yield) as a tan solid. An analytical sample was recrystallized from hexane to give white needles: mp 71−72.5 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.16 (s, 1H), 4.09 (s, 2H), 4.02 (s, 2H), 2.21 (s, 3H), 1.44 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.8, 163.6, 147.9, 139.4, 135.2, 120.8, 81.7, 54.7, 44.5, 28.0, 15.3. Anal. ($C_{13}H_{18}N_2O_5$) C, H, N.

A solution of *tert*-butyl 2-(6-methoxy-5-methyl-3-nitropyridin-2-yl)acetate from the previous step (83.0 g, 294 mmol) in trifluoroacetic acid (200 mL) was heated in a hot water bath for 1 h. The solution was concentrated in vacuo to give a brown oil. The oil was diluted with hexane and stirred. The resulting solid was collected by filtration and air-dried to give 2-(6-methoxy-5-methyl-3-nitropyridin-2-yl)acetic acid (62.8 g, 94% yield) as a tan solid. An analytical sample was recrystallized from hexane: mp 135−137 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.70 (s br, 1H), 8.20 (s, 1H), 4.25 (s, 2H), 4.03 (s, 3H), 2.24 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.6, 163.8, 146.5, 139.2, 135.3, 121.5, 54.86, 42.9, 15.3. Anal. ($C_9H_{10}N_2O_5$) C, H, N.

A mixture of 2-(6-methoxy-5-methyl-3-nitropyridin-2-yl)-acetic acid from the previous step (62.5 g, 276 mmol), potassium carbonate (20.0 g, 145 mmol), and DMF (100 mL) was heated to 90 °C with stirring in a hot water bath for 1 h. Gas evolution was noted during the heating period and had ceased after 1 h. The mixture was poured into stirred ice water (600 mL) with washing of the reaction flask with a small volume of acetone. The resulting precipitate was collected by filtration and air-dried to give **32** (48.5 g, 96% yield) as a tan solid. An analytical sample was recrystallized from hexanes to give tan needles: mp 85.9−90.5 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.08 (s, 1H), 4.02 (s, 3H), 2.76 (d, 3H), 2.19 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.4, 151.6, 139.4, 135.0, 119.4, 54.5, 24.2, 15.2. Anal. ($C_8H_{10}N_2O_3$) C, H, N.

2-Methoxy-3,6-dimethyl-5-nitropyridine (32). Method B: Dimethyl sulfoxide (35 mL) was added to a dry round-bottomed flask containing sodium hydride (1.82 g, 45.5 mmol, 60% in mineral oil). The resulting suspension was heated at 70 °C for 35 min, during which time the suspension became a solution. The reaction mixture was cooled to room temperature, trimethylsulfoxonium iodide (10.0 g, 45.5 mmol) was added, and the mixture was stirred at room temperature for 30 min. Compound **29** (4.50 g, 26.80 mmol) was added, and the resulting dark-red solution was stirred at room temperature for 30 min, at which time TLC showed complete consumption of starting material. The reaction mixture was transferred to a separatory funnel containing water (30 mL), and the aqueous layer was extracted with ethyl acetate (3 × 100 mL). The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, and concentrated. The residue was purified by column chromatography on silica gel (20% ethyl acetate in hexanes) to afford **32** (2.00 g, 41% yield) as a colorless solid identical to that prepared by Method A: mp 85.5−86.2 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.08 (s, 1H), 4.02 (s, 3H), 2.77 (s, 3H), 2.20 (s, 3H).

3,6-Dimethyl-5-nitropyridin-2-ol (33). A solution of **32** (32.3 g, 182 mmol) in conc hydrochloric acid (300 mL) was heated at 100 °C for 1 h. Analysis by TLC indicated that some starting material remained. The reaction was heated at 110 °C for an additional 45 min. The reaction mixture was cooled to room temperature and poured onto ice (400 g). When the ice had melted and the temperature of the resulting thick brown suspension was still less than 0 °C, the mixture was filtered. The solid cake was washed with water (100 mL) and allowed to dry on the filter for 30 min. The solid was then resuspended in cold (−10 °C) ethanol (150 mL), filtered, washed with cold ethanol (50 mL), and air-dried on the filter for 1 h to afford **33** (28.0 g, 94% yield) as a tan powder: mp 263 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.42 (s br, 1 H), 8.03 (s, 1 H), 2.61 (s, 3 H), 2.01 (s, 3 H). LRMS (ESI) *m*/*e* 169.3 [(M + H)$^+$, calcd for $C_7H_9N_2O_3$ 169.1]. Anal. ($C_7H_8N_2O_3$) C, H, N.

2-(Difluoromethoxy)-3,6-dimethyl-5-nitropyridine (34). Method A: Sodium hydride (6.63 g, 166 mmol, 60% in mineral oil) was washed with hexanes (100 mL) to remove the mineral oil and was then suspended in dry acetonitrile (1500 mL) at room temperature. Compound **33** (27.9 g, 166 mmol) was added in portions over 30 min to give a yellow suspension. During the addition, there was some bubbling but negligible temperature change. Cesium fluoride (2.50 g, 16.6 mmol) was then added followed by the slow addition of trimethylsilyl 2,2-difluoro-2-(fluorosulfonyl)acetate (36.0 mL, 182 mmol) over 30 min. (Caution: trimethylsilyl 2,2-difluoro-2-(fluorosulfonyl)acetate can cause skin damage and should always be handled with gloves in a fume hood.) During the addition there was some bubbling, the temperature rose from 23 to 30 °C, and the suspension became noticeably less turbid. After stirring for 15 min, TLC indicated that starting material still remained, so additional trimethylsilyl 2,2-difluoro-2-(fluorosulfonyl)acetate (6.5 mL, 33 mmol) was added over 10 min. After an additional 15 min, TLC indicated consumption of starting material. The reaction was quenched by the addition of water (20 mL) dropwise at such a rate that the bubbling did not become too vigorous. After bubbling ceased, additional water (200 mL) was added. Most of the solvent was removed in vacuo, and the aqueous residue was extracted with ethyl acetate (3 × 200 mL). The combined organic layers were dried over Na_2SO_4 and evaporated to a brown syrup which solidified upon standing. This residue was dissolved in ethanol (400 mL), and decolorizing charcoal (15 g) was added. The suspension was heated at 70 °C for 20 min and then filtered through a pad of celite and sand. The filtrate was collected, and the solvent was evaporated. The residue was dissolved in methylene chloride, and the solution was evaporated to give **34** (33.4 g, 92% yield) as a pale-yellow solid identical to that prepared by method B: mp 51−52 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.21 (s, 1 H), 7.55 (t, J = 72.0 Hz, 1 H), 2.76 (s, 3 H), 2.30 (s, 3 H). Anal. ($C_8H_8N_2O_3F_2$) C, H, N.

2-(Difluoromethoxy)-3,6-dimethyl-5-nitropyridine (34). Method B: To a suspension of **33** (700 mg, 4.17 mmol) in acetonitrile (70 mL) was added sodium hydride (450 mg, 11.3 mmol, 60% in mineral oil). After stirring at room temperature for 15 min, 2,2-difluoro-2-(fluorosulfonyl)acetic acid (0.73 mL, 7.09 mmol) was added dropwise over several minutes. (Caution: 2,2-difluoro-2-(fluorosulfonyl)acetic acid can cause skin damage and should always be handled with gloves in a fume hood.) Some bubbling occurred during the addition. After stirring the reaction mixture at room temperature for 15 min, the reaction was quenched by the slow addition of water (10 mL). The acetonitrile was removed in vacuo, and the residue was transferred to a separatory funnel containing water (50 mL). The aqueous layer was extracted with ethyl acetate (3 × 50 mL). The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, and concentrated. The residue was purified by column chromatography on silica gel (10% ethyl acetate in hexanes) to afford **34** (870 mg, 96% yield) as a colorless solid: mp 48−49 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.21 (s, 1 H), 7.54 (t, J = 72.4 Hz, 1 H), 2.76 (s, 3 H), 2.30 (s, 3 H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 158.1, 151.0, 142.0, 137.0, 120.0, 113.9 (t, J = 255.8 Hz), 23.5, 14.7.

6-(Difluoromethoxy)-2,5-dimethylpyridin-3-amine (35). To a solution of **34** (33.4 g, 153 mmol) in methylene chloride (100 mL) and ethanol (600 mL) was added 10% palladium on carbon (3.3 g). The resulting suspension was hydrogenated on a Parr shaker at 40 psi H_2 for 1 h. TLC was used to monitor the reaction. An additional 3.3 g of palladium on carbon was added hourly until no starting material remained. A total of 13.2 g of Pd/C was added. The reaction mixture was kept under an H_2 atmosphere for 2 h after the last addition of catalyst. The reaction mixture was filtered through celite and sand and the collected solids were washed with ethyl acetate (2 × 100 mL). The filtrate was concentrated in vacuo to give a gray oil, which was purified by

column chromatography on silica gel (35% → 50% ethyl acetate in hexanes) to furnish **35** (25.7 g, 89% yield) as a pale-yellow oil, which solidified upon cooling in a refrigerator. The product was recrystallized from hexanes below 0 °C to afford white needles: mp 40−42 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35 (t, J = 74.0 Hz, 1 H), 6.84 (s, 1 H), 2.27 (s, 3 H), 2.15 (s, 3 H). LRMS (ESI) m/e 189.2 [$(M+H)^+$, calcd for $C_8H_{11}N_2OF_2$ 189.1]. Anal. ($C_8H_{10}N_2OF_2$) C, H, N.

Biology. Metabolic Stability Assay. Estimates of rates of compound turnover (pmol/min/mg protein) were generated using pooled human and rat liver microsomes (BD Biosciences, Woburn, MA). The stability in liver microsomes was determined by a high throughput in-house assay in which 3 μM of compound was incubated with 1.3 mM NADPH and 1 mg/mL of microsomal protein at 37 °C. Incubations were performed in sodium phosphate buffer (100 mM), pH 7.4 and were terminated at 0 and 10 min following the start of the incubations. The final organic concentration of solvent was 0.015% DMSO and 0.985% acetonitrile. Compound concentrations were determined by LC/MS/MS.

Protein Binding Studies. Unbound fraction in human serum was determined in vitro by equilibrium dialysis using the Dianorm dialysis system or HTDialysis LLC HTD 96B system. Human serum was spiked with compound and equilibrated against isotonic phosphate buffer for 3 or 7 h at 37 °C for the different systems, respectively. Following the incubation period, plasma and buffer samples were analyzed for compound concentrations using LC/MS/MS. Unbound fraction was calculated based on the ratio between buffer concentration and the serum concentration.

Rat Pharmacokinetic Studies. Pharmacokinetic parameters were estimated in Spague−Dawley rats following intravenous (2 mg/kg; n = 3) and oral (10 mg/kg; n = 3) dosing. Intravenous doses were prepared in a vehicle consisting of PEG:ethanol, 90:10 (v/v) at a volume of 1 mL/kg. The oral doses were prepared in a vehicle consisting of 1% polysorbate 80 in 0.5% methylcellulose suspension or Labrafil M 1944CS/DMAC/polysorbate 80, 85:10:5 (v/v) at a volume of 3 mL/kg. Blood samples were collected via a jugular vein catheter at 0, 0.17, 0.25, 0.5, 1, 2, 4, 6, 8, and 24 h postdose for the intravenous experiment, and at 0, 0.25, 0.5, 1, 2, 4, 6, 8, and 24 h postdose for the oral experiment. Plasma was separated by centrifugation and stored frozen at −20 °C until analysis. Concentrations were determined by LC/MS/MS.

Beagle Dog Discrete Studies. Pharmacokinetic properties were estimated in male beagle dogs (n = 4) following a 2 mg/kg intravenous dose. Intravenous doses were prepared in a vehicle consisting of propylene glycol:ethanol, 90:10 (v/v) at a volume of 1 mL/kg over 5 min at a constant rate of 0.2 mL/kg/min. Pharmacokinetic properties were estimated in male beagle dogs (fasted) following a 2 mg/kg oral dose in 0.2% polysorbate 80 in 0.5% methylcellulose suspension (n = 4) or following a 3 mg/kg oral dose in Labrafil M 1944CS/DMAC/polysorbate 80, 85:10:5 (v/v) (n = 2). The dose volume was 1 mL/kg. Blood samples were collected via a femoral vein at 0, 0.17, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, and 24 h postdose for the intravenous experiment and at 0, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, and 24 h postdose for the oral experiments. Plasma was separated by centrifugation and stored frozen at −20 °C until analysis. Plasma concentrations were determined by LC/MS/MS.

LC/MS/MS Conditions. Sample preparation was conducted as follows. Aliquots (typically, 50 μL) of the biological matrix from in vivo study and standard/QC samples were treated with acetonitrile (200 μL) containing an appropriate internal standard, followed by vortex mixing for 2 min. The supernatant was then separated from the precipitated proteins after a 20 min centrifugation at 3000 rpm and 200 μL was transferred to a 96-well plate. The supernatant was evaporated under nitrogen using a TurboVap, with the plate heater set at 37 °C, and then reconstituted using 75 μL of 0.1% formic acid.

An aliquot (5 μL) was injected onto a Synergi Fusion-RP column (2 mm × 50 mm, 4 μm) (Phenomenex, Torrance, CA) at room temperature for LC/MS/MS-based analysis (mobile phase = 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B); flow rate = 0.4 mL/min). A combination of isocratic and linear gradients were used for peak separation. The HPLC was interfaced to a Micromass Quattro Ultima LC/MS/MS tandem mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray interface operating in the positive ionization mode. Detection of each analyte was achieved through selected reaction monitoring.

In Vitro Metabolism Using Liver Microsomes. In vitro metabolic reaction mixtures (1 mL) contained 100 mM of potassium phosphate buffer (pH 7.4), 10 μM of a substrate, and pooled rat or human liver microsomes (final concentration as 1 mg/mL; BD Biosciences, Bedford, MA). The reactions were initiated by adding NADPH-regenerating reagents (final concentrations as 1.3 mM $NADP^+$, 3.3 mM β-D-glucose-6-phosphate, 3.3 mM magnesium chloride, and 0.5 unit/mL of glucose-6-phosphate dehydrogenase) and carried out at 37 °C. At the time of 0 and 30 min, an aliquot (300 μL) of reaction mixture was collected and reactions were terminated by adding an equal volume of acetonitrile. Following centrifugation at 10000 rpm for 5 min using an Eppendorf 5415C centrifuge (Hamburg, Germany), 30 μL of the supernatant was injected into HPLC/UV/MS for metabolite profiling. In separate glutathione trapping experiments, 5 mM of GSH was added to the reaction mixtures while the other reaction components and sample preparation procedures remained the same mentioned above. The relative abundance of each drug-related component detected in liver microsomal incubations was estimated based upon the ratio of the UV peak area (330−340 nm) of each particular component vs the combined UV peak areas (330−340 nm) of all recovered drug-related components at 30 min.

In Vivo Metabolism Using Bile Duct Cannulated (BDC) Rats. All animal procedures were reviewed and approved by the Animal Care and Use Committee at Bristol-Myers Squibb Co. BDC rats (male Sprague−Dawley) were obtained from Hilltop Lab Animals (Scottdale, PA). The dose was formulated in 90% poly ethylene glycol (PEG) and 10% ethanol (v/v). Three BDC rats were orally administered with **19e** at a dose of 20 mg/kg of body weight. Bile and urine samples were collected between 0 and 24 h, and plasma samples were collected at 0, 0.25, 0.5, 1, 2, 4, 6, 8, and 24 h. Three additional BDC rats were administered with the dosing vehicle (orally) as a control, and bile, urine, and plasma were collected accordingly. An aliquot (150 μL) of pooled bile or urine samples was mixed with an equivalent volume of acetonitrile. After precipitation by centrifugation at 10000 rpm for 5 min, the supernatant (30 μL) was injected directly onto the HPLC/UV/MS. For plasma sample preparation, three volumes of acetonitrile were added to one volume of pooled plasma (250 μL) to precipitate proteins. Following centrifugation at 10000 rpm for 5 min, the supernatant was collected and dried using a N_2 stream. The residues were redisolved in 150 μL of 50% acetonitrile and 50% H_2O containing 0.1% formic acid, and a 30 μL aliquot was introduced onto the HPLC/UV/MS for analysis. The relative abundance of each drug-related component detected in plasma, bile, or urine samples was estimated based upon the ratio of the UV peak area (330−340 nm) of each particular component vs the combined UV peak areas (330−340 nm) of all recovered drug-related components in the particular matrix.

HPLC/UV/MS for Metabolite Identification. The HPLC/UV/MS system consisted of a Waters 2695 Separations Module (Waters, Milford, MA), which was connected with a Surveyor PDA detector (ThermoFisher Scientific, Waltham, MA) and an LCQ-Deca XP ion trap mass spectrometer (ThermoFisher Scientific) equipped with an electrospray ionization source (ESI). Separation of antagonists and their metabolites was achieved on a Synergi Hydro-RP column (4 μm, 3 mm × 150 mm;

Phenomenex, Torrance, CA) using solvent A (5% acetonitrile and 95% H_2O containing 0.1% formic acid) and solvent B (100% acetonitrile). At a flow rate of 0.3 mL/min, the initial gradient (98% A and 2% of solvent B) was held constant for the first 2 min followed by a linear increase to 70% of solvent B over the next 8 min and finally ramped up to 100% of solvent B over an additional 15 min. The gradient was maintained for 5 min before returning to the initial condition for a 10 min equilibration period. The eluate from the column was routed to the PDA detector scanning from 200 to 550 nm. The mass spectrometer was set for a full scan and data- or list-dependent scan modes and was operated in a positive ion mode. Adjusted to achieve the best sensitivity, the electrospray voltage was 5.0 kV, heated capillary temperature was 350 °C, sheath gas was 40 (arbitrary unit), auxiliary gas was 90 (arbitrary unit), and normalized collision energy (NCE for MS^n) was 45%.

Metabolite Identification. UV and mass spectrometry data were acquired and analyzed using Xcalibur version 1.3 (ThermoFisher Scientific). Metabolite ID (ThermoFisher Scientific) and Mass Frontier (ThermoFisher Scientific) were also employed to help identify metabolites according to a predicted metabolite list derived from an in-house biotransformation database at Bristol-Myers Squibb Co. and the metabolite prediction software, Meteor (Lhasa Limited, Leeds, UK).[60]

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Supporting Information Available: Tables of elemental analysis data and high-resolution mass spectral data with HPLC purity data for compounds lacking elemental analysis data. This material is available free of charge via the Internet at http://pubs.acs.org.

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(49) Experimental procedures for the CRF binding assay, functional assay (Y-79 cells), behavioral studies, and receptor occupancy measurements are described in reference 33.

(50) The logD measurement was determined by partitioning of the compound between octanol and water at pH = 7.4.

(51) The pK_a values were determined in the pH range between 2 and 11, and the data were obtained using a Sirius GLpKa instrument. The pK_a was measured using the spectrophotometric titration method and the compound was dissolved in varying concentrations of acetonitrile/water with extrapolation to 0% cosolvent.

(52) Oleoyl macrogolglycerides, also known as Labrafil M 1944CS, was purchased from Gattefossé, 36 Chemin de Genas, BP 603, 69804 Saint-Priest, France (http://www.gattefossecanada.ca/en/products/pharmaceutical/gattefosse_oral.shtml).

(53) The unbound fractions for the compounds in Table 2 are as follows: **17a**, 2.1%; **19b**, 1.9%; **17i**, 0.8%; **17j**, 0.8%; **19d**, 1.6%; **19e**, 3.5%. The unbound fractions were determined by equilibrium dialysis.

(54) The relative abundance of each drug-related component detected in liver microsomal incubations was estimated based upon the ratio of the UV peak area (330−340 nm) of each particular component vs the combined UV peak areas (330−340 nm) of all recovered drug-related components after an incubation time of 30 min.

(55) The relative abundance of each drug-related component detected in plasma, bile, or urine samples was estimated based upon the ratio of the UV peak area (330−340 nm) of each particular component vs the combined UV peak areas (330−340 nm) of all recovered drug-related components in the particular matrix.

(56) IC_{50} of sauvagine = 0.58 ± 0.28 nM, $n = 2$ (0.77 nM, 0.38 nM) in the CRF_2 receptor binding assay.

(57) Screening was preformed by MDS Pharma Services, 158 Li-Teh Road, Peitou Taipei, Taiwan.

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(59) Compound **19e** is also known as BMS-764459.

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