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Amide-containing diketoacids as HIV-1 integrase inhibitors: Synthesis, structure–activity relationship analysis, and biological activity

Hongcai Li [a], Chao Wang [a], Tino Sanchez [b], Yanmei Tan [a], Chunying Jiang [a], Nouri Neamati [b,*], Guisen Zhao [a,*]

[a] School of Pharmaceutical Sciences, Shandong University, Ji'nan, Shandong Province 250012, PR China
[b] Department of Pharmaceutical Sciences, School of Pharmacy, University of Southern California, Los Angeles, CA 90089, USA

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ABSTRACT

HIV-1 integrase, which catalyzes the integration of the viral genome into the cellular chromosome, is an essential enzyme for retroviral replication, and represents an attractive and validated target in the development of therapeutics against AIDS. In this paper, 17 amide-containing novel diketoacids were designed and synthesized, and their ability to inhibit HIV-1 integrase was tested. The structure–activity relationships were also analyzed.

1. Introduction

Human immunodeficiency virus type-1 (HIV-1) is the etiological agent of acquired immunodeficiency syndrome (AIDS). Three different classes of chemotherapeutic agents are actually available to block the replication of HIV-1, namely reverse transcriptase inhibitors, protease inhibitors, and fusion inhibitors.[1] Highly active anti-retroviral therapy (HAART) based on the use of the above drugs effectively inhibits the replication cycle of HIV-1. However, HAART fails to eradicate viral replication, and the emergence of multi-drug-resistant viral strains in infected patients can complicate the response to the treatment.[2,3]

In addition to reverse transcriptase and protease, another target useful for chemotherapeutic intervention is the HIV-1 integrase (IN), an essential enzyme that catalyzes the insertion of the viral DNA into the genome of the host cell through a complex process, which consists of three biochemical steps: (i) cleavage of a dinucleotide pair from the 3′-end of the viral DNA (termed '3′-processing', 3′-P), (ii) insertion of the resulting shortened strands into the host-cell chromosome (termed 'strand transfer', ST), and (iii) removal of the two unpaired nucleotides at the 5′-end of the viral DNA and gap-filling process.[4] Due to the absence of any known human homolog,[5] IN is considered as an attractive and validated target for the development of novel anti-HIV drugs.

Among the reported HIV-1 IN inhibitors, diketo-containing compounds, mainly aryl diketoacids (ADK) and their derivatives, represent a most promising class of compounds.[6] The mechanism appears to involve the binding of the diketoacid portion to the Mg^{2+} or Mn^{2+} co-factor located in the active site of the enzyme.[7] Several IN inhibitors containing the aryl diketo moiety have been

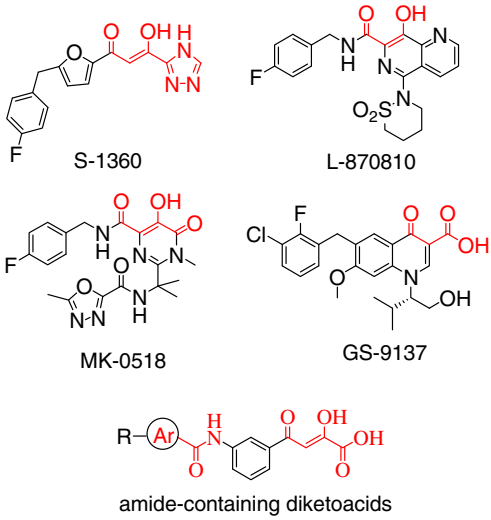

Figure 1. The structure of selected ADK-based clinically studied IN inhibitors and amide-containing diketoacids.

* Corresponding authors. Tel.: +1 323 442 2341; fax: +1 323 442 1390 (N.N.); tel.: +86 531 88382009; fax: +86 531 88382835 (G.Z.).
E-mail addresses: neamati@usc.edu (N. Neamati), guisenzhao@sdu.edu.cn (G. Zhao).

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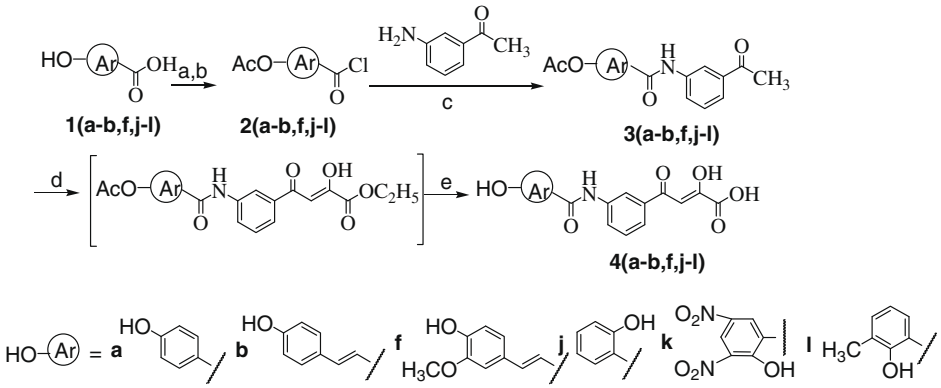


Scheme 1. Reagents and conditions: (a) Ac_2O, pyridine, rt, 24 h; (b) $SOCl_2$, 80 °C, 5 h; (c) THF, rt, <18 h; (d) $(CO_2C_2H_5)_2$, $NaOCH_3$, THF, rt, <18 h, then 1 M HCl; (e) 1 M NaOH, THF/CH_3OH (1:1), rt, 1 h, then 1 M HCl.

tested in the clinic, including S-1360, L-870810, GS-9137, and MK-0518 (Fig. 1). Especially MK-0518, also known as raltegravir, has been approved by the FDA as the first IN inhibitor among the clinical anti-HIV drugs.[8] Previous studies in this field,[7,9] have suggested that an H-bond donating group is required for activity. Further studies with the chemical structures of many potent IN inhibitors have documented that an amide group is well tolerated.[7,10–12] In fact L-870810 and MK-0518 (Fig. 1) also contain one or two amide groups in their templates. In this paper, seventeen amide-containing novel diketoacids (Fig. 1) were designed and synthesized and their inhibitory activities on HIV-1 IN were tested, and the structure–activity relationships were analyzed.

2. Results and discussion

2.1. Chemistry

The chemical synthetic routes of target compounds are presented in Schemes 1 and 2. Analogues containing hydroxyl groups on the terminal aryl ring (**4a–b**, **4j–l**, and **4f**) were prepared as shown in Scheme 1. Various phenolic hydroxyl groups of carboxylic acids were protected with ethanoyl in the presence of pyridine, and then reacted with thionyl chloride under reflux at 80 °C to give the substituted acyl chloride (**2**). Using 3-aminoacetophenone as raw material, compound (**3**) was synthesized by acylation with the substituted acyl chloride (**2**) in THF at room temperature. Treatment of compound (**3**) with diethyl oxalate and sodium methoxide provided an intermediate as an orange oil, which was hydrolyzed with aqueous sodium hydroxide, and then treated with hydrochloric acid to afford β-diketo acids (**4**) with various substituents on the phenyl ring in good yield (Scheme 1).

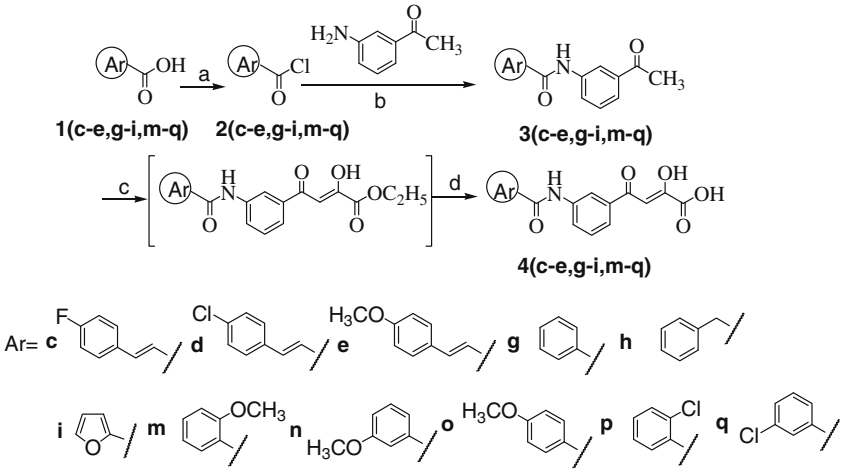
Most of the compounds reported in this paper were prepared as depicted in Scheme 2. Different from Scheme 1, the various acyl chlorides (**2**) were obtained directly by treating substituted aromatic acids with thionyl chloride at 80 °C. Then, a similar method was applied to the synthesis of compounds **3** and **4**, as shown in Scheme 2.

2.2. Inhibitory activity of target compounds (4) against HIV-1 integrase in vitro

All synthesized compounds were tested for their ability to inhibit 3′-processing (3′-P) and strand transfer (ST). The data are summarized in Table 1. Compound **4k** had the best efficacy on both 3′-P and ST with an IC_{50} value of 8 and 2 μM, respectively. The other compounds showed moderate ST inhibitory potency with IC_{50} values from 4 to 33 μM.

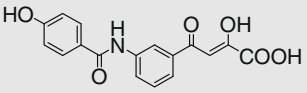
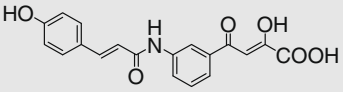
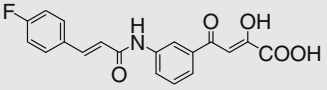
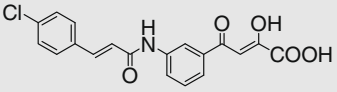
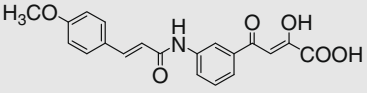
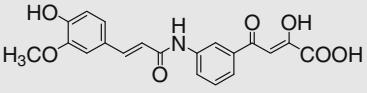
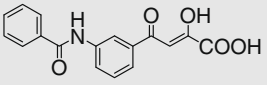
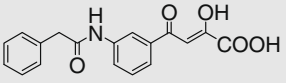
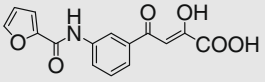
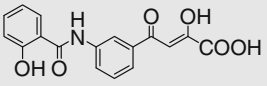
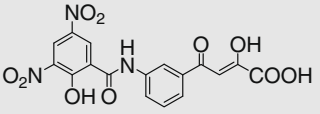
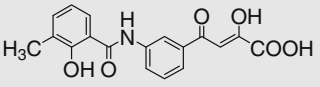
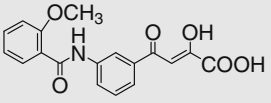
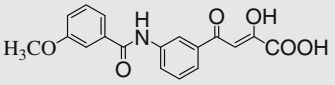
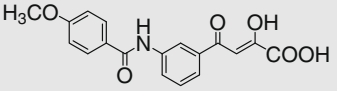
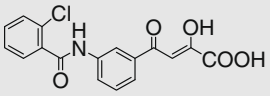
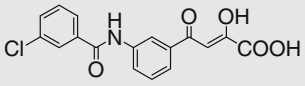
The structure–activity relationships (SAR) in these novel compounds were investigated by introducing substituents on the terminal aryl ring, and increasing the length of the carbon chain between the terminal benzene ring and the amide group.

We started our work focusing on extending the chain length between the terminal aromatic ring and the amide group, to enhance the binding interaction between the compounds and IN, and thus



Scheme 2. Reagents and conditions: (a) $SOCl_2$, 80 °C, 5 h; (b) THF, rt, <18 h; (c) $(CO_2C_2H_5)_2$, $NaOCH_3$, THF, rt, <18 h, then 1 M HCl; (d) 1 M NaOH, THF/CH_3OH (1:1), rt, 1 h, then 1 M HCl.

Table 1
Structures and HIV IN inhibitory activities of target compounds

Code	Structure	3′-P IC_{50}[a] (μM)	ST IC_{50}[a] (μM)
4a		81 ± 11	13 ± 2
4b		26 ± 6	7 ± 2
4c		22 ± 4	7 ± 2
4d		18 ± 6	4 ± 1
4e		30 ± 5	11 ± 4
4f		42 ± 8	11 ± 1
4g		>100	31 ± 2
4h		56 ± 6	7 ± 1
4i		>100	33 ± 3
4j		41 ± 12	11
4k		8 ± 2	2 ± 0.4
4l		48 ± 22	10 ± 4
4m		73 ± 2	13
4n		69 ± 10	12 ± 1
4o		95 ± 5	19 ± 1
4p		92 ± 9	18 ± 2
4q		57 ± 5	15 ± 5

[a] IC_{50}: Inhibitory concentration 50% (inhibition of purified integrase).

improve their activities. As shown in Table 1, appending an ethylenic linkage resulted in a two to threefold increase in both 3′-P and ST inhibitory potency (**4b** vs **4a** and **4e** vs **4o**). Compound **4b** showed enzyme inhibitory activities with IC_{50} values of 26 μM and 7 μM for 3′-P and ST, respectively. Furthermore, addition of a methylene between the terminal aromatic ring and the amide group also provided a twofold enhancement in 3′-P inhibitory potency and a fourfold higher inhibitory activity against ST (**4h** vs **4g**).

Encouraged by the improved potency of compound **4b** versus **4a**, we continued our optimization effort by changing the phenyl tail group with various substituents. As shown by compounds **4b–f**, both electron-withdrawing and electron-donating substituents on the terminal benzene ring were tolerated. Compared to the parent compound **4b**, substitution of the 4-OH with a methoxyl (**4e**) decreased IN inhibition activities, whereas fluorine (**4c**) showed similar activity and chlorine (**4d**) enhanced the 3′-P and ST inhibitory potency. The chlorine-substituted compound **4d** was the most active among the four analogues **4b–e**. Moreover, it was found that introducing a methoxyl at the 3′-position (**4f**) had little impact on activity. A similar observation was made with compounds **4j** and **4l**, where addition of a methoxyl at the 3-position had minimal change in activity. Compound **4k** with a 3,5-dinitrophenyl group showed the best inhibitory profile, with IC_{50} values of 8 μM and 2 μM for 3′-P and ST, respectively.

Utilizing the strategy of the bioisosterism principle, we replaced the terminal benzene group with a furan ring. As exemplified by compound **4i**, replacement of the benzene ring with a furan group displayed HIV-1 IN inhibitory activities similar to **4g**.

Continuing SAR studies of the substituted groups with different locations on the terminal aryl ring, we investigated compounds **4a**, **4j**, and **4m–q**. It was noted that 3-methoxy and 3-chloro substituted compounds (**4n** and **4q**) showed a slight improvement in 3′-P inhibitory potency, but had similar levels of ST activity relative to the compounds with 2-substitutions (**4m** and **4p**). A methoxyl group in the 4-position (**4o**) exhibited a little loss in the IN inhibitory activities compared to **4m** and **4n**. However, it is worth noting that compound **4j**, with a hydroxyl substituent at C-2, showed a twofold increase in 3′-P inhibition and similar ST activity versus the C-4 hydroxyl substituent **4a**. Moreover the C-2 hydroxyl substituent **4k** and **4l** also exhibited noticeable 3′-P and ST inhibitory potency. The results imply that another diketo moiety was formed between the benzamide group and the C-2 hydroxyl group, which might bind to two divalent metal ions on the active site of IN.

In conclusion, 17 novel amide-containing diketoacids were designed and synthesized as HIV-1 IN inhibitors. The experiments on the inhibition on HIV-1 IN indicated that (1) the chain length between the terminal aromatic ring and the amide group had a large influence on the IN activities. With the exception of C-2 hydroxy-substituted benzamide compounds (**4j–l**), introducing an

ethylenic linkage or methylene could enhance the IN inhibitory activity; (2) both electron-withdrawing and electron-donating substituents on the terminal aromatic ring were tolerated, but mostly electron-withdrawing substituents showed better potency; (3) the terminal aromatic group could be replaced by a furan ring, and it showed little impact on activities; (4) changing the positions of the substituents on the terminal aryl ring slightly affected activities, except for the hydroxy-substituted benzamide compounds. Because a novel diketo moiety could be formed in benzamide compounds with the 2-hydroxy group, improved activities were observed.

3. Experimental

Unless otherwise noted, all materials were obtained from commercial suppliers and used without further purification. Melting points were determined on a Büchi capillary melting point apparatus. Infrared (IR) spectra were measured on KBr pellets, using a Nicolet Nexus 470FT-IR and were expressed in cm^{-1}. Proton nuclear magnetic resonance (^1H NMR) spectra were recorded with a Bruker Avance DRX600 spectrometer with DMSO-d_6 as the solvent and tetramethyl-silane (TMS) as the internal standard. The chemical shifts were reported in δ (ppm). Mass spectra (MS) were measured with an API 4000 mass spectrometer, and the high resolution mass spectra data were obtained using an Accela UPLC-LTQ Orbitrap Mass Spectrometer. Thin-layer chromatography (TLC) was performed on silica gel GF254 plates (layer thickness, 0.2 mm) and the compounds were visualized using UV light. Petroleum ether used for TLC had a boiling range of 60–90 °C.

3.1. General methods for preparing substituted acyl chlorides 2

Substituted aromatic acid (15 mmol), acetic anhydride (20 mmol) and two drops of pyridine were placed in a 50 mL three-necked flask equipped with an electromagnetic stirrer. The reaction proceeded at room temperature with stirring for 24 h. The solvent was evaporated under reduced pressure to obtain acylation products. Then, sulfone chloride (20 mmol) was added to the flask and the reaction was allowed to reflux at 80 °C overnight. The solvent was evaporated under reduced pressure at 50 °C to obtain the products **2(a–b, f, j–l**). The products were diluted with 10 mL anhydrous tetrahydrofuran and kept on standby.

Other acyl chlorides **2(c–e, g–i, m–q**) were synthesized similarly by the following procedures. Sulfone chloride (20 mmol) was added to a 50 mL one-necked flask that contained the substituted aromatic acids **1(c–e, g–i, m–q**) (0.06 mol). The reaction was left to reflux at 80 °C overnight. The solvent was evaporated under reduced pressure at 50 °C to obtain the product, which was then diluted with 10 mL anhydrous tetrahydrofuran and kept on standby.

3.2. General methods for preparing amide compounds 3[13]

In a 100 mL three-necked flask equipped with an electromagnetic stirrer, dropping funnel, and a reflux condenser bearing a calcium chloride drying tube, was placed 3-aminoacetophenone (20 mmol), which was dissolved in anhydrous tetrahydrofuran (15 mL). The acyl chloride solution (compound **2**) (20 mmol) was added dropwise with stirring in an ice bath. The reaction was continued at room temperature with stirring overnight. The solution was filtered and the filter cake was washed twice with tetrahydrofuran. The filtrate and washings of tetrahydrofuran were mixed and diluted with dichloromethane, and then washed with 1 M hydrochloric acid solution (10 mL × 3), distilled water (10 mL × 3), 5% sodium bicarbonate solution (10 mL × 3), and saturated sodium chloride solution (10 mL × 3). The organic phase was dried over Na_2SO_4 overnight. After filtering, the solvent was evaporated under reduced pressure to obtain products **3**.

3.2.1. 4-(3-Acetylphenylcarbamoyl)phenyl acetate (3a)

White powder, yield 76.2%, mp 138–139 °C, TLC R_f = 0.50 (petroleum ether/acetic ether 1:1, v/v). ^1H NMR (DMSO-d_6) δ: 10.48 (s, 1H), 8.36 (s, 1H), 8.07 (d, J = 7.80 Hz, 1H), 8.04 (d, J = 7.20 Hz, 2H), 7.72 (d, J = 7.80 Hz, 1H), 7.52 (t, J = 7.80 Hz, 1H), 7.32 (d, J = 7.20 Hz, 2H), 2.58 (s, 3H), 2.32 (s, 3H). MS (ESI) m/z 298.6 $[M+H]^+$.

3.2.2. 4-(3-(3-Acetylphenylamino)-3-oxoprop-1-enyl)phenyl acetate (3b)

White powder, yield 58.2%, mp 118–119 °C, TLC R_f = 0.42 (petroleum ether/acetic ether 1:1, v/v). ^1H NMR (DMSO-d_6) δ: 10.47 (s, 1H), 8.26 (s, 1H), 7.98 (d, J = 7.80 Hz, 1H), 7.72 (d, J = 7.80 Hz, 1H), 7.69 (m, 2H), 7.63 (d, J = 16.20 Hz, 1H), 7.32 (d, J = 7.20 Hz, 2H), 7.23 (d, J = 8.40 Hz, 1H), 6.80 (d, J = 15.60 Hz, 1H), 2.56 (s, 3H), 2.31 (s, 3H). MS (ESI) m/z 324.5 $[M+H]^+$.

3.2.3. *N*-(3-Acetylphenyl)-3-(4-fluorophenyl)acrylamide (3c)

White powder, yield 52.4%, mp 158–160 °C, TLC R_f = 0.49 (petroleum/acetic ether 1:1, v/v). ^1H NMR (DMSO-d_6) δ: 10.44 (s, 1H), 8.27 (s, 1H), 7.97 (d, J = 8.40 Hz, 1H), 7.77 (m, 1H), 7.72 (m, 2H), 7.62 (d, J = 16.20 Hz, 1H), 7.30 (t, J = 8.00 Hz, 2H), 7.25 (t, J = 8.00 Hz, 1H), 6.78 (d, J = 15.60 Hz, 1H), 2.58 (s, 3H). MS (ESI) m/z 284.5 $[M+H]^+$.

3.2.4. *N*-(3-Acetylphenyl)-3-(4-chlorophenyl)acrylamide (3d)

White powder, yield 55.1%, mp 114–115 °C, TLC R_f = 0.46 (petroleum ether/acetic ether 1:1, v/v). ^1H NMR (DMSO-d_6) δ: 10.46 (s, 1H), 8.26 (s, 1H), 7.97 (d, J = 7.80 Hz, 1H), 7.70 (d, J = 7.80 Hz, 1H), 7.67 (d, J = 9.00 Hz, 2H), 7.61 (d, J = 15.60 Hz, 1H), 7.52 (d, J = 8.40 Hz, 2H), 7.50 (d, J = 7.80 Hz, 1H), 6.83 (d, J = 15.60 Hz, 1H), 2.58 (s, 3H). MS (ESI) m/z 300.6 $[M+H]^+$.

3.2.5. *N*-(3-Acetylphenyl)-3-(4-methoxyphenyl)acrylamide (3e)

White powder, yield 50.3%, mp 143–145 °C, TLC R_f = 0.45 (petroleum ether/acetic ether 1:1, v/v). ^1H NMR (DMSO-d_6) δ: 10.46 (s, 1H), 8.27 (s, 1H), 7.98 (d, J = 7.80 Hz, 1H), 7.69 (d, J = 8.40 Hz, 3H), 7.63 (d, J = 16.20 Hz, 1H), 7.50 (t, J = 7.80 Hz, 1H), 7.22 (d, J = 8.40 Hz, 2H), 6.80 (d, J = 15.60 Hz, 1H), 2.58 (s, 3H), 2.28 (s, 3H). MS (ESI) m/z 296.6 $[M+H]^+$.

3.2.6. 4-(3-(3-Acetylphenylamino)-3-oxoprop-1-enyl)-2-methoxyphenyl acetate (3f)

White powder, yield 51.6%, mp 160–162 °C, TLC R_f = 0.45 (petroleum ether/acetic ether 1:1, v/v). ^1H NMR (DMSO-d_6) δ: 10.47 (s, 1H), 8.28 (s, 1H), 7.90 (m, 2H), 7.69 (d, J = 7.80 Hz, 1H), 7.63 (d, J = 16.80 Hz, 1H), 7.42 (m, 2H), 7.18 (d, J = 7.80 Hz, 1H), 6.82 (d, J = 15.60 Hz, 1H), 3.84 (s, 3H), 2.58 (s, 3H), 2.28 (s, 3H). MS (ESI) m/z 354.4 $[M+H]^+$.

3.2.7. *N*-(3-Acetylphenyl)benzamide (3g)

White powder, yield 79.8%, mp 99–101 °C, TLC R_f = 0.58 (petroleum ether/acetic ether 1:1, v/v). ^1H NMR (DMSO-d_6) δ: 10.46 (s, 1H), 8.38 (s, 1H), 8.08 (d, J = 7.80 Hz, 1H), 7.98 (d, J = 7.20 Hz, 2H), 7.72 (d, J = 7.80 Hz, 1H), 7.62 (t, J = 7.20 Hz, 1H), 7.53 (m, 3H), 2.59 (s, 3H). MS (ESI) m/z 240.4 $[M+H]^+$.

3.2.8. *N*-(3-Acetylphenyl)-2-phenylacetamide (3h)

White powder, yield 64.2%, mp 125–126 °C, TLC R_f = 0.54 (petroleum ether/acetic ether 1:1, v/v). ^1H NMR (DMSO-d_6) δ: 10.47 (s, 1H), 8.32 (s, 1H), 7.93 (d, J = 8.40 Hz, 1H), 7.74 (d,

J = 7.80 Hz, 1H), 7.62 (m, 1H), 7.50 (t, J = 7.80 Hz, 2H), 7.25 (t, J = 7.20 Hz, 1H), 7.06 (t, J = 7.20 Hz, 2H), 3.69 (s, 2H), 2.47 (s, 3H). MS (ESI) m/z 254.3 $[M+H]^+$.

3.2.9. *N*-(3-Acetylphenyl)furan-2-carboxamide (3i)

White powder, yield 41.1%, mp 132–134 °C, TLC R_f = 0.43 (petroleum ether/acetic ether 1:1, v/v). ^{1}H NMR (DMSO-d_6) δ: 10.40 (s, 1H), 8.34 (s, 1H), 8.05 (d, J = 7.80 Hz, 1H), 7.97 (s, 1H), 7.71 (d, J = 7.80 Hz, 1H), 7.51 (t, J = 7.80 Hz, 1H), 7.38 (s, 1H), 6.72 (s, 1H), 2.58 (s, 3H). MS (ESI) m/z 230.2 $[M+H]^+$.

3.2.10. 2-(3-Acetylphenylcarbamoyl)phenyl acetate (3j)

White powder, yield 62.8%, mp 82–83 °C, TLC R_f = 0.52 (petroleum ether/acetic ether 1:1, v/v). ^{1}H NMR (DMSO-d_6) δ: 10.55 (s, 1H), 8.31 (s, 1H), 7.97 (m, 2H), 7.75 (d, J = 7.80 Hz, 1H), 7.53 (t, J = 7.80 Hz, 1H), 7.45 (t, J = 7.80 Hz, 1H), 6.98 (m, 2H), 2.59 (s, 3H), 2.18 (s, 3H). MS (ESI) m/z 298.6 $[M+H]^+$.

3.2.11. 2-(3-Acetylphenylcarbamoyl)-4,6-dinitrophenyl acetate (3k)

Yellow power, yield 47.6%, mp 200–201 °C, TLC R_f = 0.43 (petroleum ether/acetic ether 1:1, v/v). ^{1}H NMR (DMSO-d_6) δ: 11.08 (s, 1H), 9.08 (s, 1H), 8.86 (s, 1H), 8.28 (s, 1H), 7.93 (d, J = 8.40 Hz, 1H), 7.79 (d, J = 8.40 Hz, 1H), 7.57 (t, J = 7.80 Hz, 1H), 2.60 (s, 3H), 2.16 (s, 3H). MS (ESI) m/z 388.5 $[M+H]^+$.

3.2.12. 2-(3-Acetylphenylcarbamoyl)-6-methylphenyl acetate (3l)

White powder, yield 51.8%, mp 124–126 °C, TLC R_f = 0.48 (petroleum ether/acetic ether 1:1, v/v). ^{1}H NMR (DMSO-d_6) δ: 10.52 (s, 1H), 8.40 (s, 1H), 8.10 (d, J = 7.80 Hz, 1H), 7.97 (d, J = 7.80 Hz, 1H), 7.88 (d, J = 8.40 Hz, 1H), 7.60 (t, J = 8.10 Hz, 1H), 7.40 (d, J = 7.20 Hz, 1H), 7.08 (t, J = 7.80 Hz, 1H), 2.59 (s, 3H), 2.21 (s, 3H), 2.12 (s, 3H). MS (ESI) m/z 312.5 $[M+H]^+$.

3.2.13. *N*-(3-Acetylphenyl)-2-methoxybenzamide (3m)

White powder, yield 59.7%, mp 72–73 °C, TLC R_f = 0.52 (petroleum ether/acetic ether 1:1, v/v). ^{1}H NMR (DMSO-d_6) δ: 10.32 (s, 1H), 8.36 (s, 1H), 7.97 (d, J = 8.40 Hz, 1H), 7.70 (d, J = 7.80 Hz, 1H), 7.63 (d, J = 7.80 Hz, 1H), 7.51 (m, 2H), 7.19 (d, J = 8.40 Hz, 1H), 7.07 (t, J = 7.20 Hz, 1H), 3.89 (s, 3H), 2.58 (s, 3H). MS (ESI) m/z 270.5 $[M+H]^+$.

3.2.14. *N*-(3-Acetylphenyl)-3-methoxybenzamide (3n)

White powder, yield 63.7%, mp 105–106 °C, TLC R_f = 0.50 (petroleum ether/acetic ether 1:1, v/v). ^{1}H NMR (DMSO-d_6) δ: 10.43 (s, 1H), 8.37 (s, 1H), 8.08 (d, J = 8.40 Hz, 1H), 7.72 (d, J = 7.80 Hz, 1H), 7.57 (d, J = 7.80 Hz, 1H), 7.52 (t, J = 7.80 Hz, 2H), 7.47 (t, J = 7.80 Hz, 1H), 7.18 (d, J = 8.40 Hz, 1H), 3.85 (s, 3H), 2.59 (s, 3H). MS (ESI) m/z 270.5 $[M+H]^+$.

3.2.15. *N*-(3-Acetylphenyl)-4-methoxybenzamide (3o)

White powder, yield 70.4%, mp 167–168 °C, TLC R_f = 0.49 (petroleum ether/acetic ether 3:1, v/v). ^{1}H NMR (DMSO-d_6) δ: 10.29 (s, 1H), 8.36 (s, 1H), 8.08 (d, J = 8.40 Hz, 1H), 7.99 (d, J = 8.40 Hz, 2H), 7.69 (d, J = 7.20 Hz, 1H), 7.50 (t, J = 7.80 Hz, 1H), 7.09 (d, J = 8.40 Hz, 2H), 3.85 (s, 3H), 2.59 (s, 3H). MS (ESI) m/z 270.6 $[M+H]^+$.

3.2.16. *N*-(3-Acetylphenyl)-2-chlorobenzamide (3p)

White powder, yield 67.4%, mp 95–97 °C, TLC R_f = 0.55 (petroleum ether/acetic ether 1:1, v/v). ^{1}H NMR (DMSO-d_6) δ: 10.46 (s, 1H), 8.38 (s, 1H), 8.08 (d, J = 7.80 Hz, 1H), 7.98 (d, J = 7.80 Hz, 2H), 7.72 (d, J = 7.80 Hz, 1H), 7.62 (t, J = 7.20 Hz, 1H), 7.55 (m, 1H), 7.51 (s, J = 7.80 Hz, 1H), 2.59 (s, 3H). MS (ESI) m/z 274.4 $[M+H]^+$.

3.2.17. *N*-(3-Acetylphenyl)-3-chlorobenzamide (3q)

White powder, yield 65.8%, mp 138–139 °C, TLC R_f = 0.53 (petroleum ether/acetic ether 1:1, v/v). δ: 10.55 (s, 1H), 8.36 (s, 1H), 8.08 (d, J = 7.80 Hz, 1H), 8.06 (s, 1H), 7.95 (d, J = 7.80 Hz, 1H), 7.74 (d, J = 7.80 Hz, 1H), 7.69 (d, J = 7.80 Hz, 1H), 7.59 (t, J = 7.80 Hz, 1H), 7.53 (t, J = 7.80 Hz, 1H), 2.59 (s, 3H). MS (ESI) m/z 274.3 $[M+H]^+$.

3.3. General methods for preparing amide-containing diketoacids 4[14]

Cleanly cut sodium (0.69 g, 0.03 mol) was placed in a 100 mL three-necked flask, equipped with an electromagnetic stirrer, dropping funnel, and a reflux condenser bearing a calcium chloride drying tube. Absolute methanol was added dropwise. After all the sodium had dissolved, the excess methanol was removed by reduced pressure distillation. Anhydrous tetrahydrofuran (15 mL) was added when the sodium methoxide was cooled to room temperature. A mixture of compounds **3** (10 mmol) and diethyl oxalate (20 mmol) was added dropwise with stirring, over an ice bath. The reaction proceeded at room temperature with stirring overnight. A solution of hydrochloric acid (1 M, 30 mL) and ice water was added into the reaction solution. The solution was extracted with dichloromethane (10 mL × 3). The extract liquid was washed with saturated sodium chloride solution (10 mL × 3), and was then dried over Na_2SO_4. After filtering, the solvent was evaporated under reduced pressure to obtain an orange-red oil.

The oil was dissolved in a mixed solution of methanol and tetrahydrofuran (1:1, 15 mL). Sodium hydroxide solution (1 M, 20 mL) was added dropwise with stirring. The reaction proceeded at room temperature for 1 h. The reaction solution was washed with diethyl ether (10 mL × 3), and the water phase was acidified with hydrochloric acid solution (1 M) to pH 1–2. The resulting solid was collected, washed by diethyl ether and acetic ether, and dried to obtain compounds **4**.

3.3.1. 2-Hydroxy-4-(3-(4-hydroxybenzamido)phenyl)-4-oxobut-2-enoic acid (4a)

White powder, yield 57.1%, mp 192–193 °C. ^{1}H NMR (DMSO-d_6) δ: 10.24 (s, 1H), 10.17 (s, 1H), 8.46 (s, 1H), 8.15 (d, J = 7.84 Hz, 1H), 7.89 (d, J = 8.53 Hz, 2H), 7.77 (d, J = 7.77 Hz, 1H), 7.54 (t, J = 7.96 Hz, 1H), 7.05 (s, 1H), 6.88 (d, J = 8.53 Hz, 2H). IR (KBr, cm^{-1}): ν_{OH}: 3387.37, 3245.76; $\nu_{C=O}$: 1722.99, 1631.00, 1610.39; $\nu_{C=C}$: 1653.80, 1545.37, 1511.98, 1488.29, 1439.01, 1424.80; ν_{C-O}: 1256.12, 1213.92, 1175.04; $\gamma_{=C-H}$: 847.10, 776.72. MS (ESI) m/z 328.4 $[M+H]^+$; HRMS (ESI) m/z for $C_{17}H_{14}NO_6$ $[M+H]^+$: calcd 328.0816, found 328.0821.

3.3.2. 2-Hydroxy-4-(3-((*E*)-3-(4-hydroxyphenyl)acrylamido)phenyl)-4-oxobut-2-enoic acid (4b)

Yellowish powder, yield 47.6%, mp 186–188 °C. ^{1}H NMR (DMSO-d_6) δ: 14.22 (br, 1H), 10.43 (s, 1H), 9.99 (s, 1H), 8.40 (s, 1H), 8.01 (d, J = 7.80 Hz, 1H), 7.74 (d, J = 7.20 Hz, 1H), 7.53 (m, 2H), 7.48 (d, J = 8.40 Hz, 2H), 7.01 (s, 1H), 6.84 (d, J = 8.40 Hz, 2H), 6.63 (d, J = 15.60 Hz, 1H). IR (KBr, cm^{-1}): ν_{OH}: 3358.42, 3246.83; $\nu_{C=O}$: 1719.43, 1675.42, 1625.15; $\nu_{C=C}$: 1604.67, 1585.84, 1548.95, 1514.72; ν_{C-O}: 1256.54, 1216.93, 1168.95; $\gamma_{=C-H}$: 825.14, 760.76. MS (ESI) m/z 354.4 $[M+H]^+$; HRMS (ESI) m/z for $C_{19}H_{16}NO_6$ $[M+H]^+$: calcd 354.0972, found 354.0975.

3.3.3. 2-Hydroxy-4-(3-((*E*)-3-(4-fluorophenyl)acrylamido)phenyl)-4-oxobut-2-enoic acid (4c)

Yellowish powder, yield 69.4%. mp 189–190 °C. ^{1}H NMR (DMSO-d_6) δ: 14.22 (br, 1H), 10.50 (s, 1H), 8.40 (s, 1H), 8.00 (d, J = 7.80 Hz, 1H), 7.78 (d, J = 7.80 Hz, 1H), 7.64 (d, 1H), 7.57 (m, 2H), 7.55 (t, J = 7.80 Hz, 1H), 7.30 (t, J = 8.40 Hz, 2H), 7.04 (s, 1H),

6.76 (d, J = 15.60 Hz, 1H). IR (KBr, cm^{-1}): ν_{OH}: 3333.41; $\nu_{=CH}$: 3072.27; $\nu_{C=O}$: 1731.58, 1661.62, 1627.36; $\nu_{C=C}$: 1599.63, 1547.87, 1509.46; ν_{C-O}: 1287.74, 1264.71, 1228.28, 1159.64; $\gamma_{=C-H}$: 829.87, 777.74. MS (ESI) m/z 356.5 $[M+H]^+$; HRMS (ESI) m/z for $C_{19}H_{15}NO_5F$ $[M+H]^+$: calcd 356.0929, found 356.0931.

3.3.4. 2-Hydroxy-4-(3-((*E*)-3-(4-chlorophenyl)acrylamido)phenyl)-4-oxobut-2-enoic acid (4d)

White powder, yield 75.2%, mp 188–190 °C. ^1H NMR (DMSO-d_6) δ: 10.51 (s, 1H), 8.38 (s, 1H), 8.00 (d, J = 7.80 Hz, 1H), 7.76 (d, J = 7.80 Hz, 1H), 7.68 (d, J = 8.40 Hz, 2H), 7.63 (d, J = 15.60 Hz, 1H), 7.54 (m, 3H), 6.98 (br, 1H), 6.83 (d, J = 16.20 Hz, 1H). IR (KBr, cm^{-1}): ν_{NH}: 3527.55; ν_{OH}: 3414.76, 3263.68; $\nu_{=CH}$: 3124.02, 3063.42; $\nu_{C=O}$: 1733.01, 1657.61, 1622.60; $\nu_{C=C}$: 1592.78, 1545.00, 1490.30; ν_{C-O}: 1339.05, 1265.86, 1218.09; $\gamma_{=C-H}$: 819.05, 781.47. MS (ESI) m/z 372.4 $[M+H]^+$; HRMS (ESI) m/z for $C_{19}H_{15}NO_5Cl$ $[M+H]^+$: calcd 372.0633, found 372.0631.

3.3.5. 2-Hydroxy-4-(3-((*E*)-3-(4-methoxyphenyl)acrylamido)phenyl)-4-oxobut-2-enoic acid (4e)

White powder, yield 71.6%, mp 154–155 °C. ^1H NMR (DMSO-d_6) δ: 14.26 (br, 1H), 10.46 (s, 1H), 8.39 (s, 1H), 8.01 (d, J = 7.80 Hz, 1H), 7.74 (d, J = 7.20 Hz, 1H), 7.68 (m, 3H), 7.58 (s, 1H), 7.53 (t, J = 7.80 Hz, 1H), 7.02 (d, J = 8.40 Hz, 2H), 6.71 (d, J = 15.60 Hz, 1H), 3.81 (s, 3H). IR (KBr, cm^{-1}): $\nu_{=CH}$: 3005.75; ν_{CH}: 2961.29, 2936.56, 2838.60; $\nu_{C=O}$: 1685.74, 1624.64, 1601.33; $\nu_{C=C}$: 1546.88, 1513.15, 1426.36; ν_{C-O}: 1256.66, 1219.25, 1173.83; $\gamma_{=C-H}$: 827.02, 775.09. MS (ESI) m/z 368.4 $[M+H]^+$; HRMS (ESI) m/z for $C_{20}H_{18}NO_6$ $[M+H]^+$: calcd 368.1129, found 368.1157.

3.3.6. 2-Hydroxy-4-(3-((*E*)-3-(4-hydroxy-3-methoxyphenyl)acrylamido)phenyl)-4-oxobut-2-enoic acid (4f)

Yellow powder, yield 61.7%, mp 185–186 °C. ^1H NMR (DMSO-d_6) δ: 14.19 (br, 1H), 10.39 (s, 1H), 9.59 (s, 1H), 8.42 (s, 1H), 8.00 (d, J = 8.40 Hz, 1H), 7.76 (d, J = 7.80 Hz, 1H), 7.53 (m, 2H), 7.21 (s, 1H), 7.09 (d, J = 7.80 Hz, 1H), 7.05 (s, 1H), 6.84 (d, J = 7.80 Hz, 1H), 6.63 (d, J = 15.00 Hz, 1H), 3.84 (s, 3H). IR (KBr, cm^{-1}): ν_{OH}: 3314.90; $\nu_{C=O}$: 1735.07, 1699.98, 1589.49; $\nu_{C=C}$: 1547.36, 1514.52, 1430.64; ν_{C-O}: 1288.13, 1265.64, 1209.22, 1175.66; $\gamma_{=C-H}$: 842.24, 807.91, 773.84. MS (ESI) m/z 384.6 $[M+H]^+$; HRMS (ESI) m/z for $C_{20}H_{18}NO_7$ $[M+H]^+$: calcd 384.1078, found 384.1082.

3.3.7. 4-(3-Benzamidophenyl)-2-hydroxy-4-oxobut-2-enoic acid (4g)

White powder, yield 62.7%, mp 188–189 °C. ^1H NMR (DMSO-d_6) δ: 14.25 (br, 1H), 10.51 (s, 1H), 8.49 (s, 1H), 8.17 (d, J = 7.80 Hz, 1H), 8.00 (d, J = 7.20 Hz, 2H), 7.81 (d, J = 7.80 Hz, 1H), 7.62 (t, J = 7.50 Hz, 1H), 7.57 (m, 3H), 7.06 (s, 1H). IR (KBr, cm^{-1}): ν_{NH}: 3530.83; ν_{OH}: 3450.33, 3251.78; $\nu_{=CH}$: 3094.66, 3067.41; $\nu_{C=O}$: 1645.72, 1626.87; $\nu_{C=C}$: 1547.81, 1447.65; ν_{C-O}: 1298.08, 1262.03, 1201.15; $\gamma_{=C-H}$: 778.87, 679.31. MS (ESI) m/z 312.4 $[M+H]^+$; HRMS (ESI) m/z for $C_{17}H_{14}NO_5$ $[M+H]^+$ calcd 312.0866, found 312.0872.

3.3.8. 2-Hydroxy-4-oxo-4-(3-(2-phenylacetamido)phenyl)but-2-enoic acid (4h)

Yellowish powder, yield 32.4%, mp 174–176 °C. ^1H NMR (DMSO-d_6) δ: 10.57 (s, 1H), 8.32 (s, 1H), 7.93 (d, J = 8.40 Hz, 1H), 7.74 (d, J = 7.80 Hz, 1H), 7.50 (t, J = 7.80 Hz, 1H), 7.34 (m, 4H), 7.25 (t, J = 6.90 Hz, 1H), 7.00 (s, 1H), 3.69 (s, 2H). IR (KBr, cm^{-1}): ν_{NH}: 3539.03; ν_{OH}: 3248.31; $\nu_{=CH}$: 3142.47, 3087.86, 3063.18, 3030.78; ν_{CH}: 2928.19; $\nu_{C=O}$: 1728.21, 1658.32, 1626.34; $\nu_{C=C}$: 1597.77, 1552.01, 1494.66, 1454.15; ν_{C-O}: 1288.45, 1265.15; $\gamma_{=C-H}$: 777.48, 694.55. MS (ESI) m/z 326.5 $[M+H]^+$; HRMS (ESI) m/z for $C_{18}H_{16}NO_5$ $[M+H]^+$: calcd 326.0817, found 326.0823.

3.3.9. 4-(3-Furan-2-carboxamido)phenyl)-2-hydroxy-4-oxobut-2-enoic acid (4i)

White powder, yield 73.2%, mp 180–181 °C. ^1H NMR (DMSO-d_6) δ: 14.89–14.21 (br, 2H), 10.48 (s, 1H), 8.47 (s, 1H), 8.16 (d, J = 7.80 Hz, 1H), 7.98 (s, 1H), 7.80 (d, J = 7.80 Hz, 1H), 7.56 (t, J = 7.80 Hz, 1H), 7.41 (s, 1H), 7.08 (s, 1H), 6.74 (s, 1H). IR (KBr, cm^{-1}): ν_{OH}: 3353.96, 3118.15; $\nu_{C=O}$: 1742.07, 1652.59, 1614.41; $\nu_{C=C}$: 1587.55, 1548.88, 1473.12; ν_{C-O}: 1308.86, 1264.86, 1228.36; $\gamma_{=C-H}$: 783.47, 758.24. MS (ESI) m/z 302.5 $[M+H]^+$; HRMS (ESI) m/z for $C_{15}H_{12}NO_6$ $[M+H]^+$: calcd 302.0659, found 302.0667.

3.3.10. 2-Hydroxy-4-(3-(2-hydroxybenzamido)phenyl)-4-oxobut-2-enoic acid (4j)

Yellowish powder, yield 43.6%, mp 178–180 °C. ^1H NMR (DMSO-d_6) δ: 11.62 (s, 1H), 10.57 (s, 1H), 8.43 (s, 1H), 8.05 (d, J = 8.02 Hz, 1H), 7.95 (d, J = 7.74 Hz, 1H), 7.84 (d, J = 7.81 Hz, 1H), 7.58 (t, J = 7.98 Hz, 1H), 7.46 (t, J = 7.59 Hz, 1H), 7.07 (s, 1H), 7.00 (d, J = 7.96 Hz, 1H), 6.97 (d, J = 7.51 Hz, 1H). IR (KBr, cm^{-1}): ν_{OH}: 3404.36; $\nu_{=CH}$: 3066.96; $\nu_{C=O}$: 1728.95, 1640.21, 1607.10; $\nu_{C=C}$: 1549.25, 1491.35, 1457.00; ν_{C-O}: 1268.72, 1216.51; $\gamma_{=C-H}$: 775.42, 750.97. MS (ESI) m/z 328.4 $[M+H]^+$; HRMS (ESI) m/z for $C_{17}H_{14}NO_6$ $[M+H]^+$: calcd 328.0816, found 328.0822.

3.3.11. 2-Hydroxy-4-(3-(2-hydroxy-3,5-dinitrobenzamido)phenyl)-4-oxobut-2-enoic acid (4k)

Yellow powder, yield 36.8%, mp 186–187 °C. ^1H NMR (DMSO-d_6) δ: 14.25 (br, 1H), 13.43 (s, 1H), 8.87 (s, 1H), 8.60 (s, 1H), 8.50 (s, 1H), 7.94 (d, J = 7.80 Hz, 1H), 7.79 (d, J = 7.80 Hz, 1H), 7.56 (t, J = 7.80 Hz, 1H), 7.10 (s, 1H). IR (KBr, cm^{-1}): ν_{OH}: 3365.73; $\nu_{=CH}$: 3099.25; $\nu_{C=O}$: 1753.38, 1673.56, 1610.61; $\nu_{C=C}$: 1544.96, 1439.92; ν_{C-O}: 1346.20, 1272.08; $\gamma_{=C-H}$: 787.05, 740.11. MS (ESI) m/z 418.4 $[M+H]^+$; HRMS (ESI) m/z for $C_{17}H_{12}N_3O_{10}$ $[M+H]^+$: calcd 418.0517, found 418.0525.

3.3.12. 2-Hydroxy-4-(3-(2-hydroxy-3-methylbenzamido)phenyl)-4-oxobut-2-enoic acid (4l)

Yellowish powder, yield 59.2%, mp 186–188 °C. ^1H NMR (DMSO-d_6) δ: 14.20 (br, 1H), 12.36 (s, 1H), 10.62 (s, 1H), 8.40 (s, 1H), 8.10 (d, J = 7.80 Hz, 1H), 7.93 (d, J = 7.80 Hz, 1H), 7.88 (d, J = 8.40 Hz, 1H), 7.60 (t, J = 8.10 Hz, 1H), 7.40 (d, J = 7.20 Hz, 1H), 7.08 (s, 1H), 6.90 (m, 1H), 2.21 (s, 3H). IR (KBr, cm^{-1}): ν_{OH}: 3416.18; $\nu_{C=O}$: 1728.82, 1646.95, 1583.69; $\nu_{C=C}$: 1547.67, 1486.27, 1435.38; ν_{C-O}: 1295.25, 1249.97; $\gamma_{=C-H}$: 778.68, 744.30. MS (ESI) m/z 342.3 $[M+H]^+$; HRMS (ESI) m/z for $C_{18}H_{16}NO_6$ $[M+H]^+$: calcd 342.0972, found 342.0981.

3.3.13. 2-Hydroxy-4-(3-(2-methoxybenzamido)phenyl)-4-oxobut-2-enoic acid (4m)

White powder, yield 60.6%, mp 173–175 °C. ^1H NMR (DMSO-d_6) δ: 15.00 (br, 1H), 14.19 (br, 1H), 10.36 (s, 1H), 8.48 (s, 1H), 8.04 (d, J = 8.40 Hz, 1H), 7.79 (d, J = 7.80 Hz, 1H), 7.63 (d, J = 7.50 Hz, 1H), 7.54 (m, 2H), 7.19 (d, J = 8.40 Hz, 1H), 7.07 (m, 2H), 3.89 (s, 3H). IR (KBr, cm^{-1}): ν_{OH}: 3371.43; $\nu_{C=O}$: 1728.22, 1626.89, 1600.26; $\nu_{C=C}$: 1556.09, 1488.73, 1466.82, 1439.13; ν_{C-O}: 1294.23, 1272.81, 1246.87; $\gamma_{=C-H}$: 780.17, 753.28. MS (ESI) m/z 342.3 $[M+H]^+$; HRMS (ESI) m/z for $C_{18}H_{16}NO_6$ $[M+H]^+$: calcd: 342.0972, found 342.0979.

3.3.14. 2-Hydroxy-4-(3-(3-methoxybenzamido)phenyl)-4-oxobut-2-enoic acid (4n)

White powder, yield 69.1%, mp 114–115 °C. ^1H NMR (DMSO-d_6) δ: 15.05 (br, 1H), 14.22 (br, 1H), 10.47 (s, 1H), 8.48 (s, 1H), 8.16 (d, J = 8.40 Hz, 1H), 7.81 (d, J = 7.80 Hz, 1H), 7.57 (t, J = 8.10 Hz, 2H), 7.52 (s, 1H), 7.47 (m, 1H), 7.19 (d, J = 8.40 Hz, 1H), 7.06 (s, 1H), 3.85 (s, 3H). IR (KBr, cm^{-1}): ν_{OH}: 3439.39; $\nu_{C=O}$: 1729.32, 1649.30, 1626.58; $\nu_{C=C}$: 1589.66, 1544.10, 1488.08; ν_{C-O}:

1292.21, 1265.58, 1200.56; $\gamma_{=C-H}$: 778.59, 747.62, 682.41. MS (ESI) m/z 342.3 $[M+H]^+$; HRMS (ESI) m/z for $C_{18}H_{16}NO_6$ $[M+H]^+$: calcd 342.0972, found 342.0978.

3.3.15. 2-Hydroxy-4-(3-(4-methoxybenzamido)phenyl)-4-oxobut-2-enoic acid (4o)

White powder, yield 52.8%, mp 110–112 °C. ^1H NMR (DMSO-d_6) δ: 15.04 (br, 1H), 14.21 (br, 1H), 10.35 (s, 1H), 8.47 (s, 1H), 8.16 (d, J = 7.80 Hz, 1H), 7.99 (d, J = 9.00 Hz, 2H), 7.78 (d, J = 7.80 Hz, 1H), 7.55 (t, J = 7.80 Hz, 1H), 7.08 (d, J = 8.40 Hz, 2H), 7.06 (d, J = 7.20 Hz, 1H), 3.85 (s, 3H). IR (KBr, cm^{-1}): ν_{OH}: 3530.43, 3269.09; $\nu_{C=O}$: 1686.66, 1643.08, 1604.57; $\nu_{C=C}$: 1578.62, 1510.58, 1427.91; ν_{C-O}: 1304.64, 1261.46, 1171.08; $\gamma_{=C-H}$: 844.37, 774.75. MS (ESI) m/z 342.3 $[M+H]^+$; HRMS (ESI) m/z for $C_{18}H_{16}NO_6$ $[M+H]^+$: calcd 342.0972, found 342.0981.

3.3.16. 2-Hydroxy-4-(3-(2-chlorobenzamido)phenyl)-4-oxobut-2-enoic acid (4p)

White powder, yield 63.4%, mp 138–140 °C. ^1H NMR (DMSO-d_6) δ: 14.20 (br, 1H), 10.77 (s, 1H), 8.44 (s, 1H), 8.02 (d, J = 7.80 Hz, 1H), 7.82 (d, J = 7.80 Hz, 1H), 7.64 (d, J = 7.50 Hz, 1H), 7.56 (m, 3H), 7.48 (t, J = 7.50 Hz, 1H), 7.03 (s, 1H). IR (KBr, cm^{-1}): ν_{OH}: 3447.02, 3236.54; $\nu_{C=O}$: 1715.43, 1659.78; $\nu_{C=C}$: 1592.20, 1547.44, 1487.42, 1434.60; ν_{C-O}: 1305.19, 1265.20, 1206.34; $\gamma_{=C-H}$: 777.12, 750.22. MS (ESI) m/z 346.4 $[M+H]^+$; HRMS (ESI) m/z for $C_{17}H_{13}NO_5Cl$ $[M+H]^+$: calcd 346.0477, found 346.0475.

3.3.17. 2-Hydroxy-4-(3-(3-chlorobenzamido)phenyl)-4-oxobut-2-enoic acid (4q)

White powder, yield 55.8%, mp 187–188 °C.^1H NMR (DMSO-d_6) δ: 14.92 (br, 1H), 14.22 (br, 1H), 10.60 (s, 1H), 8.47 (s, 1H), 8.16 (d, J = 7.80 Hz, 1H), 8.06 (s, 1H), 7.96 (d, J = 7.80 Hz, 1H), 7.83 (d, J = 7.80 Hz, 1H), 7.70 (d, J = 7.80 Hz, 1H), 7.60 (m, 2H), 7.07 (s, 1H). IR (KBr, cm^{-1}): ν_{NH}: 3531.51; ν_{OH}: 3454.57, 3270.01; $\nu_{=CH}$: 3066.85; $\nu_{C=O}$: 1729.75, 1646.98, 1626.48; $\nu_{C=C}$: 1546.21, 1488.41, 1449.44; ν_{C-O}: 1293.95, 1260.36, 1201.54; $\gamma_{=C-H}$: 778.15, 698.12. MS (ESI) m/z 346.4 $[M+H]^+$; HRMS (ESI) m/z for $C_{17}H_{13}NO_5Cl$ $[M+H]^+$: calcd 346.0477, found 346.0484.

3.4. Biological activity methods

3.4.1. Materials, chemicals, and enzymes

All compounds were dissolved in DMSO, and the stock solutions were stored at −20 °C. The $\gamma[^{32}P]$ATP was purchased from MP Biomedical. The expression systems for the wild-type IN and soluble mutant $IN^{F185KC280S}$ were generous gifts of Dr. Robert Craigie, Laboratory of Molecular Biology, NIDDK, NIH, Bethesda, MD.

3.4.2. Preparation of oligonucleotide substrates

The oligonucleotides 21top, 5′-GTGTGGAAAATCTCTAGCAGT-3′ and 21bot, 5′-ACT-GCTAGAGATTTTCCACAC-3′ were purchased from the Norris Cancer Center Core Facility (University of Southern California), and purified by UV shadowing on a polyacrylamide gel. To analyze the extent of 3′-processing and strand transfer using 5′-end-labeled substrates, 21top was 5′-endlabeled using T_4 polynucleotide kinase (Epicenter, Madison, WI) and $\gamma[^{32}P]$ATP (MP Biomedical). The kinase was heat-inactivated, and 21bot was added in 1.5-M excess. The mixture was heated to 95 °C, allowed to cool slowly to room temperature, and run through a spin 25 mini-column (USA Scientific) to separate the annealed double-stranded oligonucleotide from unincorporated material.

3.4.3. Integrase assays

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 the 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% dimethyl sulfoxide, and 25 mM MOPS, pH 7.2) at 30 °C for 30 min. Then, 20 nM of the 5′-end ^{32}P-labeled linear oligonucleotide substrate was added, and incubation was continued for an additional 1 h. Reactions were quenched by the addition of an equal volume (16 μL) of loading dye (98% deionized formamide, 10 mM EDTA, 0.025% xylene cyanol and 0.025% bromophenol blue). 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 the concentration that produced 50% inhibition.

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