Imidazo[1,2-*b*]pyridazines, Novel Nucleus with Potent and Broad Spectrum Activity against Human Picornaviruses: Design, Synthesis, and Biological Evaluation

Chafiq Hamdouchi,*,† Concha Sanchez-Martinez,‡ Joseph Gruber,† Miriam del Prado,‡ Javier Lopez,‡ Almudena Rubio,† and Beverly A. Heinz†

Lilly Research Laboratories, A Division of Eli Lilly and Company, Lilly Corporate Center, DC: 0540, Indianapolis, Indiana 46285, and Centro de Investigación Lilly, Avenida de la Industria, 30.28108 Alcobendas, Madrid, Spain

Received December 27, 2002

A novel structural class of picornavirus inhibitors comprising an imidazo[1,2-*b*]pyridazine nucleus was discovered. 2-Aminoimidazo[1,2-*b*]pyridazines (**6d**, (*E*/*Z*)-**7b**, (*E*)-**7d**, (*Z*)-**7d**, (*E*/*Z*)-**8b**, (*E*)-**10b**, (*E*)-**13a**, (*Z*)-**13a**, (*E*)-**13b**, (*Z*)-**13b**, (*E*)-**13c**, and (*Z*)-**13c**) were designed and synthesized in an effort to identify potent broad spectrum antirhinoviral agents. A practical synthetic route to this chemical scaffold has been developed. The target compounds were evaluated in a plaque reduction assay and in a cytopathic effect assay. Our preliminary SAR studies highlight the minimum structural features required for antirhinovirus activity. Our data suggest that the nature of the linker between the phenyl and the imidazopyridazine moieties has a significant influence on the activity of these compounds. Oximes are slightly better than vinyl carboxamides at this position. The oximes are the most potent analogues against human rhinovirus 14 (HRV-14), and at the concentrations evaluated, no apparent cellular toxicity is noted. Furthermore, the *E* geometry appears to be a key element for activity; the *Z* isomer leads to a considerable loss in potency. Of particular interest, analogue **7b** exhibits potent broad-spectrum antirhinoviral and antienteroviral activity when evaluated against a panel of seven additional rhino- and enteroviruses. The chemistry and the biological evaluations are discussed.

Introduction

Picornaviruses cause numerous human diseases, including poliomyelitis, acute hepatitis, myocarditis, and the common cold. Family members include several well-known human pathogens such as polioviruses, hepatitis A virus, coxsackieviruses, and over 110 serotypes of human rhinoviruses (HRV). Despite the fact that some picornaviral infections are mild and self-limiting in healthy adults, serious sequelae can occur in children or patients with pre-existing medical problems. Therefore, the need for new prevention and treatment options for picornaviral infections has focused on the discovery of effective vaccines and potent antiviral agents.

The human rhinoviruses are recognized as the most important etiologic agents of the common cold in adults and children.[1] In addition to causing the common cold, these viruses also typically precipitate or exacerbate several chronic conditions such as bronchitis, otitis media, sinusitis, emphysema, and asthma. Because of their significant association with disease, the search for an effective treatment for rhinovirus infections has attracted considerable attention in the scientific community. Since the large number of serotypes makes the development of a broad-spectrum vaccine unlikely, efforts have focused on the development of effective antivirals. Several classes of antiviral drugs have been studied for the treatment of rhinovirus colds, including those that bind directly to the virus and inhibit virus uncoating, compounds that block the attachment of rhinovirus to ICAM-1, and compounds that inhibit activity of the viral protease. However, despite good in vitro activity, the majority of these compounds have been ineffective when dosed by either oral or intranasal administration, the routes preferred for the treatment of common colds. Antiviral compounds have been rejected as clinical candidates because of problems with toxicity, unfavorable pharmacology, or insufficient potency. Thus, the treatment of the common cold remains an elusive goal.

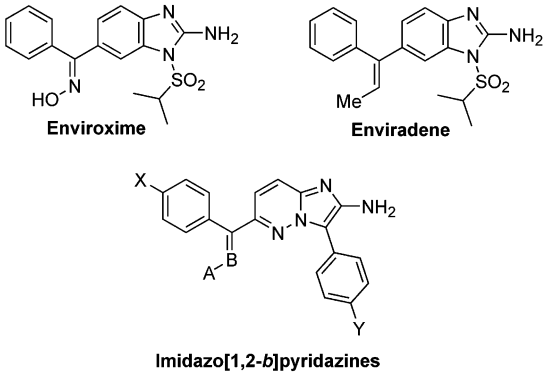


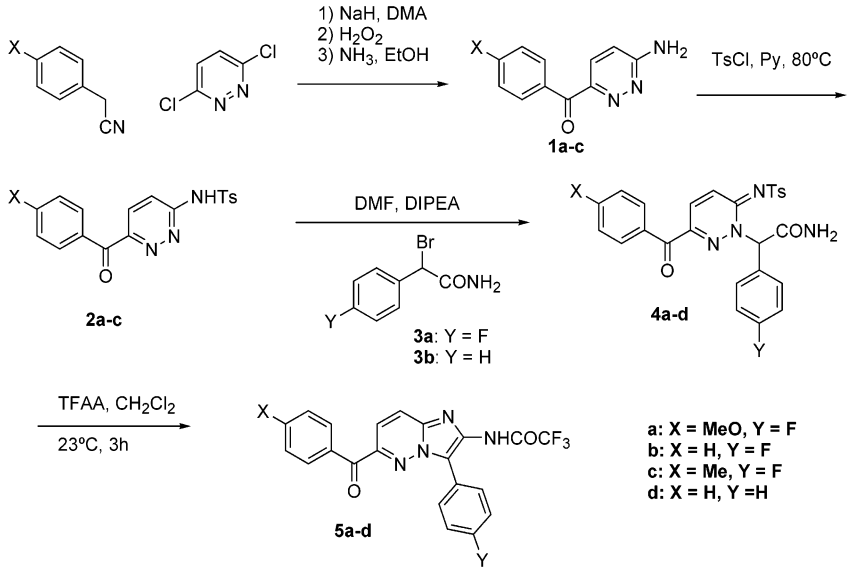
Figure 1.

Enviroxime (Figure 1), a benzimidazole derivative with strong in vitro antirhinoviral activity, is one of the more extensively studied synthetic agents.[2] Enviroxime and related benzimidazoles[3] showed potent broad-

* To whom correspondence should be addressed. E-mail: Hamdouchi_Chafiq@Lilly.com. Phone: 317-276-9582. Fax: 317-277-3652.
† Lilly Research Laboratories.
‡ Centro de Investigación Lilly.

10.1021/jm020583i CCC: $25.00 © 2003 American Chemical Society
Published on Web 08/21/2003

Scheme 1



spectrum antiviral activity against a wide range of both rhinoviruses and enteroviruses. Noncytotoxic concentrations of enviroxime are associated with complete inhibition of replication of 81 rhinovirus serotypes, with 50% inhibitory concentrations (IC_{50}) ranging from 0.05 to 0.12 μg/mL. Although the mechanism of action of enviroxime is not completely understood, it is believed that the drug inhibits the formation of the viral RNA polymerase replication complex.[4]

The major shortcomings of enviroxime are its poor oral bioavailability and undesirable side effects. Studies of oral enviroxime indicated low drug levels in blood and nasal secretions and an unacceptably high frequency of nausea and vomiting.[5] Thus, despite its potent in vitro antirhinovirus activity, significant therapeutic benefit was not found with either oral or intranasal administration, and enviroxime clinical studies were terminated. Enviradene, a related benzimidazole, showed improved pharmacokinetics in dogs and caused no emesis.[6] However, the peak plasma levels did not surpass the antiviral IC_{50} value, and the studies on enviradene were discontinued.

In our laboratory, a series of 3-substituted imidazo[1,2-*a*]pyridines were recently found to have strong activity against human rhinovirus.[7] Structure−activity relationship (SAR) studies of imidazo[1,2-*a*]pyridines demonstrated that substitution at the 3-position is essential for antiviral activity and that the isopropyl sulfonyl group present in enviroxime is not critical for activity.

As part of our continuous efforts toward the identification of potent broad-spectrum antirhinoviral agents, structurally related imidazo[1,2-*b*]pyridazine analogues were examined (Figure 1). This series has shown significant improvement in potency relative to the imidazo[1,2-*a*]pyridines series as well as broad-spectrum activity against a variety of rhinovirus serotypes.

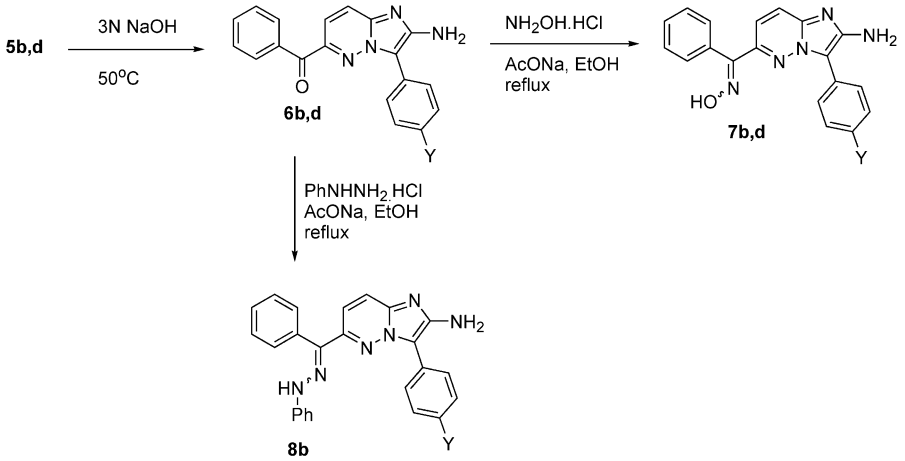
Chemistry

We initiated an SAR exploration of imidazo[1,2-*b*]pyridazine derivatives as potential antirhinoviral agents. We made the initial assumption that structural information gained from the benzimidazole[3] and imidazo[1,2-*a*]pyridine[7] SARs would be transferable to the imidazo[1,2-*b*]pyridazine series. On the basis of this analysis, we undertook the synthesis of 2-aminoimidazo[1,2-*b*]pyridazines (**7**−**13**) that would offer the following potential advantages: (a) potent antiviral activity (structurally related to benzimidazoles), (b) broad spectrum of activity, (c) enhanced oral bioavailability, and (d) fewer side effects.

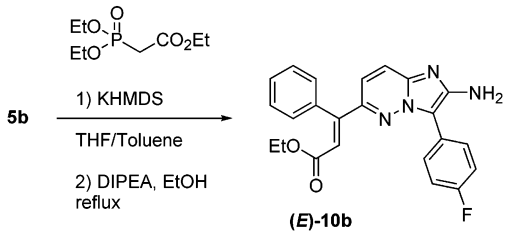
The synthesis started with the condensation of a series of para-substituted phenylacetonitriles with 3,6-dichloropyridazine under basic conditions (Scheme 1). The reaction accommodated a variety of commercially available para-substituted phenylacetonitriles. Subsequent oxidation with hydrogen peroxide afforded the corresponding 6-chloro-3-benzoylpyridazines, which were treated with ammonia in ethanol to yield the desired 6-amino-3-benzoylpyridazines **1a**−**c**. Tosylation of the amino group with *p*-tolylsulfonyl chloride in pyridine and subsequent reaction with the 2-bromo-2-arylacetamides **3a**,**b** in the presence of Hünig's base in DMF provided the corresponding pyridazinyl acetamides **4a**−**d** in good yield. The 2-bromo-2-arylacetamides **3a**,**b** were prepared from their corresponding 2-arylacetic acid by bromination in the presence of *N*-bromosuccinimide and subsequent amidation. Conversion of **4a**−**d** to the desired 2-(*N*-trifluoracetylamino)imidazopyridazine ketones **5a**−**d** was accomplished by treatment with trifluoroacetic anhydride.

We next turned our efforts to the derivatization of the ketone moiety. The trifluoroacetamide group in compounds **5b**,**d** was hydrolyzed with 3 N NaOH to give the corresponding aminoimidazopyridazines **6b**,**d** in quantitative yield. Compounds **6b**,**d** were then treated with $NH_2OH{\cdot}HCl$ to yield the desired oximes **7b**,**d** as mixtures of *E* and *Z* isomers. The *Z* isomers were typically isolated after recrystallization from a mixture of ethyl acetate/hexane while the *E* isomers were separated from the remaining mother liquors by chromatography. The assignment of the geometry of the double bond was based on the following nuclear Overhauser enhancement (NOE) results. Irradiation of HO at δ 11.87 in (*E*)-**7** gave an enhancement of the aryl protons. No enhancement was observed for the py-

Scheme 2



Scheme 3



ridazine protons. A stability study on the final oximes **7b**,**d** showed an equilibrium between the *Z* and *E* isomers forms in polar solvents such as DMSO and TFA at room temperature.

Treatment of the ketone **6b** with $PhNHNH_2 \cdot HCl$ in refluxing ethanol led to the corresponding phenylhydrazone **8b** that was shown to exist as a rapidly equilibrating mixture of *E* and *Z* geometric isomers (Scheme 2).

Efforts to overcome the configurational instability suggested the need to evaluate additional unsaturated linkers. Treatment of the ketone **5b** with the Horner–Wadsworth–Emmons reagent, potassium salt of triethyl phosphonoacetate, afforded the corresponding α,*β*-unsaturated ester **9b** as a mixture (about 13:1) of *E* and *Z* isomers. Hydrolysis of the trifluoroacetamide group with DIPEA in refluxing ethanol furnished the final aminoimidazo[1,2-*b*]pyridazine (*E*)-**10b** (Scheme 3).

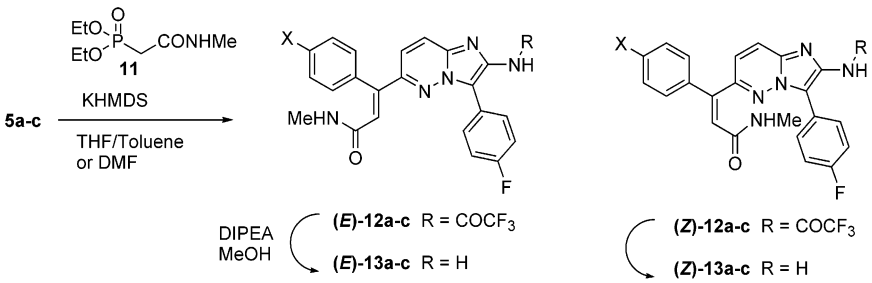
The incorporation of the vinyl carboxamide group was achieved using the protocol outlined in Scheme 4. When ketones **5a**–**c** were subjected to the Horner–Wadsworth–Emmons reaction with the potassium salt of diethyl (*N*-methylcarbamoylmethyl)phosphonate **11**,[7] the desired vinyl carboxamides **12a**–**c** were isolated as mixtures of *E* and *Z* isomers.

The stereospecificity of the reaction depends on the substrate, the solvent, and the reaction time. In THF (35 h), compound **5b** gave a mixture of *E* and *Z* isomers (about 4:1). In DMF, the reaction was much faster (5 h) but the *E*/*Z* ratio decreased to 1:5. Interestingly, equilibrium was produced in favor of the *E* isomer (about 3:1) when the reaction was performed in DMF and quenched after 48 h. The isomers were separated either by crystallization or by chromatography. In the case of compound **12b**, simple crystallization from ethyl acetate afforded (*E*)-**12b** in its pure form. The assignment of the geometry of the double bond was based on the following NOE results (Scheme 5). Irradiation of H_a at δ 7.0 in (*E*)-**12b** gave an enhancement of the vinyl proton (H_d). No enhancement was observed for the proton (H_e). Irradiation of H_d at δ 6.7 gave enhancement of the pyridazine proton (H_a) and the aromatic proton of the 4-fluorphenyl. Conversion of **12a**–**c** to their corresponding final targets **13a**–**c** was performed by cleavage of the trifluoroacetamide group with DIPEA in refluxing methanol.

Structure–Activity Relationship Analysis

2-Aminoimidazo[1,2-*b*]pyridazines **6d**, (*E*/*Z*)-**7b**, (*E*)-**7d**, (*Z*)-**7d**, (*E*/*Z*)-**8b**, (*E*)-**10b**, (*E*)-**13a**, (*Z*)-**13a**, (*E*)-**13b**, (*Z*)-**13b**, (*E*)-**13c**, and (*Z*)-**13c** were subjected to biological evaluation following the procedures described in the Experimental Section. The activity of new analogues was compared to that of enviroxime used as a reference standard. Human rhinovirus 14 (HRV-14) was selected as the prototype virus for testing because of the amount of information known about this serotype. Plaque reduction assays were used to determine the antiviral activity reported as the IC_{50} (Table 1). This family of compounds exhibited potent antirhinoviral activity. Our

Scheme 4



Scheme 5

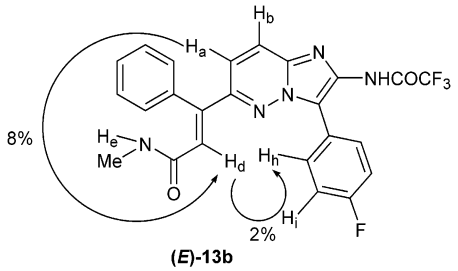


Table 1. Antiviral Activity Evaluation of Imidazopyridazines

compd	X	Y	B−A	IC_{50} (μg/mL) PRA[a]	TC_{50} (μg/mL) CPE/XTT[b]
enviroxime				0.05	
6d	H	H	O	7.19	
(*E*/*Z*)-**7b**	H	F	N−OH	0.05	>10
(*E*)-**7d**	H	H	N−OH	0.04	>10
(*E*/*Z*)-**8b**	H	F	N−NHPh	>10	>10
(*E*)-**10b**	H	F	CH−CO_2Et	>10	
(*E*)-**13a**	MeO	F	CH−CONHMe	0.6	>10
(*Z*)-**13a**	MeO	F	CH−CONHMe	4.2	>10
(*E*)-**13b**	H	F	CH−CONHMe	0.11	4.06
(*Z*)-**13b**	H	F	CH−CONHMe	>10	
(*E*)-**13c**	Me	F	CH−CONHMe	0.23	>10
(*Z*)-**13c**	Me	F	CH−CONHMe	>10	>10

[a] PRA assay using rhinovirus 14. [b] CPE/XTT assay using rhinovirus 14. The concentration of test compound required to cause 50% cytotoxic effect (TC_{50}) relative to a control with no drug and no virus present and that inhibits the development of virus cytopathic effect (CPE) by 50% (IC_{50}) was determined from the linear portion of each dose−response curve.

data suggest that the nature of the linker between the phenyl and the imidazopyridazine moieties has a significant influence on the activity of these compounds. The oximes (*E*)-**7d** and (*E*/*Z*)-**7b** showed potency (IC_{50} = 0.04 and 0.05 μg/mL) comparable to that of enviroxime. The vinylcarboxamides that would be expected to participate in hydrogen-bond formation with their target in an analogous way to the oximes[8] were also found to be potent ((*E*)-**13b**, IC_{50} = 0.11 μg/mL). A complete loss of activity was observed with the vinyl esters and with larger groups such as phenylhydrazones. Furthermore, the *E* geometry appears to be a key element for activity; the *Z* isomer led to a considerable loss in potency.

To establish a general cellular toxicity of this chemical platform and to determine whether it correlates to the antiviral activity, imidazo[1,2-*b*]pyridazines were also tested in a cytopathic effect (CPE) assay. The CPE assay was performed as described previously[9] with a modification to the method of the quantification of the cytopathic effect of virus on the cells. Instead of using crystal violet staining, XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide) was used as a substrate to quantify the surviving host cells. We were pleased to find that, in general, the concentration of test compound that resulted in a 50% cytotoxic effect (TC_{50}) relative to a control with no drug and no virus present was considerably high. Our data indicate that there are no apparent associations between cellular toxicity and antiviral activity.

In addition to screening imidazopyridazines against HRV-14, we examined the broad-spectrum nature of this chemical platform. Compound **7d** was selected for screening against a panel of seven additional viruses that were chosen as representative of the large number of human rhinoviruses and human enteroviruses. Included in this panel were two viruses from the minor rhinovirus receptor subgroup, human rhinovirus 1A (HRV-1A) and human rhinovirus 2 (HRV-2); one additional virus from the major rhinovirus receptor subgroup, human rhinovirus 16 (HRV-16); a prototypical enterovirus, poliovirus 1 (PV-1); and three nonpolio enteroviruses, coxsackievirus A21 (CA21), coxsackievirus A21 mouse muscle adapted (CA21M), and coxsackievirus B3 (CB3). We were pleased to find that compound **7d** exhibited potency and broad-spectrum activity against all these viruses (Table 2).

Table 2. Broad-Spectrum Antiviral Activity Evaluation of **7d**[a]

assay	IC_{50} (μg/mL)
PR-HRV14	0.04
PR-HRV1A	0.05
PR-HRV2	0.03
PR-HRV16	0.06
PR-PV1	0.04
PR-CA21C	0.04
PR-CA21M	0.02
PR-CB3	0.05
average	0.04

[a] PRA assay, IC_{50} (μg/mL), using four human rhinoviruses (HRV-14, HRV-1A, HRV-2, and HRV-16), a prototypical enterovirus (poliovirus 1 (PV-1)), coxsackievirus A21 (CA21), coxsackievirus A21 mouse muscle adapted (CA21M), and coxsackievirus B3 (CB3).

Conclusion

Enviroxime and related benzimidazoles with potent broad-spectrum antirhinoviral and antienteroviral activity represent a unique opportunity for the treatment of the common cold. These compounds are believed to inhibit the formation of the viral RNA polymerase replication complex. However, despite good in vitro activity, they have been ineffective when given in a manner that would be acceptable for the treatment of colds (namely, by either oral or intranasal administration). Therefore, the identification of a novel nucleus that maintains the potency and broad-spectrum activity of enviroxime and offers new physiochemical properties is highly desirable. The compounds described here represent a novel lead series for the development of antipicornavirus agents. Members of this series of inhibitors have shown potency that is comparable to that of enviroxime. 2-Aminoimidazo[1,2-*b*]pyridazines showed a high potency when evaluated against HRV-14 with no apparent associations between cellular toxicity and antiviral activity. Furthermore, compound **7d** has shown broad-spectrum activity against a panel of seven additional viruses that were chosen as representative of the large number of human pathogens. Finally, a practical synthetic route to this chemical scaffold has been developed.

Experimental Section

General Methods. All reagents were purchased from Aldrich and used without further purification unless stated otherwise. Column chromatography was carried out on flash silica gel (Merck 230−400 mesh). TLC analysis was conducted on Whatman silica gel plates. Melting points were determined with a Thomas-Hoover melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded in the solvent indicated on a Bruker spectrometer at 200 MHz, a Varian Mer-

cury spectrometer at 400 MHz, or a GE QE-300 spectrometer at 300.15 MHz. ^{13}C NMR spectra were recorded in the solvents indicated on the previously mentioned spectrometers at 50 Mz, 100 MHz, and 75 MHz, respectively. IR spectra were recorded on a Nicolet 510P FT-IR spectrometer; UV spectra were recorded on a Shimadzu UV-2101 PC spectrometer; and field desorption (FD) mass spectra were recorded on either a VG ZAB-3F or VG 70-SE instrument. High-resolution mass spectra were recorded on a Micromass QTOF mass spectrometer. IR spectra, UV spectra, FDMS spectra, elemental analyses, and some ^{1}H NMR spectra were provided by the Physical Chemistry Department at Lilly Research Laboratories.

6-Amino-3-(4-methoxybenzoyl)pyridazine (1a). Step 1: 6-Chloro-3-(4-methoxybenzoyl)pyridazine. Potassium *tert*-butoxide (37.66 g, 335.62 mmol) was added to a stirred solution of 4-methoxyphenylacetonitrile (167.81 mmol, 22.76 mL) in 200 mL of *N*,*N*-dimethylacetamide (DMA) at 0 °C under nitrogen.. The reaction mixture was stirred for 30 min, and 3,6-dichloropyridazine (25 g, 167.81 mmol) was added in one portion. The ice bath was then removed, and the reaction mixture was stirred at room temperature for 1 h. The reaction mixture was again cooled to 0 °C, and hydrogen peroxide (30 mL of a 30% solution, 326 mmol) was added dropwise. The reaction mixture was stirred for an additional hour, then poured onto 1 N HCl (1 L), and extracted with ethyl acetate (3 × 300 mL). The organic layers were combined and washed with 1 N NaOH (3 × 200 mL) and brine (3 × 150 mL). After drying over $MgSO_4$, the solvents were removed in vacuo and the remaining solid recrystallized from ethyl acetate/hexane to yield 21.92 g (53%) of the desired product: ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.17 (AB system, 2H, J = 8.9, Het), 7.05 (AA′BB′ system, 4H, Ar), 3.85 (s, 3H, OMe); FDMS *m/z* 250/248 (M^+). Anal. Calcd for $C_{12}H_9N_2O_2Cl$: C, 57.96; H, 3.65; N, 11.26. Found: C, 57.98; H, 3.64; N, 11.29.

Step 2: 6-Amino-3-(4-methoxybenzoyl)pyridazine (1a). To a solution of 6-chloro-3-(4-methoxybenzoyl)pyridazine (21.92 g, 88.15 mmol) in 350 mL of EtOH was added 125 mL of anhydrous ammonia. The mixture was then placed in a bomb and heated to 145 °C for 16 h. The solvents were removed, and the solids were recrystallized from ethyl acetate to yield 15.76 g (78%) of 6-amino-3-(4-methoxybenzoyl)pyridazine **1a**: mp 136 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.05 (AA′ of AA′BB′ system, 2H, J = 8.9, Ar), 7.8 (A of AB system, 1H, J = 12, Het), 7.16 (bs, 2H, exchange D_2O, NH_2), 7.05 (BB′ of AA′BB′ system, 2H, J = 8.9, Ar), 6.85 (B of AB system, 1H, J = 12, Het), 3.82 (s, 3H, Me); HRMS calcd for $C_{12}H_{11}N_3O_2$ 229.0851, found 353.0846.

6-Amino-3-benzoylpyridazine (1b). Compound **1b** was prepared according to the procedure for **1a**: 13.35 g (75%); mp 122 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.95−7.92 (m, 2H, Ar), 7.34 (AB system, 2H, J = 9.2, Het), 7.60−7.51 (m, 1H, Ar), 7.48−7.46 (m, 2H, Ar), 7.23 (bs, 2H, exchange D_2O, NH_2); FDMS *m/z* 200 $(M + H)^+$. Anal. Calcd for $C_{11}H_9N_3O$: C, 66.32; H, 4.55; N, 21.09. Found: C, 66.06; H, 4.52; N, 21.30.

6-Amino-3-(4-methylbenzoyl)pyridazine (1c). Compound **1c** was prepared according to the procedure for **1a**: 12.64 g (58%); UV, EtOH, λ = 295 (ϵ = 17 622); IR (KBr): 3354, 3170, 1660, 1616, 1458, 1301, 1163, 927, 783 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.64 (AA′BB′ system, 4H, Ar), 7.37 (AB system, 2H, J = 12, Het), 7.21 (bs, 2H, exchange D_2O, NH_2), 2.41 (s, 3H, Me); FDMS *m/z* 214 $(M + H)^+$. Anal. Calcd for $C_{12}H_{11}N_3O$: C, 67.59; H, 5.20; N, 19.71. Found: C, 67.03; H, 4.80; N, 19.16.

***N*-[6-(4-Methoxybenzoyl)-2*H*-pyridazin-3-ylidene)-4-methylbenzenesulfonamide (2a).** To a suspension of 6-aminopyridazine **1a** (21.80 mmol, 1.0 equiv) in dry pyridine (35 mL) was added *p*-toluenesulfonyl chloride (34.88 mmol, 1.6 equiv) portionwise. The reaction mixture was heated at 80−90 °C for 24 h. The pyridine was removed in vacuo, and the remaining residue was mixed with 100 mL of ice/water and stirred for 1 h. The solid was collected and recrystallized from ethyl acetate to yield **2a** (85%) as a white solid: mp 148 °C; ^{1}H NMR (200 MHz,$CDCl_3$) δ 8.16 (AA′BB′ system, 2H, Ar), 8.02 (d, J = 9.6, 1H, Het), 7.88 (AA′BB′ system, 2H, Ar), 7.50 (d, J = 9.6, 1H, Het), 7.29 (AA′BB′ system, 2H, Ar), 6.97 (AA′BB′ system, 2H, Ar), 3.90 (s, 3H, OMe) 2.42 (s, 3H, Me); ^{13}C NMR (DMSO-d_6) δ 187.5, 163.7, 156.0, 142.7, 139.7, 133.4, 132.5, 129.7, 127.0, 126.4, 124.1, 113.9, 55.8, 21.1; MS (EI^+) *m/z* 383 M^+ (9), 318 (63), 229 (10), 135 (100), 107 (6), 91 (34), 65 (12); HRMS calcd for $C_{19}H_{17}N_3O_3S$ 383.093 98, found 383.094 24.

***N*-(6-Benzoyl)-2*H*-pyridazin-3-ylidene-4-methylbenzenesulfonamide (2b).** Compound **2b** was prepared according to the procedure for **2a**: 14.72 g (83%); mp 136 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.11 (d, J = 9.8, 1H, Het), 7.93 (m, 3H, Ar + Het), 7.55 (AA′BB′ system, 2H), 7.64 (m,1H), 7.49 (m, 2H, Ar), 2.32 (s,3H); HRMS calcd for $C_{18}H_{15}N_3O_3S$ 353.0834, found 353.0829.

***N*-[6-(4-Methylbenzoyl)-2*H*-pyridazin-3-ylidene)-4-methylbenzenesulfonamide (2c).** Compound **2c** was prepared according to the procedure for **2a**: 11.5 g (76%); mp 155 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.12 (d, J = 9.7, 1H, Het), 7.95 (d, J = 9.7, 1H, Het), 7.86 (AA′BB′ system, 2H, Ar), 7.78 (AA′BB′ system, 2H, Ar), 7.36 (AA′BB′ system, 2H, Ar), 7.32 (AA′BB′ system, 2H, Ar), 2.38 (s, 3H, Me), 2.35(s, 3H, Me); ^{13}C NMR (75 MHz, DMSO-d_6) δ 188.2, 155.4, 143.5, 142.1, 139.0, 131.9, 131.6, 129.0, 128.7, 128.3, 125.8, 123.4, 114.2, 20.7, 20.4; MS (EI^+) *m/z* 367 M^+ (12), 302 (100), 119 (83), 91 (84), 65 (31); HRMS calcd for $C_{19}H_{17}N_3O_3S$ 367.099 61, found 367.099 15.

2-Bromo-2-(*p*-fluorophenyl)acetamide (3a). Step 1: 4-Fluorophenylacetamide. A solution of oxalyl chloride (200 mmol) in CH_2Cl_2 (25 mL) was added dropwise over 25 min to a stirred solution of 4-fluorophenylacetic acid (21 g, 100 mmol) and DMF (3 drops) in dry CH_2Cl_2 (170 mL) at 0 °C under nitrogen. The ice bath was removed, and the reaction mixture was stirred for 3 h. The solvents were removed under vacuo and then azeotroped with toluene (3 × 25 mL). The remaining oil was dissolved in 300 mL of toluene and 300 mL of hexane, and the mixture was stirred vigorously with a mechanical stirrer. Ammonia gas was then blown through a gas dispersion tube over the top of this solution for 1 h. The resulting solid was filtered and then dissolved in EtOAc/H_2O. The organic layer was washed with 1 N HCl, saturated $NaHCO_3$, and brine and was dried over sodium sulfate. The solvent was removed in vacuo and the remaining solid was recrystallized from EtOAc/hexane to yield 4-fluorophenylacetamide (87%) as a white solid: ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.29 (dd, J = 8.9, J = 5.6, 2H, Ar), 7.12 (dd, J = 9.0, J = 8.9, 2H, Ar), 6.00 (bs, 1H, NH_2), 5.35 (bs, 1H, NH_2) 3.59 (s, 2H, CH_2).

Step 2: 2-Bromo-2-(*p*-fluorophenyl)acetamide (3a). To a suspension of 4-fluorophenylacetamide (6.5 mmol, 1 equiv) in 25 mL of CCl_4 was added NBS (6.5 mmol, 1 equiv). The mixture was refluxed under UV radiation for 6 h. The reaction mixture was cooled to room temperature and then washed with water and brine. The residue was purified by flash chromatography (ethyl acetate/hexane, 1:3): mp 112 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.5 (dd, J = 8.8, J = 5.5, 2H, Ar), 7.12 (dd, J = 8.8, J = 8.9, 2H, Ar), 6.6 (bs, 1H, NH_2), 6.1 (bs, 1H, NH_2), 5.43 (s, 1H, CH); ^{13}C NMR (DMSO-d_6) δ 168.9, 164.7 134.0, 131.0, 130.8, 115.7, 115.3, 48.5; MS (EI^+) *m/z* 230 M^+ (65), 214 (100), 188 (22),186 (21), 152 (77), 136 (24), 124 (56), 109 (100), 108 (64), 107 (48), 85 (47), 83 (74), 80 (15).

2-Bromo-2-phenylacetamide (3b). Step 1: 2-Bromo-2-phenylacetic Acid. A mixture of phenylacetic acid (0.13 mol), benzoyl peroxide (0.54 mmol), and *N*-bromosuccinimide (0.130 mol) in 500 mL of CCl_4 was refluxed under UV radiation for 5 h. The reaction mixture was cooled to room temperature, and the succinimide was filtered away. The CCl_4 was removed in vacuo and the remaining oil was recrystallized from hexane, yielding the desired product as a yellow solid (82%).

Step 2: 2-Bromo-2-phenylacetamide (3b). A solution of oxalyl chloride (200 mmol) in dry CH_2Cl_2 (25 mL) was added dropwise over 25 min to a solution of 2-bromo-2-phenylacetic acid (21 g, 100 mmol) and DMF (3 drops) in CH_2Cl_2 (170 mL) at 0 °C under. The ice bath was removed, and the reaction mixture was stirred for 3 h. The solvents were removed in vacuo and then azeotroped with toluene (3 × 25 mL). The

remaining oil was dissolved in 300 mL of toluene and 300 mL of hexane, and the mixture was stirred vigorously with a mechanical stirrer. Ammonia gas was then blown through a gas dispersion tube over the top of this solution for 1 h. The resulting solid was filtered and then dissolved in ethyl acetate/H_2O. The organic layer was washed with 1 N HCl, saturated $NaHCO_3$, and saturated NaCl and was then dried over sodium sulfate. Solvent was removed in vacuo and the remaining solid was recrystallized from ethyl acetate/hexane to yield 16.8 g (68%) of the desired product: mp 138−141°C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 7.80 (bs, CONH), 7.54 (dd, $J_1 = 7.8$, $J_2 = 2.3$, 2H, Ar), 7.41 (bs, 1H, CONH), 7.41−7.30 (m, 3H, Ar), 5.54 (s, 1H, ArC*H*BrCONH$_2$); ^{13}C NMR (50 MHz, DMSO-d_6) δ 169.1 (CONH$_2$), 129.0 (ArCH), 133.8 (C), 128.8, 128.7, 49.9 (Ph*C*H-BrCONH$_2$); MS (EI$^+$) *m/z* 91.11 (100) [M$^+$ − 122], 134.13 (89.09) [M$^+$ − 79], 169.05 (15.00) [M$^+$ − 44], 170.06 (10.97) [(M$^+$ − 44) + 1], 171.05 (15.51) [(M$^+$ − 44) + 2], 172.05 (10.49) [(M$^+$ − 44) + 3], 213.06 (0.38) [M$^+$].

2-[(4-Fluorophenyl)-2-[3-(4-methoxybenzoyl)-6-(toluene-4-sulfonylimino)-6*H*-pyridazin-1-yl]acetamide (4a). To a suspension of *N*-[6-(4-methoxybenzoyl)-2*H*-pyridazin-3-ylidene)-4-methylbenzenesulfonamide **2a** (12.74 mmol, 1 equiv) in 60 mL of dry DMF was added 3.1 mL of DIPEA (17.19 mmol, 1.3 equiv) under an argon atmosphere. The mixture was stirred for 30 min, and 2-bromo-2-(*p*-fluorophenyl)acetamide (15.28 mmol, 1.2 equiv) was added. After being stirred for 24 h, the solution was poured onto 600 mL of water and stirred for 1 h. Solids were collected and air-dried to give **4a** in 92% yield: mp 245 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 8.08 (s, 2H, Ar), 7.93−6.80 (m, 12H, Ar), 6.64 (s, 1H, CH), 3.83 (s, 3H, OMe), 2.48 (s, 3H, Me); ^{13}C NMR (50 MHz, DMSO-d_6) δ 185.7, 168.2, 163.6, 162.5, 155.0, 144.4, 142.5, 140.1, 133.3, 133.1, 131.4, 129.8, 129.6, 127.0, 126.2, 125.9, 115.6, 113.5, 68.7, 55.7, 21.1; MS (EI$^+$) *m/z* 535 M$^+$ (1), 517 (10), 399 (8), 362 (11), 171 (14), 155 (31), 135 (100), 107 (16), 91 (73), 65 (18); HRMS calcd for $C_{27}H_{23}FN_4O_5S$ 534.137 32, found 534.136 66.

2-[3-Benzoyl-6-(toluene-4-sulfonylimino)-6*H*-pyridazin-1-yl]-2-(4-fluorophenyl)acetamide (4b). To a suspension of *N*-(6-benzoyl)-2*H*-pyridazin-3-ylidene-4-methylbenzenesulfonamide **2b** (10.59 g, 30 mmol) in 50 mL of dry DMF, was added DIPEA (5.75 mL, 33 mmol) followed by α-bromo-*p*-fluorophenylacetamide **3a** (9.66 g, 41.7 mmol). The reaction mixture was stirred for 72 h, then poured into 3 L of H_2O, and stirred vigorously for 1 h. The precipitate was collected and recrystallized from ethyl acetate to yield 12.66 g (84%) of 2-[3-benzoyl-6-(toluene-4-sulfonylimino)-6*H*-pyridazin-1-yl]- 2-(4-fluorophenyl)acetamide: mp 226 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 8.09 (AB system, 2H, $J = 8.9$ Hz, Het), 7.789 (bs, 1H, CONH$_2$), 7.77 (d, 2H, $J = 8.2$, Ar), 7.59−7.53 (m, 2H, Ar), 7.36−7.24 (m, 7H, Ar), 7.13−7.07 (m, 2H, Ar), 6.62 (bs, 1H, CONH$_2$), 2.34 (s, 3H, Me); FDMS *m/z* 504 (M$^+$). Anal. Calcd for $C_{26}H_{21}FN_4O_4S$: C, 61.90; H, 4.20; N, 11.10. Found: C, 62.11; H, 4.34; N, 10.97.

2-[3-(4-Methylbenzoyl)-6-(toluene-4-sulfonylimino)-6*H*-pyridazin-1-yl]-2-(4-fluorophenylacetamide (4c). Compound **4c** was prepared according to the procedure for **4a.** Starting from the tosylate **2c** and 2-bromo-2-(4-fluorphenyl)-acetamide **3a**, compound **4c** was obtained in 74% yield: mp 237 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 8.09−7.12 (m, 14H, Ar), 6.64 (s, 1H, CH), 2.37 (s, 3H, Me), 2.35 (s, 3H, CH$_3$); ^{13}C NMR (50 MHz, DMSO-d_6) δ 186.4, 167.7, 161.8, 154.3, 143.4, 143.2, 141.8, 139.3, 132.3, 131.2, 130.5, 130.1, 128.9, 128.1, 125.5, 125.2, 114.7, 67.9, 20.7, 20.4; MS (EI$^+$) *m/z* 519 M$^+$(2), 501 (15), 399 (13), 320 (37), 155 (38), 119 (87), 91 (100); HRMS calcd for $C_{27}H_{23}FN_4O_4S$ 518.142 404, found 518.142 820.

2-[3-Benzoyl-6-(toluene-4-sulfonylimino)-6*H*-pyridazin-1-yl]-2-phenylacetamide (4d). Compound **4d** was prepared according to the procedure for **4a.** Starting from the tosylate **2d** and 2-bromophenylacetamide **3b**, compound **4d** was obtained in 89% yield: mp 211 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 8.09 (AB system, 2H, Het), 7.90 (bs, 1H, CONH$_2$), 7.77 (d, 2H, $J = 8.1$, Ar), 7.57−7.54 (m, 4H, Ar), 7.34−7.25 (m, 8H, Ar), 6.68 (bs, 1H, CONH$_2$), 2.35 (s, 3H, Me); HRMS calcd for $C_{26}H_{22}N_4O_4S$ 486.1362, found 486.1365.

2,2,2-Trifluoro-*N*-[3-(4-fluorophenyl)-6-(4-methoxybenzoyl)imidazo[1,2-*b*]pyridazin-2-yl]acetamide (5a). To a suspension of 2-[(4-fluorophenyl)-2-[3-(4-methoxybenzoyl)-6-(toluene-4-sulfonylimino)-6*H*-pyridazin-1-yl]acetamide **4a** (3.32 mmol, 1 equiv) in 20 mL of dry CH_2Cl_2 in a flame-dried two-neck round-bottomed flask fitted with a reflux condenser, was added trifluoroacetic anhydride until a clear solution was obtained. The mixture was stirred for 3 h at room temperature. The solvents were removed, and the residue was taken up in ethyl acetate, washed with $NaHCO_3$ (2 × 20 mL) and saturated NaCl (2 × 20 mL), and dried over sodium sulfate. Ethyl acetate was removed in vacuo, and the residue was crystallized from ethyl acetate to yield 2,2,2-trifluoro-*N*-[3-(4-fluorophenyl)-6-(4-methoxybenzoyl)imidazo[1,2-*b*]pyridazin-2-yl]acetamide **5a** as a white solid (82% yield): mp 230−232 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 11.95 (bs, 1H, NH), 8.35 (d, $J = 9.4$, 1H, Het), 8.15 (m, 2H, Ar), 7.80 (m, 3H, Ar), 7.34 (m, 2H, Ar), 7.13 (m, 2H, Ar), 3.88 (s, 3H, OMe); ^{13}C NMR (50 MHz, DMSO-d_6) δ 188.2, 163.7, 161.5, 149.0, 137.5, 136.2, 133.4, 130.6, 130.4, 127.6, 125.7, 123.2, 120.6, 117.9, 115.7, 115.4, 55.6; HRMS calcd for $C_{22}H_{14}F_4N_4O_3$ 458.1002, found 458.1000.

2,2,2-Trifluoro-*N*-[3-(4-fluorophenyl)-6-benzoylimidazo-[1,2-*b*]pyridazin-2-yl]acetamide (5b). To a solution of 2-[3-benzoyl-6-(toluene-4-sulfonylimino)-6*H*-pyridazin-1-yl]-2-(4-fluorophenyl)acetamide (12.66 g, 25.12 mmol) in 150 mL of CH_2Cl_2 was added 100 mL of TFAA, and the mixture was refluxed under N_2 for 5 h. Solvents were removed, and the resulting foam was diluted with ethyl acetate, washed with saturated $NaHCO_3$ (3 × 100 mL) and brine (3 × 100 mL), and then dried over sodium sulfate. Ethyl acetate was removed and solids were recrystallized from ethyl acetate to yield 8.4 g (78%) of 2,2,2-trifluoro-*N*-[3-(4-fluorophenyl)-6-benzoylimidazo[1,2-*b*]pyridazin-2-yl]acetamide: mp 277 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 11.96 (bs, 1H, NHCOCF$_3$), 8.39 (d, $J = 9.4$, 1H, Het), 8.12 (dd, $J = 7.0$, $J = 1.6$, 2H, Ar), 7.90 (d, $J = 9.4$, 1H, Het), 7.78 (dd, $J = 8.6$, $J = 5.5$, 2H, Ar), 7.69 (dd, $J = 7.0$, $J = 1.6$, 1H, Ar), 7.58 (t, $J = 7.0$, 2H, Ar), 7.31 (t, $J = 8.6$, Ar); FDMS *m/z* 428 (M$^+$); Anal. Calcd for $C_{21}H_{12}F_4N_4O_2$: C, 58.75; H, 3.05; N, 13.05. Found: C, 59.03; H, 3.12; N, 13.03.

2,2,2-Trifluoro-*N*-[3-(4-fluorophenyl)-6-(4-methylbenzoyl)imidazo[1,2-*b*]pyridazin-2-yl]acetamide (5c). Compound **5c** was prepared according to the procedure for **5a**. Starting from compound **4c**, the imidazopyridazine **5c** was obtained in 85% yield: mp 220 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 11.96 (bs, 1H, NH), 8.36 (d, $J = 9.4$, 1H, Het), 8.04 (AA′BB′ system, 2H, Ar), 7.80 (m, 3H, Ar), 7.34 (m, 4H, Ar), 2.42 (s, 3H, Me); ^{13}C NMR (50 MHz, DMSO-d_6) δ 189.5, 159.4, 146.1, 137.7, 136.2, 132.5, 131.0, 130.5, 128.8, 125.7, 123.1, 122.9, 121.5, 118.6, 117.7, 115.3, 21.2; MS (EI$^+$) *m/z* 442 M$^+$ (52) 119 (100), 91 (54), 84 (10), 65 (18); HRMS calcd for $C_{22}H_{14}F_4N_4O_2$ 442.1053, found 442.1060.

2-*N*-[6-Benzoyl-3-phenylimidazo[1,2-*b*]pyridazin-2-yl]-2,2,2-trifluoroacetamide (5d). Compound **5d** was prepared according to the procedure for **5a**. Starting from compound **4d**, the imidazopyridazine **5d** was obtained in 92% yield: mp 248 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 11.83 (bs, 1H, NHCOCF$_3$), 8.31 (d, $J = 9.2$, 1H, Het), 7.90 (d, $J = 9.2$, 1H, Het), 7.80−7.65 (m, 2H, Ar), 7.60−7.51 (m, 1H, Ar), 7.48−7.46 (m, 2H, Ar), 7.78 (m, 2H, Ar), 7.69 (m, 1H, Ar), 7.58 (m, 2H, Ar), HRMS calcd for $C_{21}H_{13}F_3N_4O_2$ 410.0991, found 410.0981.

2-Amino-3-(4-fluorophenyl)-6-benzoylimidazo[1,2-*b*]-pyridazine (6b). A solution of 2,2,2-trifluoro-*N*-[3-(4-fluorophenyl)-6-benzoylimidazo[1,2-*b*]pyridazin-2-yl]acetamide **5b** (204 mg, 0.476 mmol) in 3 N NaOH (3.2 mL) was heated at 50 °C for 72 h. The reaction mixture was poured into ethyl acetate (150 mL) and washed with saturated NaCl (2 × 100 mL). The organic layer was dried (sodium sulfate) and evaporated. The residue was triturated with Et_2O (7 mL) for 2 h to give 144 mg (93%) of the desired amine (**6b**): mp 218 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.06 (AA′ of AA′BB′ system, 2H, Ar), 7.9 (m, 3H), 7.7 (m, 2H), 7.58 (m, 2H), 7.22 (m, 2H), 6.05(b, 2H); HRMS calcd for $C_{21}H_{13}F_3N_4O_2$ 410.0991, found 4102.0980.

2-Amino-3-phenyl-6-benzoylimidazo[1,2-*b*]pyridazine (6d). Compound **6d** was prepared according to the procedure for **6b**. Starting from compound **5d**, the aminoimidazopyridazine **6d** was obtained in 87% yield: mp 199−201 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.05 (d, 2H, Ar), 7.9 (A of an AB system, 1H, Het), 7.85 (d, 2H, Ar),7.68 (m, 2H), 7.58 (m, 2H), 7.4 (m, 2H), 7.22 (m, 1H), 6.1(b, 2H, NH_2); HRMS calcd for $C_{19}H_{14}N_4O$ 314.1168, found 314.1177.

2-Amino-3-(4-fluorophenyl)-6-benzoylimidazo[1,2-*b*]pyridazine Oxime [(*E*)-7b] and [(*Z*)-7b]. A suspension of **6b** (129 mg, 0.399 mmol), $NH_2OH{\cdot}HCl$ (339 mg, 4.878 mmol), and NaOAc (408 mg, 4.973 mmol) in 80% EtOH (15 mL) was refluxed under argon atmosphere for 24 h. The reaction mixture was monitored by TLC (neutral aluminum oxide, CH_2Cl_2/CH_3CN). The ethanol was evaporated, and the crude residual solid was dissolved in ethyl acetate (150 mL). The organic layer was washed with 5% $NaHCO_3$ (2 × 100 mL) and saturated NaCl (4 × 100 mL) and dried over anhydrous sodium sulfate, and the solvent was evaporated. ^{1}H NMR analysis of the crude material showed compound **7b** to be a mixture of *E* and *Z* isomers in a 1:1 ratio. The residue was recrystallized from hot ethyl acetate/hexane, affording 48 mg (36%) of the *Z* isomer in its pure form. The mother liquor was concentrated and purified by flash column chromatography (CH_2Cl_2/CH_3CN, 2:1, neutral aluminum oxide) to yield 63 mg (47%) of a solid that was shown by ^{1}H NMR analysis to be predominantly (90:10) the *E* isomer.

(*E*)-7b: ^{1}H NMR (200 MHz, DMSO-d_6) δ 11.87 (s, 1H, OH), 7.77 (d, J = 9.3, 1H), 7.68 (dd, J = 8.9, J = 5.5, Ar), 7.64 (d, J = 9.3, 1H), 7.53−7.36 (m, 5H, Ar), 7.00 (t, J = 8.9, 2H), 5.77 (b, 2H); ^{13}C NMR (50 MHz, DMSO-d_6) δ 166.0, 159.9, 153.3, 150.7, 146.5, 135.9, 132.1, 129.4, 128.2, 128.1, 127.7, 126.9, 125.4, 120.5, 114.8, 112.9, 106.9; MS (FAB$^+$) m/z 348.3 (M + H)$^+$.

(*Z*)-7b: ^{1}H NMR (200 MHz, DMSO-d_6) δ 11.79(s, 1H), 7.89 (d, J = 8.6, H), 7.79 (dd, J = 9, 2H), 7.53−7.36 (m, 5H), 722 (t, J_1 = 5.5, J_2= 9.0, 2H), 7.15 (d, J = 9.0, 1H), 5.8 (b, 2H); ^{13}C NMR (50 MHz, DMSO-d_6) δ 166.1, 160.4, 151.9, 151.0, 144.1, 135.7, 135.1, 129.4, 128.7, 128.6, 127.7, 126.6, 120.4, 116.6, 115.3, 107.2.

Analysis of the stability of the oxime (*E*)-**7b** demonstrated that an equilibrium between the *Z* and *E* isomers forms in polar solvents such as DMSO and TFA at room temperature.

(*E*)-2-Amino-3-phenyl-6-benzoylimidazo[1,2-*b*]pyridazine Oxime (7d). Compound **7d** was prepared according to the procedure for **7b**. Starting from compound **6d**, the aminoimidazopyridazine **7d** was obtained in 76% yield as a mixture of *E* and *Z* isomers. The mixture was separated by flash column chromatography (CH_2Cl_2/CH_3CN, neutral aluminum oxide) to yield the desired isomer (*E*)-**7d**: ^{1}H NMR (200 MHz, DMSO-d_6) δ 11.83 (s, 1H, OH), 7.79 (d, J = 9.0, 1H), 7.62 (m, 3H, Ar), 7.4(d, 5H), 7.2−7.1(m, 3H, Ar), 5.70 (b, 2H);); HRMS calcd for $C_{19}H_{15}N_5O$ 329.1277, found 329.12. Analysis of the stability of the oxime (*E*)-**7d** demonstrated that an equilibrium between the *Z* and *E* isomers forms in polar solvents such as DMSO and TFA at room temperature.

2-Amino-3-(4-fluorophenyl)-6-benzoylimidazo[1,2-*b*]pyridazine Phenylhydrazone (8b). A suspension of **6b** (143 mg, 0.422 mmol), $PhNHNH_2{\cdot}HCl$ (320 mg, 2.213 mmol), and NaOAc (183 mg, 2.231 mmol) in absolute EtOH (20 mL) was refluxed for 72 h. The reaction was monitored by ^{1}H NMR. The crude residual solid was filtered and washed with EtOH (5 mL) to yield 120 mg (64%) of **8b** as a mixture of *E* and *Z* isomers in a 1:1 ratio: ^{1}H NMR (200 MHz, DMSO-d_6) δ 5.66 (s, 2H, NH_2), 6.98 (t, J = 8.6, 2H, Ar), 7.15−7.60 (m, 10H, Ar), 7.67 (dd, J_1 = 5.5, J_2 = 8.6, 2H, Ar), 7.75 (d, J = 9.4, 1H, Het), 7.94 (d, J = 9.4, 1H, Het), 9.42 (s, 1H, NHph); FAB-MS (rel aboundance) m/z 422.23 (11.74) [M$^+$], 423.24 (14.46) [M$^+$ + 1].

3-(2′-Ethoxycarbonyl-1′-phenylvinyl)-2-(2,2,2-trifluoroacetamido-3-(4′-fluorophenyl)imidazo[1,2-*b*]pyridazine (*E*/*Z*)-9b. Triethyl phosphonoacetate (414 mg, 1.847 mmol) was placed in a round-bottom flask under an argon atmosphere. A solution of KHDMS (5.6 mL, 2.80 mmol, 0.5 M in toluene) was added. The reaction mixture was stirred for 30 min at −5 °C. A solution of the ketone **5b** (400 mg, 0.934 mmol) was added dropwise, the reaction mixture was allowed to warm to room temperature, and the mixture was stirred for 5 h. The solvents were evaporated, and the residue was dissolved in ethyl acetate (150 mL), washed with 10% NH_4Cl (4 × 100 mL) and saturated NaCl (6 × 100 mL), and dried over anhydrous sodium sulfate. Upon evaporation of the solvent, the crude material was triturated with Et_2O, yielding 250 mg (54%) of the product as an isomeric mixture (*E*/*Z*, 13:1). The mixture of isomers was used without further purification for the next step: ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.01 (t, J = 7.09, 3H, OEt), 3.99 (q, J = 7.09, 2H, OEt), 6.95 (s, 1H, $R_1R_2C{=}CHR_3$), 7.21 (t, J = 8.9, 2H, Ar), 7.29−7.26 (m, 2H, Ar), 7.44−7.40 (m, 3H, Ar), 7.51 (d, J = 9.6, 1H, Het), 7.64 (dd, J_1 = 5.56, J_2 = 8.77, 2H, Ar), 8.21 (d, J = 9.6, 1H, Het), 11.8 (s, 1H, $NHCOCF_3$); ^{13}C NMR (75 MHz, DMSO-d_6) δ 115.47 (d, J = 21.6, Ar(F)CH), 118.15, 122.91 (CH), 123.25 (d, J = 3.2, Ar(F)C), 125.55, 128.05, 128.36, 129.26 (CH), 130.15 (d, J = 8.3, Ar(F)CH), 135.73, 136.51, 149.01, 152.28 (C), 156.08 (d, J = 37.3, $NHCOCF_3$), 161.8 (d, J = 246, Ar(F)C), 165.23 (COOEt); FAB-MS (rel abundance) m/z 499.4 (100) [M$^+$ + 1], 997.6 (2.25) [2M$^+$ + 1]; HRMS calcd for $C_{25}H_{18}F_4N_4O_3$ 498.131 50, found 499.139 33 [M$^+$ + 1].

2-Amino-6-(2′-ethoxycarbonyl-1′-phenylvinyl)-3-(4′-fluorophenyl)imidazo[12-*b*]pyridazine (10b). DIPEA (4 mL) was added to a suspension of **9b** (210 mg, 0.421 mmol) (*E*/*Z*, 13:1) in 10 mL of EtOH (90%). The reaction mixture was refluxed for 48 h (TLC; CH_2Cl_2/CH_3CN, 2:1). After evaporation of the EtOH, the resulting solid was dissolved in ethyl acetate (100 mL), washed with 10% NH_4Cl (4 × 100 mL) and saturated NaCl (6 × 100 mL), and dried over sodium sulfate. Upon evaporation of the solvent, the crude residual solid was triturated in Et_2O, yielding 62 mg of the pure *E* isomer. The mother liquors were evaporated and the residue was purified by flash column chromatography (CH_2Cl_2/CH_3CN, 2:1), affording 85 mg of a solid that was shown by ^{1}H NMR analysis to be predominantly (>90:10) the *Z* isomer: total yield 147 mg (87%); ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.99 (t, J = 7.10, 3H, OEt), 3.95 (q, J = 7.10, 2H, OEt), 5.91 (s, 2H, NH_2), 7.13 (t, 2H, J = 8.8, Ar(F)H), 7.14 (d, J = 9.3, 1H, Het), 7.25−7.19 (m, 5H, Ar), 7.42−7.37 (m, 2H, Ar), 7.70 (d, J = 9.3 Hz, 1H, Het), 7.75 (dd, J_1 = 5.6, J_2 = 8.8, 2H, Ar(F)H); ^{13}C NMR (300 MHz, DMSO-d_6) δ 13.88 (OEt), 59.97 (OEt), 107.17 (C), 114.68 (CH), 115.17 (d, J = 21.3, Ar(F)CH), 119.97 (CH), 125.37 (d, J = 3.1, Ar(F)C), 127.93, 127.99 (CH), 128.58 (d, J = 7.9, Ar(F)CH), 136.13, 137.30, 148.18, 150.53, 151.77 (C), 160.31 (d, J = 244, Ar(F)C), 165.31 (COOEt); FAB-MS (rel abundance) m/z 403.0 (100) [M$^+$ + 1], 805.0 (2.12) [2M$^+$ + 1]; HRMS calcd for $C_{23}H_{19}FN_4O_2$ 402.149 204, found 402.149 210 [M$^+$], 403.157 029 [M$^+$ + 1].

Diethyl (*N*-Methylcarbamoylmethyl)phosphonate (11). The Horner−Wadsworth−Emmons reagent was prepared according to the procedure that we previously described.[6]

2-(2,2,2-Trifluoroacetamido)-3-(*p*-fluorophenyl)-6-[(*E*,*Z*)-2-*N*-methylcarbamoyl-1-(*p*-methoxyphenyl)vinyl]imidazo-[1,2-*b*]pyridazine [(*E*)-12a and (*Z*)-12a]. Method A. To a solution of KHMDS (5.27 mmol) in 5 mL of dry DMF at 0 °C under argon was added a solution of the Horner−Wadsworth−Emmons reagent **11** (1.86 mmol) in 10 mL of dry DMF. The reaction mixture was stirred at 0 °C for 45 min. The imidazopyridazine **5a** (1.55 mmol) in 10 mL of dry DMF was transferred via cannula. The bath was removed, and the resulting brown solution was allowed to warm to room temperature. After 44 h, the reaction mixture was quenched with saturated NH_4Cl (30 mL) and extracted with ethyl acetate (3 × 100 mL). The organic layers were combined and washed with saturated NH_4Cl and then with saturated NaCl. After drying over sodium sulfate, the solvent was removed in vacuo. ^{1}H NMR analysis of the crude material showed a mixture of *E* and *Z* isomers in a 1:2 ratio (67% yield). The isomers were separated by column chromatography (silica gel, ACN/CH_2Cl_2, 1:2 then 1:1 then 2:1).

(*E*)-12a: mp 247−249 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 8.16 (m, 1H, NHMe), 8.12 (d, J = 9.5, 1H, Het), 7.77 (dd, J =

5.7, J = 8.7, 2H, Ar), 7.37−7.18 (m, 4H, Ar), 7.15 (AA′BB′ system, 2H, Ar), 6.95 (d, J = 9.5, 1H, Het), 6.65 (s, 1H, CH=C), 3.75 (s, 3H, OMe), 2.56 (d, J = 4.3 Hz, 3H, NHMe); ^{13}C NMR (50MHz, DMSO-d_6) δ 164.6, 160.2, 153.7, 144.0, 135.5, 131.0, 130.1, 129.1, 128.6, 124.4, 123.4, 122.3, 121.1, 120.3, 115.8, 115.3, 114.3, 113.2, 55.2, 25.4; MS (EI$^+$) m/z 513 M$^+$ (100), 512 (12), 484 (10), 483 (38), 456 (7), 444 (15). 265 (7), 159 (8), 133 (11), 132 (9), 89 (7), 69 (7), 58 (10); HRMS calcd for $C_{25}H_{19}F_4N_5O_3$ 513.142 40, found 513.142 52.

(*Z*)-12a: mp 228−230 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 8.04−6.95 (m, 10H, Ar), 6.82 (s, 1H, CH=C), 3.79 (s, 3H, OMe), 2.48 (s, 3H, NHMe); ^{13}C NMR (50 MHz, DMSO-d_6) δ 164.2, 159.3, 159.1, 155.8, 153.6, 142.8, 135.4, 134.5, 130.2, 128.5, 126.6, 125.1, 123.3, 118.7, 118.5, 115.6, 115.2, 112.9, 55.1, 25.4.

2-(2,2,2-Trifluoroacetamido)-3-(4′-fluoromethyl)-6-(2′-*N*-methylcarbamoyl-1′-phenylvinyl)imidazo[1,2-*b*]pyridazine [(*E*)-12b]. Method A. A solution of the Horner−Wadsworth−Emmons reagent **11** (1.86 mmol) in dry DMF (10 mL) was added to a solution of KHMDS (5.27 mmol) in dry DMF (5 mL) at 0 °C under argon. The reaction mixture was stirred at 0 °C for 45 min. A solution of the imidazopyridazine **5b** (1.55 mmol)) in 10 mL of dry DMF was transferred via cannula. The bath was removed, and the resulting brown solution was allowed to warm to room temperature. After 5 h, the reaction mixture was quenched with saturated NH_4Cl (30 mL) and extracted with ethyl acetate (3 × 100 mL). The organic layers were combined and washed with saturated NH_4-Cl and then with saturated NaCl. After drying over sodium sulfate, the solvent was removed in vacuo. ^{1}H NMR analysis of the crude material showed a mixture of *E* and *Z* isomers in 1:5 ratio (59%). The reaction was repeated under the same conditions by increasing the reaction time to 48 h, resulting in a mixture of *E* and *Z* isomers in a 3:1 ratio (65% yield).

Method B. A 0.5 M solution of KHMDS (35 mL, 17.5 mmol) was added to a solution of the Horner−Wadsworth−Emmons reagent **11** (1.5 g, 7 mmol) in THF (20 mL) at −78 °C under argon. The mixture was stirred for 2 h at −78 °C followed by the dropwise addition of imidazpyridazine **5a** (1.6 g, 3.5 mmol) in 20 mL of dry THF. The resulting brown solution was allowed to warm to room temperature and then stirred at room temperature for 34 h. The reaction mixture was diluted in 400 mL of ethyl acetate, and an amount of 200 mL of 5% HCl was added. The layers were separated, and the aqueous layer was extracted with ethyl acetate (2 × 200 mL). The organic layers were combined and washed with 5% HCl (2 × 100 mL) and once with saturated NaCl. After drying over $MgSO_4$, the solvent was removed in vacuo to give a brown solid. ^{1}H NMR analysis of the crude material showed a mixture of *E* and *Z* isomers in a 4:1 ratio. Crystallization from ethyl acetate afforded the *E* isomer **12b** in its pure form in 69% yield.

(*E*)-12b: mp 243 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 11.87 (b, 1H, NHCOCF$_3$), 8.20 (d, J = 9.6, 1H, Het), 8.10 (b, 1H, NHCOMe), 7.68 (dd, J = 5.5, J = 9.0, 2H, Ar), 7.4−7.1 (m, 7H, Ar), 7.3 (d, J = 9.6, 1H, Het), 6.93 (s, 1H, CH=C), 2.54 (d, J = 4.7, 3H, NHMe); FAB-MS (rel abundance) m/z 483.25 (45.23) [M$^+$], 484.25 (100) [M$^+$ + 1], 486.25 (6.71) [M$^+$ + 2], 967.39 (3.8) [2M$^+$ + 1], 968.40 (2.45) [2M$^+$ + 2]; HRMS calcd for $C_{24}H_{17}F_4N_5O_2$ 483.131 84, found 484.139 66 [M$^+$ + 1].

2-(2,2,2-Trifluoroacetamido)-3-(4-fluorophenyl)-6-[(*E*,*Z*)-2-*N*-methylcarbamoyl-1-(4-methylphenyl)vinyl]imidazo-[1,2-*b*]pyridazine [(*E*)-12c) and (*Z*)-12c)]. 2-(2,2,2-Trifluoroacetamido)-3-(*p*-fluorophenyl)-6-(4-methylbenzoyl)imidazo[1,2-*b*]pyridazine **5c** was converted to its corresponding vinyl carboxamides **12c** in a manner substantially analogous to the preparation of **12b** (method A). Compound **12c** was isolated in 63% yield as a mixture of *E* and *Z* isomers that were separated by a column chromatography (silica gel, ACN/CH_2-Cl_2, 1:2 then 1:1 then 2:1) to give both isomers (*E*)-**12c** and (*Z*)-**12c** in their pure forms.

(*E*)-12c: mp 251 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 8.23 (m, 1H, NHMe), 8.12 (d, J = 9.3, 1H, Het), 7.65 (dd, J = 5.8, J = 8.5, 2H, Ar), 7.36−7.21 (m, 6H, Ar), 7.14 (d, J = 9.3, 1H, Het), 6.71 (s, 1H, CH=C), 2.56 (d, J = 4.4 Hz, 3H, NHMe), 2.29 (s, 3H, Me); ^{13}C NMR (50 MHz, DMSO-d_6) δ 164.5, 159.5, 153.6, 144.2, 135.6, 134.7, 130.1, 130.0, 129.5, 127.0, 124.4, 123.7, 123.4, 121.0, 120.4, 115.8, 115.3, 113.0, 25.4, 20.1; MS (EI$^+$) m/z 497 M$^+$ (100), 496 (14), 468 (11), 467 (38), 440 (8), 429 (6), 428 (20), 249 (9), 143 (7), 122 (8), 117 (9), 116 (9), 115 (18), 69 (7), 58 (12); HRMS calcd for $C_{25}H_{19}F_4N_5O_2$ 497.1475, found 497.1477.

(*Z*)-12c: mp 237−239 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 8.22 (m, 1H, NHMe), 8.13 (d, J = 9.5, 1H, Het), 7.80 (dd, J = 5.6, J = 8.5, 2H, Ar), 7.38−7.06 (m, 7H, Ar + Het), 6.84 (s, 1H, CH=C), 2.53 (d, J = 4.4, 3H, NHMe), 2.35 (s, 3H, Me).

2-Amino-3-(4-fluorophenyl)-6-[(*E*)-2-*N*-methylcarbamoyl-1-(4-methoxyphenyl)vinyl]imidazo[1,2-*b*]pyridazine [(*E*)-13a]. To a suspension of (*E*)-**12a** (250 mg, 0.517 mmol) in 90% MeOH (12 mL), DIPEA (4.8 mL) was added. The resulting orange solution was refluxed for 72 h (TLC: ACN/MeOH, 10:1). The solvents were evaporated in vacuo, and the crude mixture was subjected to radial chromatography (CH_3CN/CH_2Cl_2, 7:3) to yield the desired isomer (*E*)-**13a** as a yellow solid in 70% yield: mp 165 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.76 (dd, J = 5.3, J = 9.1, 1H, Ar), 7.55 (d, J = 9.1, 1H, Het), 7.22−7.08 (m, 4H, Ar), 6.95 (m, 2H, Ar), 6.84 (d, J = 9.1 1H, Het), 6.76 (s, 1H, CH=C), 5.36 (m, 1H, NHMe), 4.39 (bs, 2H, NH$_2$), 3.86 (s, 3H, OMe), 2.66 (d, J = 4.8, 3H, NHMe); ^{13}C NMR (50 MHz, $CDCl_3$) δ 167.0, 160.0, 161.5, 150.2, 149.6, 144.1, 130.7, 128.9, 128.8, 128.2, 125.0, 124.8, 120.9, 116.1, 115.6, 114.2, 109.2, 55.3, 26.2; MS (EI$^+$) m/z 417 M$^+$ (100), 387 (10), 360 (8), 359 (7), 266 (8), 133 (14), 132 (8), 122 (12), 89 (7), 79 (10), 58 (12); HRMS calcd for $C_{23}H_{20}FN_5O_2$ 417.160 103, found 417.160 142.

2-Amino-3-(4-fluorophenyl)-6-[(*Z*)-2-*N*-methylcarbamoyl-1-(4-methoxyphenyl)vinyl]imidazo[1,2-*b*]pyridazine [(*Z*)-13a]. Compound (*Z*)-**13a** was prepared according to the procedure described for (*E*)-**13a.** Starting from (*Z*)-**12a**, compound (*Z*)-**13a** was obtained in 82% yield: mp 140 °C; ^{1}H NMR ($CDCl_3$) δ 7.72 (dd, J = 5.4, J = 8.7, 1H, Ar), 7.56 (d, J = 9.1, 1H, Het), 7.10 (m, 6H, Ar), 6.77 (d, J = 9.1, 1H, Het), 6.31 (s, 1H, CH=C), 6.19 (m, 1H, NHMe), 4.00 (bs, 2H, NH$_2$), 3.73 (s, 3H, OMe), 2.53 (d, J = 4.8, 3H, NHMe); ^{13}C NMR ($CDCl_3$) δ 166.3, 163.9, 160.5, 149.8, 149.0, 145.0, 135.8, 131.2, 129.1, 129.0, 124.9, 123.0, 121.0, 117.6, 115.8, 114.0, 109.0, 55.3, 26.0; MS (EI$^+$) m/z 417 M$^+$ (100), 387 (10), 360 (8), 359 (7), 266 (8), 133 (14), 132 (8), 122 (12), 89 (7), 79 (10), 58 (12); HRMS calcd for $C_{23}H_{20}FN_5O_2$ 417.160 103, found 417.160 142.

2-Amino-3-(4′-fluorophenyl)-6-(2′-*N*-methylcarbamoyl-1′-phenylvinyl)imidazo[1,2-*b*]pyridazine [(*E*)-13b]. Compound (*E*)-**13b** was prepared according to the procedure for (*E*)-**13a**. Starting from (*E*)-**12b**, compound (*E*)-**13b** was obtained in 92% yield: mp 161 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.53 (d, J = 4.6, 3H, CONHMe), 5.81 (s, 2H, NH$_2$), 6.85 (s, 1H, R_1R_2C=CHR_3), 6.97 (d, J = 9.2, 1H, Het), 7.16 (t, J = 8.9, 2H, Ar), 7.40−7.20 (m, 5H, Ar), 7.70 (d, J = 9.2, 1H, Het), 7.81 (dd, J_1 = 8.9, J_2 = 5.5, 2H, Ar), 7.99 (q, J = 4.6, 1H, CONHMe); ^{13}C NMR (75 Hz, DMSO-d_6) δ 25.56 (MeNHCO), 107.19 (C), 114.62 (CH), 115.22 (d, J = 21, Ar(F)CH), 120.18, 124.88 (CH), 125.55 (d, J = 3.1, Ar(F)C), 127.62, 127.78 (CH), 128.57 (d, J = 7.9, Ar(F)CH), 129.50 (CH), 137.58, 144.63, 149.47, 151.44 (C), 160.28 (d, J = 244, Ar(F)C), 165.38 (*C*ONHMe); FAB-MS m/z 388.26 [M$^+$ + 1], 775.4 [2M$^+$ + 1].

2-Amino-3-(4′-fluorophenyl)-6-(2′-*N*-methylcarbamoyl-1′-phenylvinyl)imidazo[1,2-*b*]pyridazine [(*Z*)-13b]. Compound (*Z*)-**13b** was prepared according to the procedure described for (*E*)-**13a.** Starting from (*Z*)-**12b**, compound (*Z*)-**13b** was isolated in 86% yield: mp 145 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.57 (d, J = 4.7, 3H, CONHMe), 5.64 (s, 2H, NH$_2$), 6.63 (s, 1H, R_1R_2=CHR_3), 6.84 (d, J = 9.0, 1H, Het-H), 7.21 (t, J = 8.9, 2H, Ar), 7.45−7.25 (m, 5H, Ar), 7.69 (d, J = 9.0, 1H, Het), 7.81 (dd, J_1 = 5.6, J_2 = 8.9, 2H, Ar), 8.13 (q, J = 4.7, 1H, CONHMe); ^{13}C NMR (75 MHz, DMSO-d_6) δ 25.59 (CONH*C*H$_3$), 107.14 (C), 115.32 (d, J = 21.3, Ar(F)CH), 117.43, 120.08, 124.91 (CH), 125.89 (d, J = 3.0, Ar(F)C), 127.47 (CH), 128.75 (d, J = 7.9, Ar(F)CH), 128.89, 129.23 (CH), 135,76, 138.92, 145.02, 149.43, 150.57 (C), 160.37 (d, J = 244, Ar(F)C), 165.38 (*C*ONHMe); FAB-MS (rel abundance) m/z 387.2 (100) [M$^+$ +

1]; HRMS calcd for $C_{22}H_{18}FN_5O$ 387.149 539, found 387.149 539 [M^+], 388.157 364 [$M^+ + 1$].

2-Amino-3-(4-fluorophenyl)-6-[(*E*)-2-*N*-methylcarbamoyl-1-(4-methylphenyl)vinyl]imidazo[1,2-*b*]pyridazine [(*E*)-13c]. Compound (*E*)-**13c** was prepared according to the procedure for (*E*)-**13a**. Starting from (*E*)-**12c**, compound (*E*)-**13c** was obtained in 78% yield: mp 175 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.76 (dd, $J = 5.4$, $J = 9.1$, 1H, Ar), 7.53(d, $J = 9.1$, 1H, Het), 7.26−7.08 (m, 6H, Ar), 6.80 (d, $J = 9.1$, 1H, Het), 6.80 (s, 1H, CH=C), 5.37 (m, 1H, NHMe), 4.40 (bs, 2H, NH_2), 2.65 (d, $J = 4.8$, 3H, NHMe), 2.42 (s, 3H, Me); ^{13}C NMR (50 MHz, $CDCl_3$) δ 166.8, 161.4, 149.9, 144.4, 138.8, 136.1, 133.3, 129.5, 129.2, 128.8, 127.8, 125.6, 124.7, 124.3, 120.8, 115.8, 109.1, 26.2, 21.3; MS (EI$^+$) *m/z* 401 M^+ (100), 400 (7), 371 (9), 344 (8), 343 (7), 250 (7), 122 (13), 117 (9), 116 (9), 115 (16), 79 (9), 58 (12); HRMS calcd for $C_{23}H_{20}FN_5O$ 401.1651, found 401.1651.

2-Amino-3-(4-fluorophenyl)-6-[(*Z*)-2-*N*-methylcarbamoyl-1-(4-methylphenyl)vinyl]imidazo[1,2-*b*]pyridazine [(*Z*)-13c]. Compound (*Z*)-**13c** was prepared according to the procedure for (*E*)-**13a**. Starting from (*Z*)-**12c**, compound (*Z*)-**13c** was obtained in 68% yield: mp 137 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.80 (dd, $J = 5.4$, $J = 9.1$, 1H, Ar), 7.63(d, $J = 9.1$, 1H, Het), 7.25−7.11 (m, 6H, Ar), 6.83 (d, $J = 9.1$, 1H, Het), 6.42 (s, 1H, CH=C), 6.22 (m, 1H, NHMe), 4.31 (bs, 2H, NH_2), 2.59 (d, $J = 4.8$, 3H, NHMe), 2.36 (s, 3H, Me); ^{13}C NMR (50 MHz, $CDCl_3$) δ 166.2, 161.3, 149.7, 145.1, 139.3, 136.0, 129.4, 129.3, 129.2, 129.0, 127.7, 124.9, 124.1, 120.9, 117.6, 115.7, 109.0, 25.9, 21.1; MS (EI$^+$) *m/z* 401 M^+ (100), 400 (7), 371 (9), 344 (8), 343 (7), 250 (7), 122 (13), 117 (9), 116 (9), 115 (16), 79 (9), 58 (12); HRMS calcd for $C_{23}H_{20}FN_5O$ 401.1651, found 401.164 92.

Plaque Reduction Assay. Susceptible H-Hela cells were grown in 60 mm tissue culture dishes in medium 199 containing 10% fetal bovine serum (FBS). After overnight incubation at 37 °C to produce confluent monolayers, the growth medium was removed and 0.2 mL/well of an appropriate dilution of virus was added. After adsorption for 1 h at room temperature, the infected cell sheet was overlaid with equal parts of 1.5% sterile agarose solution and a 2× concentration of growth medium containing 5% FBS and supplemented with test compound dissolved in DMSO (final concentration of DMSO was 0.01%). Infected cultures were maintained in a CO_2 incubator at 37 °C until the DMSO control wells contained visible viral plaques. A solution containing 10% formalin was added to each well to inactivate the virus and fix the cell sheet to the plastic surface. The fixed cell sheets were stained with 0.5% crystal violet in ethanol, and the plaques were counted. Results from duplicate wells at each concentration were averaged and compared with DMSO control wells. The inhibition of plaque formation by 50% or 90% (IC_{50} or IC_{90}) was calculated from the linear region of the inhibition concentration curve using the method of Reed and Muench.[10]

Cytopathic Effect Assay. Hela cells were dispersed in 96-well microtiter plates at 10 000 cells per well with medium 199 containing 5% FBS. After overnight incubation at 37 °C, the cells were infected with virus, and medium containing serial dilutions of drug or DMSO was added to the wells. The resultant mixtures were incubated for 2−3 days (until extensive CPE was apparent in DMSO control wells). To assess the antiviral and cytotoxic effects, a fresh solution (0.4 mg/mL) of XTT [2,3-bis(methoxy-4-nitro-5-sulfophenyl)-2*H*-tetraazolium-5-carboxanilide, inner salt, sodium salt] in warm medium without FBS was prepared. For each 5 mL of the XTT solution, 25 μL of 5 mM PMS (phenazine methosulfate) in phosphate buffer saline was added. After removal of the cultured supernatant, 100 μL of the freshly prepared XTT/PMS mixture was added to each of the microtiter wells. The wells were then incubated at 37 °C (under CO_2) for 3−4 h or until a prominent color change was observed. The absorbance at 450 nm (reference was 650 nm) was read in a spectrophotometer.[11]

Acknowledgment. We are grateful to the Lilly Infectious Diseases Action Group and Lilly Research Process and Development for stimulating discussions.

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JM020583I