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Graphical Abstract

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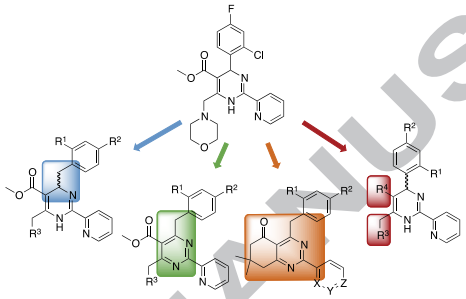

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Synthesis and antiviral evaluation of novel heteroarylpyrimidines analogs as HBV capsid effectors

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ABSTRACT

New modifications to the scaffold of previously reported HBV capsid assembly effectors such as BAY 41-4109, HAP-12 and GLS4 were explored. The anti-HBV activity in the HepAD38 system, and cytotoxicity profiles of each of the new compounds has been assessed. Among them, five new iodo- and bromo-heteroarylpyrimidines analogs displayed anti-HBV activity in the low micromolar range.

Hepatitis B virus (HBV) represents a major global health problem, with an estimated 240 million people chronically infected worldwide.[1] *Despite highly effective vaccination programs and approved treatments for chronic hepatitis B, the lack of a cure for individuals already infected with HBV, combined with the fact that people are unaware of their infection, results in continued spread of the virus. To date, there are seven FDA approved inhibitors for HBV,[2,3] but none can cure this viral infection. Moreover, these agents lack an important characteristic necessary to confer a cure, which is the ability to eliminate HBV cccDNA from the nucleus of infected hepatocytes.[4] To address this unmet clinical need, several HBV Capsid Assembly Effectors (CAE), have been developed over the years (Figure 1).[5,6,7,8] Among them, BAY 41-4109, which was studied in a phase I clinical trial,[9] was found to misdirect the HBV capsid assembly and interfere with the viral infection.[10] Recent studies have also shown that the combination of nucleoside analogs like adefovir (ADV) or tenofovir (TFV) with either one of these capsid effectors could lead to a synergistic antiviral effects.[11,12] Novira recently completed a phase Ia clinical trial with a new capsid assembly effector (NVR 3-778, later acquired by Johnson & Johnson), and revealed that combinations with Entecavir (ETV) or pegIFN had additive and/or synergistic antiviral activity leading to high viral load suppression efficacy in infected humanized mouse models.[13] Despite their mechanism of action not being completely clear, capsid assembly effectors seem to represent a promising cohort of molecules with curative potential when combined with other HBV inhibitors such as nucleoside analogs.[14]

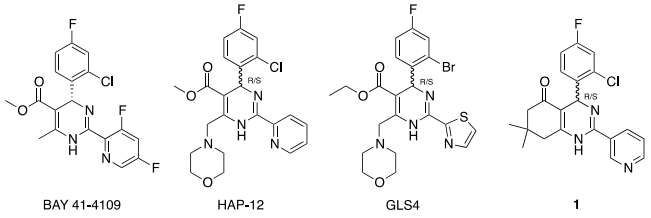

Figure 1. Chemical structure of known Capsid Assembly Effectors (CAE).

In our continuing efforts to identify more effective small antiviral molecules and based on the potential of these CAE, we report herein the synthesis and evaluation of four new series of HAP analogs (Figure 2): ***Series I*** is comprised of "flexible" HAP derivatives bearing an extra CH_2 linker between the main core and the halogenated phenyl ring. Compounds from **series *II* & *III*** are aromatic version of HAP bearing a methylene linker between the pyrimidine core and the phenyl ring. Unsaturated dihydropyrimidine can potentially be aromatized to their pyrimidine form by human liver microsomes and thus lose their potency.[15] We hypothesized that the addition of an extra methylene linker could compensate for the loss of stereochemistry in the original structure. Finally, **series *IV*** include 5- and 6-modifications that retain hydrogen bond accepting characteristics of the morpholine or the ester groups presents on the HAP scaffold. Other, less common functionalities such as a phosphonate or imidazolium groups were also evaluated.

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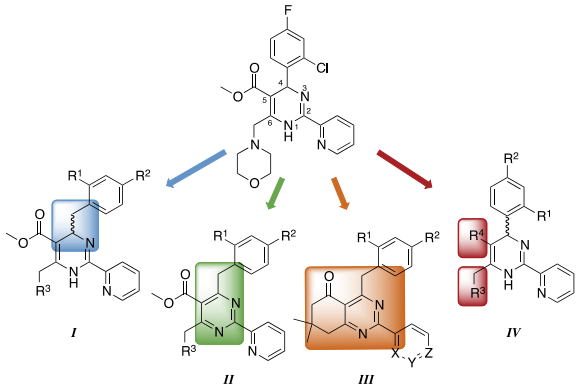
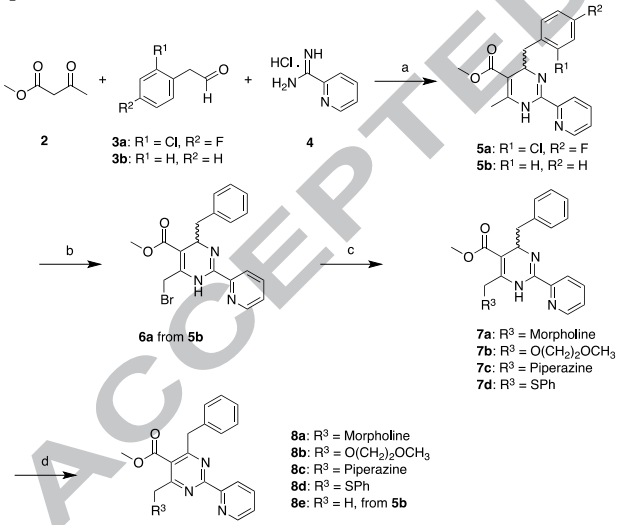
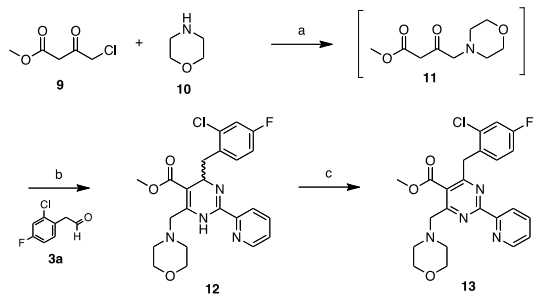


Figure 2. Proposed modifications of the known capsid assembly effectors.

A Biginelli cyclocondensation between β-ketoacetate **2**, pyridine-2-carboximidamide salt **4** and phenylacetaldehydes **3a-b** in isopropanol under microwave irradiation generated the corresponding compounds **5a** and **5b**.[16] Bromination of dihydropyrimidine **5b** using *N*-bromosuccinimide (NBS) in 1,2-dichloroethane led to the intermediate **6a**, which was easily substituted with morpholine, *N*-methylpiperazine, methoxyethanol or thiobenzene to form compounds **7a-d**. Oxidation of these dihydropyrimidines using 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) easily led to the desired pyrimidines **8a-f** (Scheme 1). It is noteworthy that the bromination of compound **5a** could not be achieved under the halogenation conditions described above and therefore, we had to prepare targeted compound **13** through the chemistry described in Scheme 2. Methyl 4-morpholino-3-acetoacetate **11**, synthesized by reaction of methyl 4-chloroacetoacetate **9** with morpholine, was quickly purified and directly reacted with aldehyde **3a** to yield dihydropyrimidine **12**. Final oxidation with DDQ gave compound **13**.

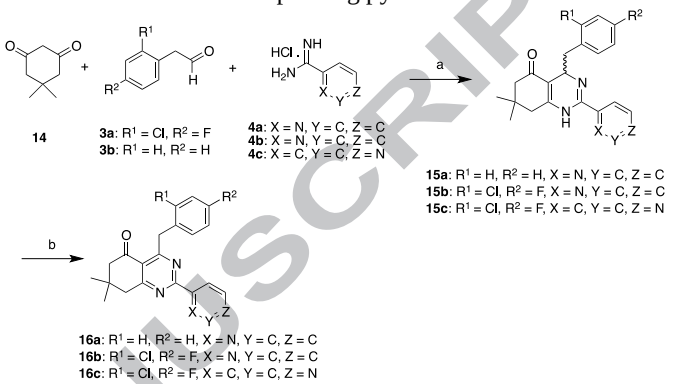


Scheme 1. Reagents and conditions: (a) piperidine, AcOH, *i*PrOH, 12 h, 11-36%; (b) NBS, 1,2-DCE, 50 °C, 30 min, 80%; (c) R^3H, NaH or Et_3N, DMF, 0 °C, 1 h, 29-72%; (d) DDQ, toluene, rt, 1 h, 42-68%.



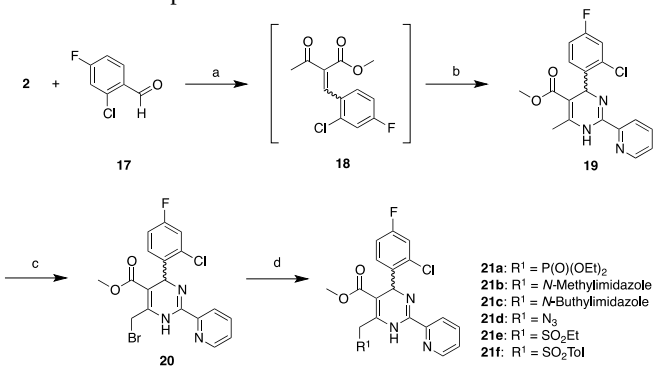
Scheme 2. Reagents and conditions: (a) NaH, DMF, 0 °C to rt, 1 h, 41-60%, (b) pyridine-2-carboximidamide, Et_3N, µW, 10 min, 14%; (c) DDQ, toluene, rt, 1 h, 20%.

Aromatic analogs of bicyclic derivative **1** were synthesized using the chemistry described in Scheme 3. Compounds **15a-c** were prepared *via* a three-component Biginelli-type cyclocondensation under microwave irradiation involving dimedone **14**, pyridine-3-carboximidamide **4a-c** and the corresponding aldehyde **3a-b**. Interestingly, all our attempts to purify and isolate compounds **15a-c** failed due to the instability of these dihydropyrimidines, which are spontaneously oxidized to their pyrimidine form. Therefore, compounds **15a-c** were directly treated with DDQ in toluene to afford the corresponding pyrimidines **16a-c**.



Scheme 3. Reagents and conditions: (a) Et_3N, 140 °C, µW, 5 min, 10-25%, (b) DDQ, toluene, rt, 1 h, 60–82%.

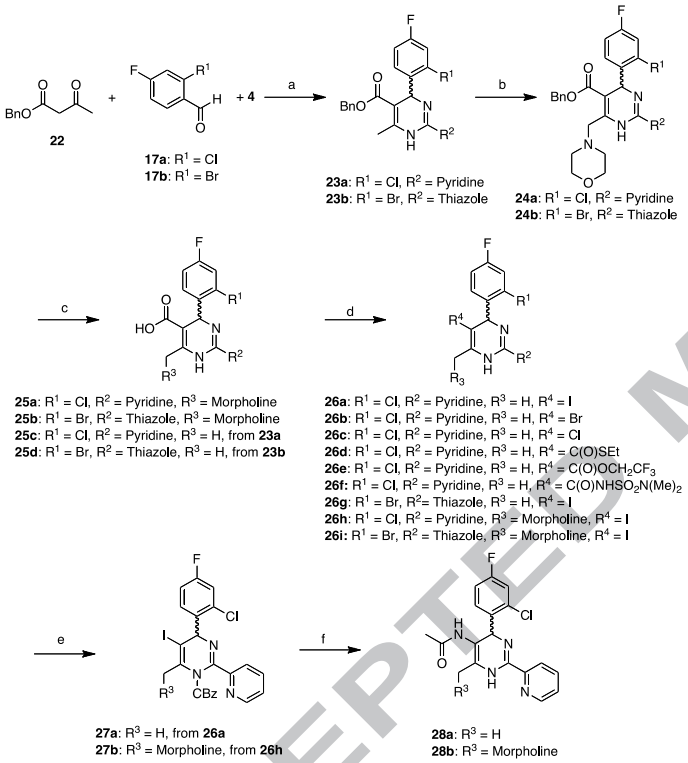
Compounds **21a-f**, 6-modified analogs of lead compound HAP-12, were prepared according to the chemistry described in Scheme 4. The one pot, 2 step, condensation of 2-chloro-4-fluorobenzaldehyde **17** with methylacetoacetate **2** and pyridine-2-carboximidamide hydrochloride **4** under microwave irradiation gave dihydropyrimidine **19** in 53% yield. Bromination of **19** followed by substitution with various nucleophiles led to the formation of compounds **21a-f**.



Scheme 4. Reagents and conditions: (a) *i*PrOH, piperidine, AcOH, 80 °C, 30 min, µW (b) **4**, 100 °C, 30 min, µW, 53%; (c) NBS, 1,2-DCE, 50 °C, 30 min, 68%; (d) $P(OEt)_3$, 140 °C, 20 min, µW for **21a**; R^1Na, Et_3N, rt, 12 h for **21d-f**; *N*-butyl or *N*-methylimidazole, DMF, rt, 12 h for **21b-c**, 36-98%.

Finally, various 5-modified HAP analogs were prepared according to chemical reactions described in Scheme 5. Compounds **23a-b** were obtained as described above by condensation of diketoester **22** with aldehyde **17a-b** and carboximidamide **4** under microwave irradiation. Bromination of compounds **23a-b** followed by nucleophilic substitution of the brominated intermediates with morpholine led to 6-methylmorpholino-HAPs **24a-b**. Dihydropyrimidine-5-carboxylic acids **25a-d**, were then acquired after hydrogenolysis of the corresponding benzylic ester (**25a** and **25c**), or by using boron trichloride (**25b** and **25d**). Thioester **26d**, ester **26e** and *N*,*N*-dimethylsulfamoyl **26f** were synthesized from **25c** under peptidic coupling conditions (EDC DMAP, DMF). Halodecarboxylation of carboxylic acids **25a-d** in presence of a source of halogen, oxone and sodium carbonate yielded halo-

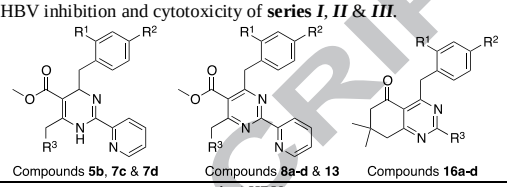
HAPs **26a-c** and **26g-i** after 20 min[17,18] It is noteworthy, that a longer reaction time or use of an excess of oxone resulted in the aromatization of compounds **26a-c**. Introduction of the amide group at the 6 position (compounds **28a-b**) was achieved by first protection of the dihydropyrimidine **26a** and **26h** with a carboxybenzyl group and reaction with acetamide in presence of a catalytic amount of palladium tris(dibenzylideneacetone) (0) and Xantphos.[19] Subsequent Cbz-deprotection using either boron trichloride or a palladium catalyzed hydrogenolysis afforded compounds **28a-b**. Interestingly, the Buchwald-Hartwig amination could not be achieved directly from 5-iododihydropyrimidines **26a** and **26h** and the choice of the correct protecting group was key. Indeed, introduction of protecting groups such as *t*-butoxylcarbonyl, tosyl or mesyl groups led to the dehalogenation and aromatization of the starting materials under palladium catalyzed amination conditions.



Scheme 5. Reagents and conditions: (a) i) *i*PrOH, piperidine, AcOH, 80 ºC, 30 min, µW ii) 100 ºC, 30 min, µW, 44-48%; (b) i) NBS, 1,2-DCE, 50 ºC, 30 min, 52%, ii) morpholine, Et_3N, DMF, rt, 12 h, 98%; (c) H_2, Pd/C, EtOH, 12 h, or BCl_3, DCM, -78 ºC to rt, 1 h, 74-95%; (d) NaR^4, Na_2CO_3, Oxone, MeOH/H_2O, R^4 = I, Br or Cl, rt, 20 min, 10-68%, for **26a-c** and **26g-i**. EDC, DMAP, CH_3CN, EtSH or CF_3CH_2OH or $H_2NSO_2N(Me)_2$, rt, 12 h, 7-76%, for **26d-f**; (e) CbzCl, KHMDS, THF, 0 ºC to rt, 1 h, 78%; (f) i) $AcNH_2$, Pd_2dba_3, XantPhos, K_2CO_3, dioxane, 105 ºC, 20 min, µW, 47-68%, ii) cyclohexadiene, Pd/C, EtOH, 3 h, or BCl_3, DCM, 0 ºC to rt, 2 h, 72-83%.

The *in vitro* anti-HBV activity of thirty new heteroarylpyrimidines (HAP) derivatives were assessed at concentration ranging from 0.001 to 10 µM in HepAD38 cells using real-time-PCR, as previously described by Stuyver *et al.*, 2002.[20] All samples were tested in duplicate and the concentration of compound that inhibited HBV DNA replication by 50% (EC_{50}) was determined using the Chou and Talalay method as previously described.[20] All data were given relative to the untreated control. In addition, cytotoxicity was determined by using the CellTiter 96 non-radioactive cell proliferation colorimetric assay (Promega) in peripheral blood mononuclear (PBM) cells and in human T lymphoblast (CEM), African green monkey kidney (Vero), and human hepatocellular carcinoma (HepG2) cells. Toxicity levels were measured as the concentration of test compound that inhibited cell growth by 50% (CC_{50}). As expected HAP-12, GLS4 and 3TC inhibited HBV DNA replication with EC_{50} values <1 µM and were used as positive control. However, it is noteworthy that compound **1**, resynthesized in our laboratory, did not express any activity against HBV in the HepAD38 system (Table 1) even though it was reported in the literature, to be a submicromolar inhibitor of HBV replication.[13] None of the compounds with an extra methylene group between the dihydropyrimidine or pyridine core (**Series *I***, ***II***, ***III***) displayed anti-HBV activity at concentrations up to 10 µM (Table 1). Interestingly, in these series, compounds **7d**, **8d-e**, **13**, **16b-c** showed single digit micromolar cytotoxicity in CEM cells.

Table 1. HBV inhibition and cytotoxicity of **series *I*, *II* & *III***.

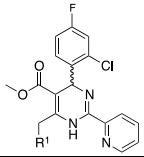


Cmpd	R^1/R^2	R^3	Anti-HBV activity, µM	Cytotoxicity, CC_{50} (µM)			
			EC_{50}	HepG2	PBM	CEM	Vero
5b	H/H	H	>10	>100	52	61	>100
7c	H/H		>10	14	>100	17	76
7d	H/H		>10	96	>100	5	>100
8a	H/H		>10	>100	81	2	59
8b	H/H		>10	≥100	99	55	87
8c	H/H		>10	>100	>100	86	>100
8d	H/H		>10	>100	65	9	12
8e	H/H	H	>10	41	67	4	61
13	Cl/F		>10	69	34	11	71
16a	H/H		>10	47	43	21	53
16b	Cl/F		>10	42	69	9	29
16c	Cl/F		>10	>100	>100	86	≥100
16d	Cl/F		>10	≥100	>100	≥100	10
HAP-12	-	-	0.5 ± 0.3	>100	>100	>100	>100
GLS4	-	-	0.3 ± 0.02	>100	28	69	18
1	-	-	>10	>100	>100	>100	>100
3TC	-	-	0.04 ± 0.03	>100	>100	>100	>100

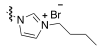
ND: not determined

As described in Table 2, replacement of the morpholine core of HAP-12 with a phosphonate moiety, an imidazolium salt or a sulfone group was counterproductive and lead to inactive compounds **21a-c** and **21e-f**. Introduction of an azido group (Compound **21d**), on the other hand, only lead to a moderate decrease of potency (EC_{50} = 7.5 µM) compared to HAP-12.

Table 2. HBV inhibition and cytotoxicity of compounds **21a-f**.



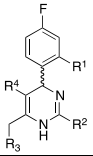
Cmpd	R^1	Anti-HBV	Cytotoxicity, CC_{50} (µM)

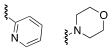
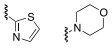
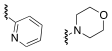
		activity, μM				
		EC_{50}	HepG2	PBM	CEM	Vero
21a		>10	>100	65	15	43
21b		>10	>100	44	23	>100
21c		>10	14	20	3	>100
21d		5.3 ± 3.2	>100	>100	>100	56
21e		>10	>100	>100	39	>100
21f		>10	>100	67	47	≥ 100
HAP-12	-	0.45	>100	>100	>100	>100
GLS4	-	0.3	>100	28	69	18
3TC	-	0.04 ± 0.03	>10	>100	>100	>100

ND: not determined

In our last series of compounds (Table 3), various modifications at the 6-position were explored. Unfortunately, replacement of the methyl or ethyl ester of HAP-12 or GLS-4 with a thioester (**26c**), a trifluoroethyl ester (**26e**), a *N*-(*N*,*N*-dimethylsulfamoyl)carboxamide (**26f**) or an acetamide (**28a-b**) was detrimental to the anti-HBV potency of these compounds and none of them showed activity at concentration up to 10 μM. Interestingly, introduction of an iodine or a bromine atom at this position lead to the discovery of compounds **26a-b** and **26g-i** which displayed EC_{50} values between 4.0 and 5.7 μM. Chlorine, however, was not a suitable modification and **26c** was found to be inactive at 10 μM.

Table 3. HBV inhibition and cytotoxicity of **series *IV***: compounds **26a-i** and **28a-b**.



Cmpd	R^1	R^2	R^3	R_4	Anti-HBV activity, μM	Cytotoxicity, CC_{50} (μM)			
					EC_{50}	HepG2	PBM	CEM	Vero
26a	Cl		H	I	4.1	>100	80	74	29
26b	Cl		H	Br	5.2 ± 1.0	79	>100	23	16
26c	Cl		H	Cl	>10	98	>100	44	13
26d	Cl		H		>10	>100	31	13	40
26e	Cl		H		>10	>100	> 100	4	65
26f	Cl		H		>10	>100	>100	>100	≥100
26g	Br		H	I	7.1	>100	80	33	>100
26h	Cl			I	4.0 ± 1.1	>100	>100	11	45
26i	Br			I	5.7	>100	75	33	>100
28a	Cl		H		>10	80	>100	>100	>100
28b	Cl				>10	>100	28	69	18
HAP-12	-	-	-	-	0.5 ± 0.3	>100	>100	>100	>100
GLS4	-	-	-	-	0.3 ± 0.02	>100	28	69	18
3TC	-	-	-	-	0.04 ± 0.03	>10	>100	>100	>100

ND: not determined

In conclusion, we have synthesized and evaluated more than thirty CAE analogs of HAP-12 and GLS4. Among them, we discovered five new 5-halogeno-heteroarylpyrimidines analogs that displayed anti-HBV activity in the low micromolar range. Despite some toxicity observed in certain cell lines, further modifications are currently being investigated and will be subject of future publications.

Acknowledgments

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[17] Representative procedure for the decarboxyhalogenation - Synthesis of **26a**: Carboxylic acid **25a** (346 mg, 1.0 mmol), sodium iodide (750 mg, 5.0 mmol) and sodium carbonate (106 mg, 1.0 mmol) were combined in a solution of water (8 mL) and methanol (8 mL). Stirring and sonication gave a clear, colorless solution to which was added Oxone (431 mg, 0.7 mmol). The solution was protected from light and stirred at room temperature for 20 min. After addition of a saturated solution of sodium thiosulfate (50 mL), the mixture was extracted with ethyl acetate (3 x 25 mL); the combined organic phases were dried over sodium sulfate and concentrated under *vacuum*. Purification by flash chromatography on silica gel (hexanes/ethyl acetate: 8/2) gave **26a** (290 mg, 68% yield) as a yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.50 (d, *J* = 3.8 Hz, 1H, ArH), 8.21 (d, *J* = 7.2 Hz, 1H, ArH), 7.74 (t, *J* = 7.6 Hz, 1H, ArH), 7.51 – 7.40 (m, 1H, ArH), 7.40 – 7.30 (m, 1H, ArH), 7.20 – 7.08 (m, 1H, ArH), 6.99 (t, *J* = 8.2 Hz, 1H, ArH), 6.05 (s, 1H, CH), 2.26 (s, 3H, CH_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.1, 160.6, 149.8, 147.8, 136.9, 133.5, 131.4, 131.3, 125.4, 121.3, 117.1, 116.8, 114.8, 114.6, 63.4, 24.7. HRMS (ESI) calcd. for $C_{16}H_{12}ClFIN_3$ $[M+H]^+$: 427.9827; Found: 427.9814.
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[19] Representative procedures for the Buchwald–Hartwig amidation – Synthesis of compound **28a**: Protected-5-iodo-dihydropyrimidines **27a** (56 mg, 0.10 mmol), acetamide (122 mg, 0.50 mmol), tris(dibenzylideneacetone) dipalladium (10 mg, 0.01 mmol), Xantphos (17 mg, 0.03 mmol), potassium

carbonate (53 mg, 0.50 mmol), and dioxane (2 mL) were combined in a sealed tube. The mixture was purged with argon for several min and stirred for 30 min at 100 ºC under microwave irradiation. The reaction was diluted with ethyl acetate (50 mL), washed with a saturated solution of ammonium chloride (50 mL). The organic phase was dried over sodium sulfate and concentrated under reduced pressure. The crude oil was then solubilized in EtOH (2.5 mL) and palladium on activated charcoal (20 mg) was added. The mixture was purged with argon for several min before cyclohexadiene (0.2 mL, 2.0 mmol) was added to the solution, and the reaction heated at 70 ºC for 15 min. The reaction mixture was then filtered on celite and concentrated under *vacuum*. Purification by flash chromatography on silica gel (hexanes/ethyl acetate: 3/7) yielded product **28a** as a brown solid (20 mg, 49% yield over 2 steps). Hydrochloride salt of **28a** was prepared for characterization. ^{1}H NMR (400 MHz, MeOD) δ 8.79 (t, *J* = 9.9 Hz, 1H, ArH), 8.18 (d, *J* = 7.9 Hz, 1H, ArH), 8.10 (t, *J* = 7.7 Hz, 1H, ArH), 7.74 (dd, *J* = 7.4, 4.8 Hz, 1H, ArH), 7.57 (dd, *J* = 8.6, 6.0 Hz, 1H, ArH), 7.32 (dd, *J* = 8.6, 2.4 Hz, 1H), ArH, 7.18 (td, *J* = 8.4, 2.4 Hz, 1H, ArH), 6.29 – 6.18 (m, 1H, CH), 2.03 (s, 3H, CH_3), 1.89 (s, 3H, CH_3). ^{13}C NMR (101 MHz, MeOD) δ 172.9, 165.5, 163.0, 155.2, 151.7, 143.9, 139.5, 135.7, 135.6, 134.3, 133.9, 133.9, 130.0, 128.4, 124.7, 118.8, 118.6, 116.3, 116.1, 114.5, 55.4, 49.9, 22.4, 13.9. HRMS (ESI) calcd. for $C_{18}H_{17}ClFN_4O$ $[M+H]^+$: 359.1075; Found: 359.1062.

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