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Efficient synthesis and utilization of phenyl-substituted heteroaromatic carboxylic acids as aryl diketo acid isosteres in the design of novel HIV-1 integrase inhibitors

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ABSTRACT

Three new types of aryl diketo acid (ADK) isosteres were designed by conversion of the biologically labile 1,3-diketo unit into heteroaromatic motif such as isoxazole, isothiazole, or 1*H*-pyrazole to improve the physicochemical property of ADK-based HIV-1 integrase (IN) inhibitors. The synthesis of the heteroaromatic carboxylic acids was established by employing phenyl β-diketoester or benzaldehyde as the starting material and 1,3-dipolar cycloaddition as the key reaction. Of the compounds tested, the 3-benzyloxyphenyl-substituted isoxazole carboxylic acid displayed the best IN inhibitory and antiviral activities, with *N*-hydroxylamidation enhancing the *in vitro* and *in vivo* potency. These findings are important for further optimization of ADK-based IN inhibitors.

The *Pol* gene of retrovirus HIV-1 encodes three essential enzymes—protease (PR), reverse transcriptase (RT), and integrase (IN). IN mediates the integration of proviral DNA into the cell genome, which is essential for retroviral replication and chronic infection, and homologous enzymes are lacking in the human host.[1] Thus IN has become an attractive target for anti-HIV therapeutics. The function of IN is achieved in a stepwise fashion by endonucleolytic processing of proviral DNA (termed 3′-processing or 3′-P) within a cytoplasmic preintegration complex, followed by translocation of the complex into the nuclear compartment where integration of 3′-processed proviral DNA into host DNA occurs in a reaction referred to as strand transfer.

Among an impressive number of synthetic IN inhibitors reported, the aryl diketo acids (ADK) are the most promising and advanced class which selectively inhibit the strand transfer reaction in extracellular assays and exhibit good antiviral effects against HIV-infected cells.[2–4] Because diketo acid is biologically labile, a variety of bioisosteres were developed such as 8-hydroxy-1,6-naphthyridine, 4-oxo-1,4-dihydroquinoline-3-carboxylic acid, and 6-oxo-1,6-dihydro pyrimidin-5-olate whose derivatives were subsequently developed into clinical candidates (Fig. 1). The application of the ADK isosteres facilitated the FDA-approval of the first IN inhibitor, MK-0518 (Raltegravir), into a successful anti-HIV drug.[5]

The emergence of viral strains resistant to clinically studied IN inhibitors and the dynamic nature of the HIV-1 genome demand a continued effort toward the discovery of novel inhibitors to keep a therapeutic advantage over the virus. A major focus of our laboratories is to design a new class of ADK bioisosteres by conversion of the biologically labile diketo moiety into metabolically stable heteroaromatic group. In this study, we designed and synthesized three types of phenyl-substituted heteroaromatic carboxylic acids (Fig. 2). The phenyl substitution and the relative orientation of the phenyl and the carboxylic acid functionality were examined with respect to the effect on the IN inhibition and HIV replication.

The phenyl-substituted isoxazole- or 1*H*-pyrazole-3-carboxylic acids were conveniently synthesized by coupling the aryl diketo

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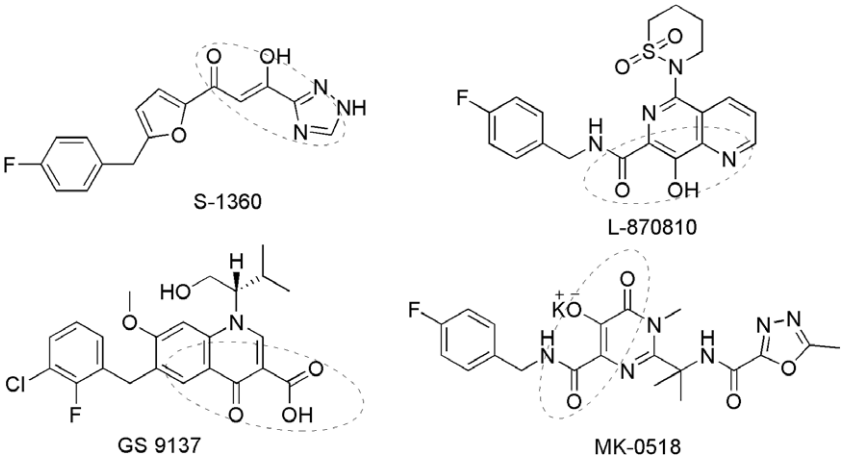


Figure 1. The structures of ADK-derived IN inhibitors clinically studied. The dashed circle indicated the moiety of diketo acid isostere.

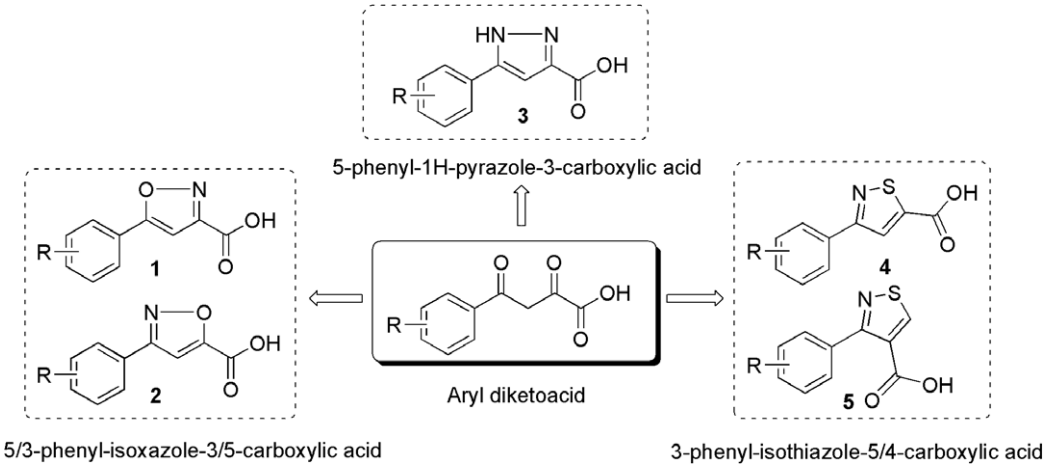
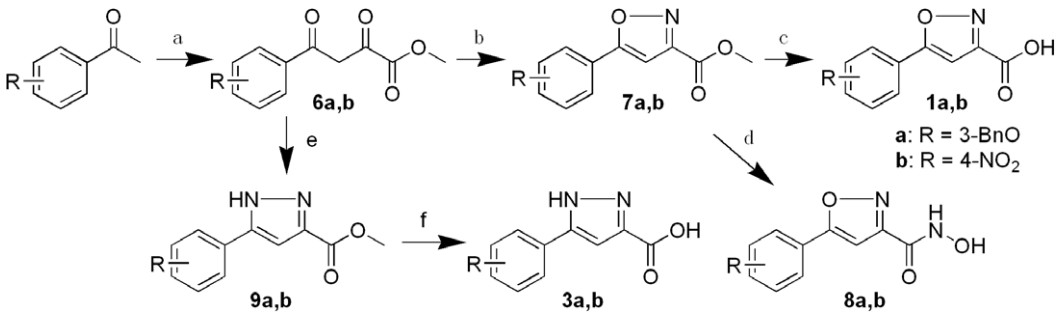


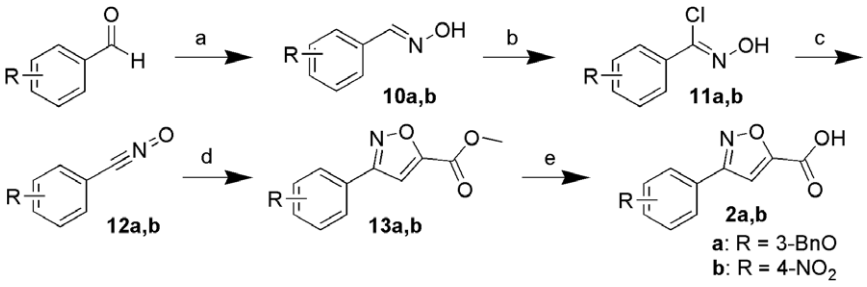
Figure 2. The design of phenyl-substituted heteroaromatic carboxylic acids as aryl diketo acid isosteres.

ester with hydroxylamine or hydrazine under different conditions. As depicted in Scheme 1, the variously substituted acetophenones were oxalylated by dimethyl oxalate in the presence of sodium *tert*-butoxide, affording β-diketoester **6a**,**b** in high yields.[6] Treatment of the aryl β-diketoester with hydroxylammonium chloride in refluxing methanol yielded isoxazole ester **7a**,**b**,[7] which was hydrolyzed to give 5-phenylisoxazole-3-carboxylic acid **1a**,**b**. Further treatment of isoxazole ester **7** with hydroxylammonium chloride and diluted solution of NaOH generated 5-phenyl-*N*-hydroxyisoxazole-3-carboxamide **8**. Toward another direction, the aryl diketo ester **6** was readily converted into pyrazole ester by coupling with hydrazine in the presence of acetic acid,[8] which was then hydrolyzed into 5-phenyl-1*H*-pyrazole-3-carboxylic acids **3a**,**b**.

Contrary to 5-phenyl-isoxazole-3-carboxylic acid, the synthesis of 3-phenyl-isoxazole-5-carboxylic acid adopted a different strategy by employing a 1,3-dipolar cycloaddition of benzonitrile *N*-oxide with methyl propiolate as the key step. As shown in Scheme 2, starting from the substituted benzaldehyde, the treatment with hydroxylammonium chloride and sodium acetate afforded benzal-



Scheme 1. General synthetic route toward 5-phenyl-isoxazole-3-carboxylic acid and 5-phenyl-1*H*-pyrazole-3-carboxylic acid. Reagents and conditions: (a) *t*-BuONa, $(CH_3OOC)_2$, THF, 0 °C–rt; (b) $NH_2OH{\cdot}HCl$, CH_3OH, reflux, 3 h; (c) 1 N NaOH, THF, 60 °C, 1 h; (d) 12.5 equiv, 1 N NaOH, 10 equiv, $NH_2OH{\cdot}HCl$, CH_3OH, rt, 2 h; (e) hydrazine, acetic acid, reflux, 1 h; (f) hydroxylammonium chloride, 2 N NaOH, methanol, 80 °C, 1 h.



Scheme 2. Synthetic approach to 3-phenyl-isoxazole-5-carboxylic acid. Reagents and condition: (a) $NH_2OH{\cdot}HCl$, CH_3COONa, methanol/H_2O, reflux, 8 h; (b) NCS, cat. pyridine, THF, 60 °C, 30 min; (c) TEA, rt, 10 min; (d) methyl propiolate, rt; (e) 2 N NaOH, methanol, 80 °C, 1 h.

dehyde oxime **10**. The oxime was chlorinated with NCS (*N*-chlorosuccinimide) and a catalytic amount of pyridine in THF at 60 °C under nitrogen atmosphere. The reaction mixture was treated with Et_3N in THF over 10 min followed by addition of methyl propiolate, during which the benzonitrile *N*-oxide **12** was generated in situ. The 1,3-dipolar cycloaddition of benzonitrile *N*-oxide with methyl propiolate yielded the isoxazole ester **13**,[9] which was hydrolyzed to produce 3-phenylisoxazole-5-carboxylic acid **2**.

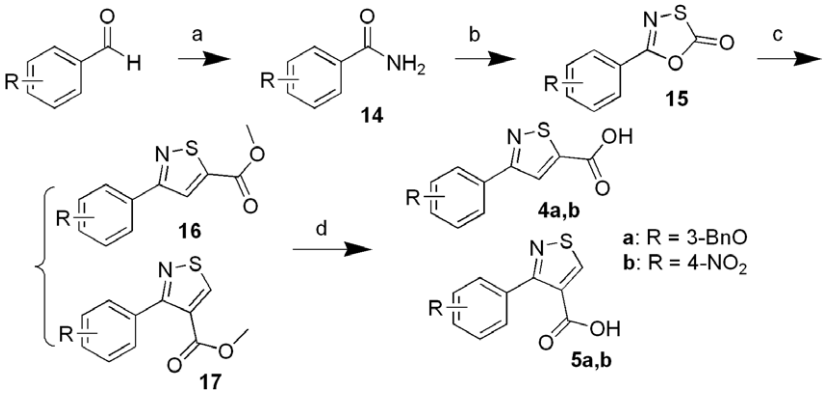
Similarly, the synthesis of isothiazole-5/4-carboxylic acid involved a 1,3-dipolar cycloaddition reaction of nitrile sulfide generated by thermal decarboxylation of the corresponding 1,3,4-oxathiazole-2-one, as shown in Scheme 3. The substituted phenyloxathiazolone **15** was readily prepared by heating substituted benzamide **14** with chlorocarbonylsulfenyl chloride in refluxing dioxane for 4.5 h. And the benzamide **14** was directly converted from the substituted benzaldehyde by one-pot tandem reaction,[10] whereby the benzaldehyde reacted with iodine in 25% ammonia water at room temperature followed by addition of urea hydrogen peroxide and K_2CO_3 to produce the corresponding amide. The coupling of nitrile sulfide with methyl propiolate afforded almost equal ratio isomers **16** and **17**, which were distinguished by the chemical shift of the hydrogen on the isothiazole ring.[11] The isothiazole-5/4-carboxylic acid esters were hydrolyzed to the corresponding acids **4** and **5**, respectively.

HIV-1 integrase inhibitory activity of phenyl-substituted heteroaromatic carboxylic acids. We examined the inhibitory activity of the new class of ADK isosteres on IN catalytic function. As shown in Table 1, no inhibition against 3′-processing reaction was observed for the three types of ADK isosteres at the concentration of 100 μM, which is consistent with the general character of aryl diketo acid functioning as selective strand transfer inhibitors. Of these heteroaromatic carboxylic acids, the phenyl-substituted isoxazole-carboxylic acids exhibited best potency against the strand transfer reaction. With respect to the substitution effect on the phenyl ring, the electron-donating group was preferred over the electron-withdrawing group for the phenyl-substituted heteroaromatic carboxylic acids as IN inhibitors. In accord, the 5-(3-(benzyloxy)phenyl)isoxazole-3-carboxylic acid (compound **1a**) exhibited an IC_{50} value of 68 μM against strand transfer reaction, while 5-(4-nitrophenyl)isoxazole-3-carboxylic acid was 1.4-fold less potent (compound **1b**, IC_{50} = 97 μM).

On the other hand, the relative location of the phenyl ring and the carboxylic acid functionality on the heteroaromatic ring was important for the potency. The 5-phenylisoxazole-3-carboxylic acid (compounds **1a**,**b**) was a superior scaffold to the 3-phenylisoxazole-5-carboxylic acid (compounds **2a**,**b**) for IN inhibition, thus the 5-(3-(benzyloxy)phenyl)isoxazole-3-carboxylic acid (**1a**, IC_{50} = 68 μM) was more potent than the 3-(3-(benzyloxy)phenyl)isoxazole-5-carboxylic acid (**2a**, IC_{50} = 81 μM) in inhibiting the strand transfer reaction. Further modification on 5-phenylisoxazole-3-carboxylic acid by carboxyl amidation with hydroxylamine resulted in a 2.5-fold enhancement on the strand transfer inhibitory potency (**8a**, IC_{50} = 27 μM) relative to the parent compound **1a**.

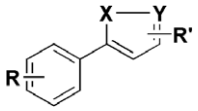
The secondary active structure in this series was 5-phenyl-1*H*-pyrazole-3-carboxylic acid, of which the 3-benzyloxyphenyl-substituted analog displayed moderate activity against the strand transfer reaction (**3a**, IC_{50} = 100 μM). However, the introduction of sulfur atom in the heteroaromatic ring caused a substantial loss of the IN inhibitory activity. The resulting isothiazole-5/4-carboxylic acids were almost inactive against IN regardless of the substitution and spatial arrangement of the phenyl ring.

Antiviral potency against HIV-1_{IIIB} in C8166 cell cultures of the phenyl-substituted heteroaromatic carboxylic acids. Although the IN inhibitory activity was not as potent as we expected, these ADK isosteres exert potent to moderate inhibition on the cytopathic effect of HIV-1 in infected C8166 cells, which indicated that the isostere surrogate did possess better bioavailability. Furthermore, the



Scheme 3. The synthetic route to 3-phenylisothiazole-5/4-carboxylic acid. Reagents and conditions: (a) 1.1 equiv I_2, 25% ammonia solution, rt, 1 h; then urea hydrogen peroxide, K_2CO_3, THF, rt, 2–4 h; (b) chlorocarbonylsulfenyl chloride, dioxane, reflux, 4.5 h; (c) methyl propiolate, xylene, reflux, 26 h; (d) THF, 1 N NaOH, rt, 2 h.

Table 1
The IN inhibitory activity and antiviral effect of the substituted phenyl heteroaromatic acids and derivatives



Compound	R	X	Y	R′	Inhibition of HIV-1 integrase (IC_{50})[a]		Anti-HIV-1 activity[b]		TI[e]
					3′-Processing (μM)	Strand transfer (μM)	EC_{50}[c] (μM)	CC_{50}[d] (μM)	
1a	3-BnO	O	N	3-COOH	>100	68	91	206	2.27
1b	4-NO_2	O	N	3-COOH	>100	97	199	>854	>4.3
8a	3-BnO	O	N	3-CONHOH	>100	27	62.8	136	2.17
2a	3-BnO	N	O	5-COOH	>100	81	**7.2**	282	39.25
3a	3-BnO	N	N	3-COOH	>100	100	277	640	2.31
3b	4-NO_2	N	N	3-COOH	>100	>100	**3.6**	>857	>238
9b	4-NO_2	N	N	3-COOMe	>100	>100	253	>809	>3.2
4a	3-BnO	N	S	5-COOH	>100	>100	51.7	246	4.76
5a	3-BnO	N	S	4-COOH	>100	>100	58	>642	>11

[a] HIV-1 integrase inhibitory activity was measured according to the procedure described in Ref. 12.
[b] Anti-HIV-1 data represent the mean values of two separate experiments.
[c] Effective concentration required to protect C8166 cells against the cytopathogenicity of HIV-1 by 50%.[13]
[d] Cytostatic concentration required to reduce C8166 cell proliferation by 50% tested by MTT method.[13]
[e] Therapeutic index (TI) is a ratio of the CC_{50} value/EC_{50} value.

three types of novel ADK isosteres displayed weak cytotoxicity (CC_{50} fell within 200–800 μM), resulting in high therapeutic index.

For the 5-phenylisoxazole-3-carboxylic acid series, the antiviral effect was correlated with the IN inhibitory activity. The best IN inhibitor, that is, 5-(3-(benzyloxy)phenyl)-*N*-hydroxyisoxazole-3-carboxamide (**8a**) exhibited an EC_{50} value of 62.8 μM. However, the 5-phenyl-1*H*-pyrazole-3-carboxylic acid and 3-phenylisoxazole-5-carboxylic acid displayed potent antiviral activity though they behaved as weak IN inhibitors. The best anti-HIV-potency was exhibited by 5-(4-nitrophenyl)-1*H*-pyrazole-3-carboxylic acid (**3b**) and 3-(3-(benzyloxy)phenyl)isoxazole-5-carboxylic acid (**2a**) with an EC_{50} value of 3.6 and 7.2 μM, respectively. More importantly, the two active compounds possessed significantly low cytotoxicities with a TI value of >238 and 39, respectively. Even the isothiazole-5/4-carboxylic acid class which was inactive against IN exerted moderate activity to protect C8166 cells from HIV-1 infection (**4a**, EC_{50} = 51.7 μM; **5a**, EC_{50} = 58 μM) with low cytotoxicity. On the one hand, the good antiviral potency of the heteroaromatic carboxylic acid series might be attributable to the improved bioavailability of the bioisosteres as we anticipated; on the other hand, the inconsistency between IN inhibitory activity and the antiviral effect might involve multiple targeting in the HIV-1 life cycle. Thus, further investigation on the mechanism of 5-phenyl-1*H*-pyrazole-3-carboxylic acid and 3-phenylisoxazole-5-carboxylic acid as anti-HIV agents is under way.

In conclusion, we designed and synthesized three types of ADK isosteres by conversion of the 1,3-diketo unit into isoxazole, 1*H*-pyrazole, and isothiazole moieties. The resulting 5-(3-(benzyloxy)phenyl)-*N*-hydroxyisoxazole-3-carboxamide (**8a**) displayed an IC_{50} value of 27 μM against IN, whereas the best antiviral effect was exhibited by 5-(4-nitrophenyl)-1*H*-pyrazole-3-carboxylic acid (**3b**) and 3-(3-(benzyloxy)phenyl)isoxazole-5-carboxylic acid (**2a**) with an EC_{50} value of 3.6 and 7.2 μM, respectively. The phenyl-substituted heteroaromatic carboxylic acids afforded advantageous features of improved antiviral potency and decreased cytotoxicity with high therapeutic index, providing promising new approach and scaffold to develop potent anti-HIV agents.

Acknowledgments

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12. *Integrase Assays procedure:* To determine the extent of 3′-processing and strand transfer, wild-type IN was preincubated at a final concentration of 200 nM with the inhibitor in reaction buffer (50 mM NaCl, 1 mM Hepes pH 7.5, 50 μM EDTA, 50 μM dithiothreitol, 10% glycerol (w/v), 7.5 mM $MnCl_2$, 0.1 mg/ml bovine serum albumin, 10 mM 2-mercaptoethanol, 10% dimethylsulfoxide, and 25 mM MOPS pH 7.2) at 30 °C for 30 min. An aliquot (5 μl) was electrophoresed on a denaturing 20% polyacrylamide gel (0.09 M tris–borate, pH 8.3, 2 mM EDTA, 20% acrylamide, 8 M urea). Gels were dried, exposed in a PhosphorImager cassette, and analyzed using a Typhoon 8610 Variable Mode Imager (Amersham Biosciences) and quantitated using ImageQuant 5.2. Percent inhibition (% I) was calculated using the following equation: $\% I = 100 \times [1 - (D - C)/(N - C)]$, where *C*, *N*, and *D* are the fractions of 21-mer substrate converted to 19-mer (3′-processing product) or strand transfer products for DNA alone, DNA plus IN, and IN plus drug, respectively. The IC_{50} values were determined by plotting the logarithm of drug concentration versus percent inhibition to obtain concentration that produced 50% inhibition.
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