Design, Synthesis, and Biological Evaluation of *Plasmodium falciparum* Lactate Dehydrogenase Inhibitors

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Received March 22, 2007

Plasmodium falciparum lactate dehydrogenase (pfLDH) is a key enzyme for energy generation of malarial parasites and is a potential antimalarial chemotherapeutic target. It is known that the oxamate moiety, a pyruvate analog, alone shows higher inhibition against pfLDH than human LDHs, suggesting that it can be used for the development of selective inhibitors. Oxamic acid derivatives were designed and synthesized. Derivatives **5** and **7** demonstrated activities against pfLDH with IC_{50} values of 3.13 and 1.75 μM, respectively, and have 59- and 7-fold selectivity over mammalian LDH, respectively. They also have micromolar range activities against *Plasmodium falciparum* malate dehydrogenase (pfMDH), which may fill the role of pfLDH when the activity of pfLDH is reduced. Thus, certain members of these oxamic acid derivatives may have dual inhibitory activities against both pfLDH and pfMDH. It is presumed that dual LDH/MDH inhibitors would have enhanced potential as antimalarial drugs.

Introduction

Malaria is a re-emerging disease in many tropical areas of the world and is often fatal due to drug resistance, leading to about a million deaths each year.[1,2] Multiple drug resistance has necessitated new efforts in drug discovery and development. Thus, the search for new drugs, which operate by novel mechanisms of action, has received increased attention. Of novel plasmodial targets, lactate dehydrogenase from *Plasmodium falciparum* (pfLDH[a]) is a potential target for the development of antimalarial drugs. Malarial parasites depend not only on anaerobic glycolysis in the cytoplasm for energy production, but also consume 30−50 times more glucose than their host cells because they lack a citric acid cycle for adenosine triphosphate (ATP) production.[3] Thus, targeting enzymes involved in glycolysis would impede ATP production and cause mortality of the parasite. Not only is pfLDH the most abundant enzyme, but it is essential for L-lactate production, a major product in glucose metabolism.[4,5] *P. falciparum* LDH reduces pyruvate to lactate using NADH, has unique residues, and has kinetic differences from the human LDH isoforms (hLDH), suggesting that pfLDH is a unique antimalarial target.

Except for the catalytic residues (Arg171, Arg109, and the dyad His195/Asp168), pfLDH has different active site and substrate specificity loop (five residue insertion, DKEWN) residues than the hLDH isoforms, which reflect the displacement of the nicotinamide ring and an increase in the volume of the active site in pfLDH.[6,7] In addition, pfLDH utilizes 3-acetylpyridine adenine dinucleotide (APAD) as a cofactor more efficiently than the hLDH isoform, suggesting a faster conformational change of the loop region and a faster release of pyruvate when NADH is replaced by APAD.[8,9]

Inhibitors have been reported to bind in three different modes in the active site of pfLDH.[10] Inhibitors either bind to the pyruvate site, the bridging site of the cofactor and pyruvate, or the adenine binding site. The natural product gossypol is a selective inhibitor of pfLDH over hLDHs, and it is presumed that gossypol derivatives bind to the bridging site for the nicotinamide of the cofactor and the pyruvate in the active site of pfLDH.[10,11] Gossypol derivatives based on the bicyclic naphthalene core, such as hemigossypols, have been reported with low micromolar inhibition of pfLDH and hLDHs without significantly improving selectivity and activity.[8,11−14] However, *p*-(trifluoromethyl)benzyl-8-deoxyhemigossypolic acid has shown selectivity over human heart and muscle LDHs (Figure 1).[9] Oxamic acid is a competitive inhibitor of pfLDH with the pyruvate substrate. Some oxamic acid derivatives have been reported but failed to show improved inhibitory activity against pfLDH and hLDHs.[15,16] Recently, azole-based inhibitors were reported to show selective inhibition of pfLDH over hLDHs at the low or submicromolar level (Figure 1).[17] These compounds bind to and show similar interactions as the pyruvate substrate, with the residues in the active site of pfLDH.

Malate dehydrogenase (MDH) reversibly converts malate to oxaloacetate using NAD(P) during asexual reproduction and growth.[18] LDHs are cytosolic enzymes found in bacteria and eukaryotes, whereas MDHs are found in cytosol, mitochondria, chloroplasts, peroxysomes, and glyoxysomes. Apicomplexan LDH is more similar to LDH-like MDH than other LDHs since Apicomplexan evolved from an ancestral LDH-like MDH.[19] It has been suggested that both pfLDH and *Plasmodium falciparum* malate dehydrogenase (pfMDH) are found in the cytosol of the parasite, and pfMDH may complement pfLDH function to play an important role in glucose consumption when pfLDH is specifically inhibited.[20] This indicates that dual inhibition of both pfLDH and pfMDH may result in death of the parasite by halting ATP production. Despite the structural similarity between pfMDH and pfLDH, they show different binding modes for the substrate. Oxamic acid shows higher inhibition of pfLDH but

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[a] Abbreviations: PfLDH, *Plasmodium falciparum* lactate dehydrogenase; LDH, lactate dehydrogenase; pfMDH, *Plasmodium falciparum* malate dehydrogenase; hLDH, human lactate dehydrogenase; CVFF, consistent valence force field; rmsd, root-mean-square deviation; mLDH, mammalian LDH; MtMDH, mitochondrial MDH; cMDH, cytosolic MDH.

10.1021/jm070336k CCC: $37.00
Published on Web 07/18/2007

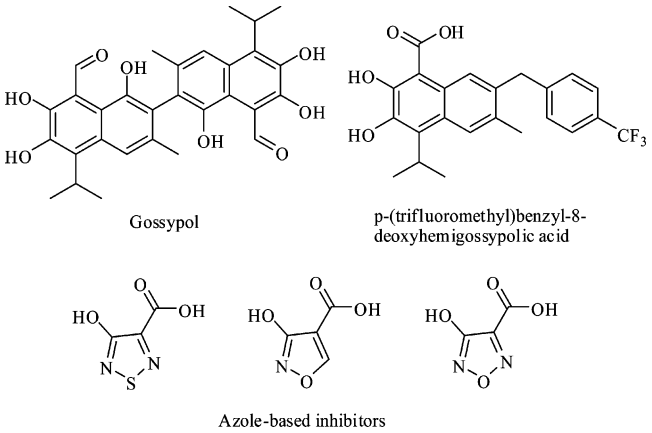


Figure 1. The structures of gossypol, hemigossypolic acid, and azoles.

does not inhibit pfMDH and porcine heart MDHs.[20] However, inhibition of both enzymes by gossypol unveils the similarity of the nucleotide binding region of the cofactor NADH. In addition, the ability to use APAD as a cofactor in pfMDH indicates that both enzymes have similar nucleotide binding pockets. Thus, it may be possible to develop new antimalarial drugs that inhibit both pfLDH and pfMDH as dual inhibitors by designing oxamic acid derivatives binding to both the pyruvate and NADH binding sites because of the presence of the typical nucleotide binding sequence, GXGXXG. We report here the design and synthesis of pfLDH inhibitors that target part of or the entire area of the active site of pfLDH. In addition to pfLDH inhibition, these inhibitors have shown inhibitory activities of MDHs.

Molecular Modeling

In receptor-based design, Van der Waals energy is considered a major factor in evaluating drug designs, indicating that the molecular and explicit shapes of the active site are major factors for design strategies. In an effort to design inhibitors that bind to the entire active site of pfLDH, we emulated the shape of the bioactive conformation of the NADH cofactor, which is covalently connected to an oxamate moiety (Figure 2A). In search of more rigid and hydrophobic groups to replace the diphosphate group of NADH, a chromene group was used in our design. A chromene group should show a similar binding mode to that of the phosphate group of the NADH. For example, in the crystal structure, the oxygen atoms of the phosphate group interact with hydrogen atoms of the nitrogen in Met30 and Ile31. Additional factors considered were synthetic viability and the number of steps for synthesis. A chromene group can easily be synthesized in two steps from commercially available 2,4,6-trihydroxyacetophenone. The hydrophobic group (chromene) has hydrogen bonding acceptors that may interact with Ile31 and Met30 at the active site of pfLDH. As seen in Figure 2A, the oxamic acid group and the nicotinamide ring of the cofactor are replaced with *N*-aryl oxamic acid groups, which are connected to the chromene moiety with an aryl alkyl linker.

For the molecular docking study, the crystal structure of pfLDH complexed with oxamate, and NADH was obtained from the PDB data bank (code 1LDG) and refined. The protocols for the refinement of the crystal structure and the docking study are described in detail in the Experimental Section. After minimization of the X-ray structure with the steepest descent and the conjugate gradient algorithm and the consistent valence force field (CVFF) potentials, a molecular dynamic simulation was performed followed by steepest descents and conjugate gradient minimization with a tethering force. A rmsd of 0.599 was obtained from the alignment of the refined structure with the crystal structure based on C-α atoms. The refined structure was validated by docking the cofactor NADH and the substrate oxamate into the active site.

To find lead structures to synthesize and test, a virtual library was built based on this novel design strategy. The library was docked into the refined protein with the 7−8 times speed-up method, followed by the standard default settings. The final selection was made based on docking scores, binding mode, and synthetic viability. The docking results showed residue interactions in the active site of pfLDH, demonstrating that the oxamate moiety hydrogen bonds with Arg 171, Arg 109, Asn 140, and His 195 and the hydrogens of the nitrogen atoms from Ile 30, Met31, Gly99 interact with the oxygen atoms of the chromene (Figure 2B).

Chemistry

The designed oxamic acid derivatives were selected based on our docking study and synthesized to explore the effect of substitution *ortho* to the *N*-acylphenyl ring and to evaluate the impact of the chromene moiety. First, two chromene groups were synthesized as outlined in Scheme 1. The acid

9 was synthesized from 2,4,6-trihydroxyacetophenone **8**, utilizing a known procedure.[21] Coupling of 4-methoxybenzyl amine with the carboxylic acid **9** using hydroxybenzotriazole (HOBt) and dicyclohexyl carbodiimde (DCC) produced amide **10** in good yield. Trivial esterification of the acid **9** with ethanol in the presence of sulfuric acid afforded ethyl ester **11**.

Oxamic acid **1** was synthesized to investigate the effect of a one-carbon linker between the chromene group and the aryl oxamate group (Scheme 2). Oxamic acid **1** was prepared from the commercially available acid **12**. *N*-Acylation with ethyl chlorooxoacetate was performed in the presence of pyridine to give acid **13**. Selective reduction of the aryl acid **13** was accomplished using BH_3−THF complex and trimethylborate to furnish the corresponding alcohol **14**. The Mitsunobu coupling of the alcohol **14** with chromene **11** using DIAD and triphenylphosphine gave ethyl ester **15**. Finally, the ester was hydrolyzed with LiOH in THF/H_2O (2/1) to give the desired product **1**.

For coupling with the chromene group(s), ethyl oxamates **20a**, **20b**, **and 20c** were synthesized from the phenethanols **16**, **17**, and the nitrile **21**, respectively, as shown in Schemes 3 and 4. Compound **17** was prepared from commercially available 4-aminophenethyl alcohol **16** in two steps. Bromination of **16** with NBS followed by conversion of the bromide using microwave conditions in the presence of nickel cyanide led to cyano compound **17**. Following protection of the hydroxyl groups of **16** and **17** with TBS-Cl, chemoselective *N*-acylation with ethyl chlorooxoacetate afforded TBS-protected **19a** and **19b** in good yields. Desilylation with TBAF gave low yields, possibly due to a competing reaction with the ethyl oxamate group. However, rapid deprotection with HF/pyridine furnished ethyl oxamates **20a** and **20b** in a reasonably good yield (Scheme 3).

As outlined in Scheme 4, ethyl oxamate **20c** was prepared by introducing a methoxy group *ortho* to the *N*-acyl moiety. Nitration of nitrile **21** with tetrabutylammonium nitrate furnished 4- or 6-nitro-substituted compounds as minor products and 2-nitro-substituted **22** as the major product.[22] Hydrolysis of **22** to the corresponding acid **23** was carried out in the presence of HCl. The acid **23** was reduced with BH_3−THF to produce alcohol **24** in good yield. After protection of the primary alcohol with 3,4-dihydro-2*H*-pyran in the presence of pyridinium

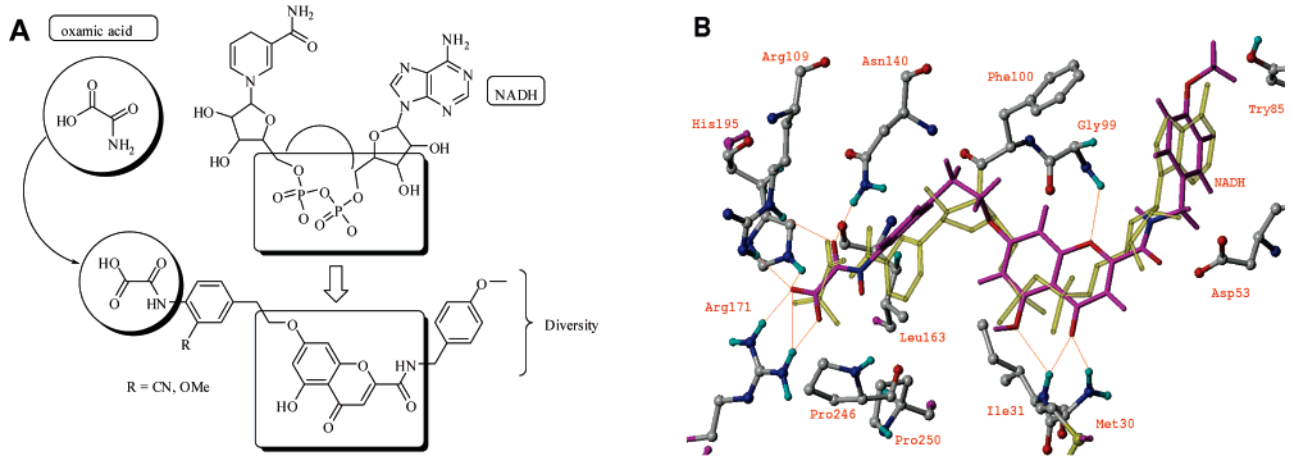
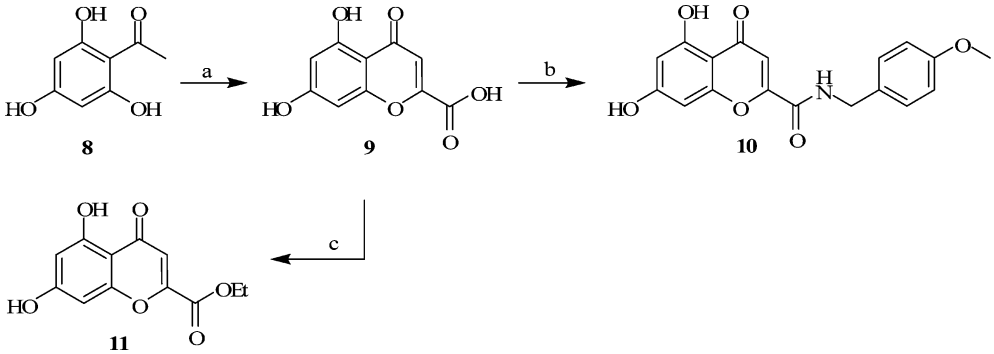


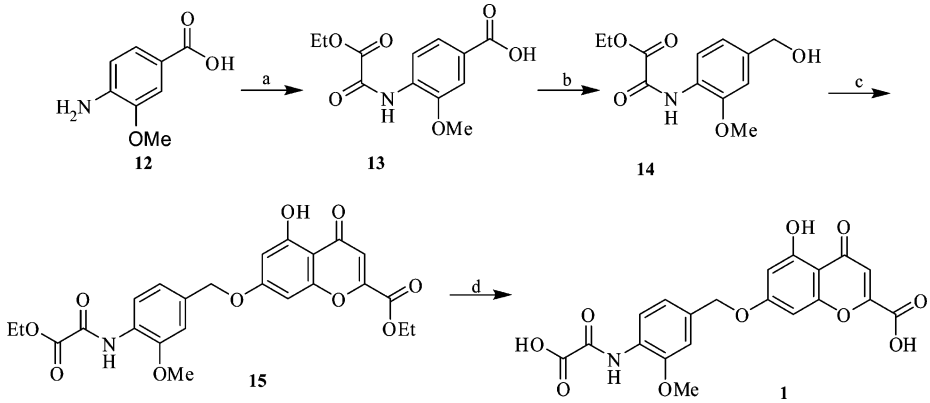
Figure 2. (A) Design of pfLDH inhibitors. (B) Molecular docking of the library. One of the designed molecules shows residue interactions in the active site of pfLDH, demonstrating that the oxamate moiety hydrogen bonds with Arg 171, Arg 109, Asn 140, and His 195 and the hydrogens of the nitrogen atoms from Ile 30, Met31, and Gly99 interact with the oxygen atoms of the chromene (purple). NADH structure from the crystal structure is overlaid in the active site (yellow).

Scheme 1. Synthesis of **10** and **11**[a]



[a] Reagents and conditions: (a) (i) ethyl chlorooxoacetate, pyridine, rt, (ii) 5% aq Na_2CO_3, EtOH, 60−70 °C, 42% (two steps); (b) HOBt, DCC, 4-methoxybenzylamine, CH_2Cl_2/DMF, rt, 1 h, 88%, (c) EtOH, H_2SO_4, reflux, 72%.

Scheme 2. Synthesis of Inhibitor **1**[a]



[a] Reagents and conditions: (a) pyridine, ethyl chlorooxoacetate, CH_2Cl_2, 0 °C to rt, 88%; (b) BH_3−THF, $B(OMe)_3$, THF, rt, 6 h, 69%; (c) DIAD, PPh_3, **11**, CH_2Cl_2, rt, 57%; (d) 1 N LiOH, THF/H_2O, rt, 2 h, 78%.

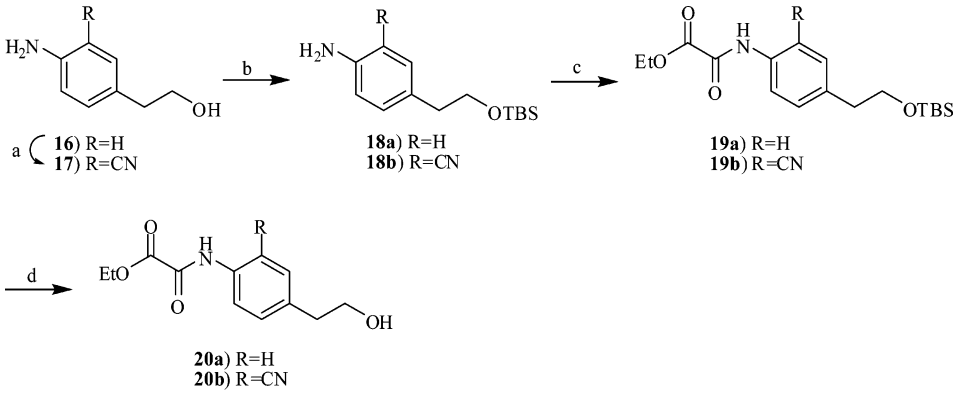
p-toluenesulfonate (PPTS), compound **25** was reduced with H_2 in the presence of Pd/C to afford the aniline **26**. Finally, *N*-acylation with ethyl chlorooxoacetate followed by deprotection of THP led to the ethyl oxamate **20c** fragment.

Oxamic acid derivatives **2**−**7** with a chromene moiety were prepared as outlined in Scheme 5. The ethyl esters **28a**−**c** and **29a**−**c** were synthesized using Mitsunobu conditions, and hydrolysis of the ethyl esters with 1 N LiOH in THF/H_2O led to oxamic acids **2**−**7** in good yields.

Results and Discussions

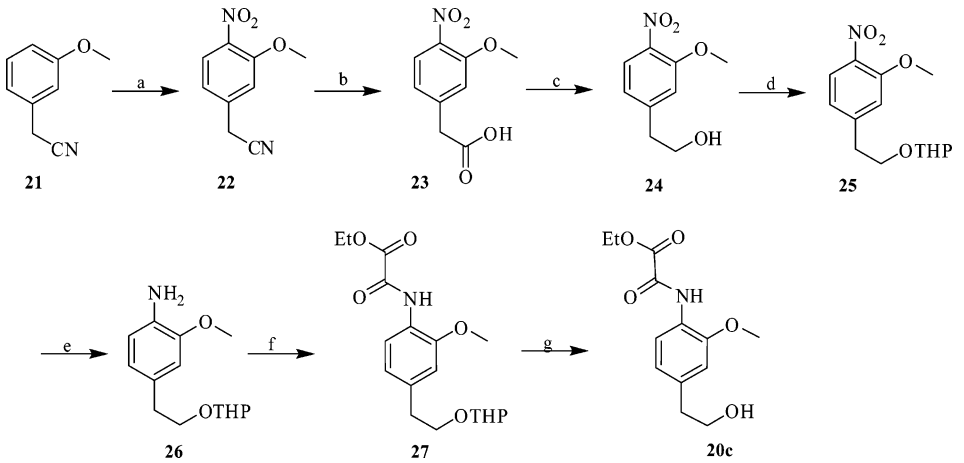
Because glucose consumption is essential for the survival of the parasite, enzymes involved in glycolysis are considered as a potential target. Inhibition of both pfMDH and pfLDH by gossypol in the low micromolar level supports the possibility of developing pfLDH/pfMDH dual inhibitors.[20] Our goal was to design and synthesize selective pfLDH inhibitors binding to the active site by introducing an oxamate group and emulating

Scheme 3. Synthesis of **20a** and **20b**[a]



[a] Reagents and conditions: (a) (i) NBS, DMF, rt, 1.5 h, 95%; (ii) $Ni(CN)_2$, microwave irradiation, NMP, 200 °C, 10 min, 62%; (b) TBS-Cl, imidazole, DMF, rt, 4 h, 88−96%; (c) ethyl chlorooxoacetate, DIPEA, CH_2Cl_2, rt, 55−70%; (d) 70% HF/pyridine, CH_2Cl_2, 0 °C, 1 h, 86−70%.

Scheme 4. Synthesis of **20c**[a]



[a] Reagents and conditions: (a) tetra-*n*-butyl ammonium nitrate, $(CF_3CO)_2O$, 18-crown-6, Ch_2Cl_2, 0 °C to rt, 1.5 h, 32%; (b) 6 N HCl, reflux, 3.5 h, 56%; (c) BH_3−THF, THF, 0 °C to rt, 1.5 h, 94%; (d) 3,4-dihydro-2*H*-pyran, PPPTS, CH_2Cl_2, rt, 50 min, 99%; (e) Pd/C, H_2, MeOH, rt, 76%; (f) ethyl chlorooxoacetate, DIPEA, CH_2Cl_2, rt, 99%; (g) *p*-TsOH, MeOH, 55 °C, 1 h, 76%.

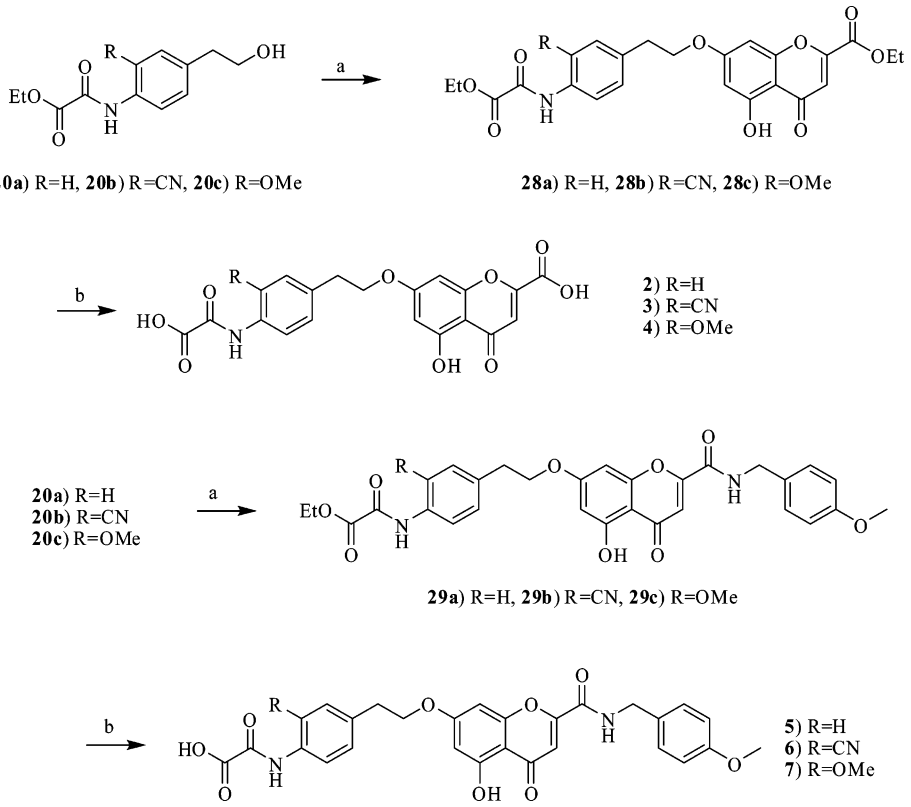
the bioactive conformation of NADH. In addition to inhibiting pfLDH, we also considered the inhibition of pfMDH. The inhibition of pfLDH, mLDH, pfMDH, MtMDH (mitochondrial MDH), and cMDH (cytosolic MDH) by oxamic acid derivatives is shown in Table 1.

Similar to the oxamate substrate, the present active oxamic acids show selective inhibition against pfLDH over mLDH. The acid **7** was the most active derivative for all LDHs and MDHs, also selectively inhibiting pfLDH (IC_{50} = 1.75 μM) by 7-fold over mLDH (IC_{50} = 11.4 μM). The acids **5** and **6**, structurally similar to **7**, showed good selective inhibitory activities against pfLDH over mLDH by almost 59- and 22-fold, respectively. These three derivatives were as effective as the natural product gossypol, but showed selectivity over mLDH, while structurally similar derivatives **1**−**4** did not show any activity against pfLDH and mLDH.

Of the oxamic acids, **7** was the most active inhibitor against malate dehydrogenases (pfMDH, MtMDH, and cMDH), with IC_{50} values of 2.66, 0.39, and 6.31 μM, respectively. The oxamic acids **5** and **7** ubiquitously inhibited all MDHs, but **6** showed good inhibition against MDHs but was not as effective as **5** and **7** against pfMDH. Interestingly, **3** only inhibited MtMDH and cMDH, with IC_{50} values of 17.1 and 13.6 μM, respectively, showing selectivity over other dehydrogenases. Despite structural similarity between pfMDH and pfLDH, activity against MDHs by two acids, **3** and **6**, indicates that the active site of pfMDH is structurally and biochemically different from other MDHs, possibly due to utilization of APAD(H) as an alternate cofactor and strict (oxaloacetate) instead of broad substrate specificity.[18,23] On the other hand, inhibition of enzymes by gossypol demonstrates the similarity of the cofactor binding site surrounded by the typical nucleotide binding sequences, GXGXXG. The acids **5** and **7** inhibited pfLDH and all MDHs as effective as gossypol but showed better selectivity over mLDH. During the catalytic cycle of pfLDH, the LDH−NADH binary complex forms first, which leads to the formation of the LDH−NADH−pyruvate ternary complex. Because inhibitors **5**−**7** were designed to bind both the substrate and the cofactor binding sites, these three oxamic acids are likely to be competitive with the cofactor NADH, acting like gossypol by binding to MDHs as well as pfLDH. However, other acids **1**−**4** may compete with the substrate for the binding site of MDHs due to the difference in the substrate binding site. Thus, the current inhibitors show the possibility to develop new antimalarial drugs that inhibit both pfLDH and pfMDH as dual inhibitors by designing oxamic acid derivatives binding to both the pyruvate and the NADH binding pockets.

Molecular docking of **5** and **7** (green) into the active site of pfLDH are shown in Figure 3. These acids fill the entire binding pocket of the cofactor NADH (orange) and the substrate oxamate in pfLDH, showing key hydrogen bonds with conserved amino acid residues and the residues lining the nucleotide binding pocket. Oxamic acids **5**−**7** display almost the same binding mode in the active site: hydrogen bonding of the oxamate

Scheme 5. Synthesis of Inhibitors **2**−**7**[a]



[a] Reagents and conditions: (a) DIAD, PPh_3, CH_2Cl_2, **10** or **11**, rt, 38−67%; (b) 1 N LiOH, THF/H_2O (2/1), rt, 46−77%.

Table 1. IC_{50} Values of Oxamate Derivatives against pfLDH,[a] mLDH,[b] pfMDH,[c] MtMDH,[d] and cMDH[e,]

	enzyme (μM)[f]				
inhibitor	pfLDH	mLDH	pfMDH	MtMDH	cMDH
oxamate	92.5 ± 10.6	123 ± 20.6	>2000	>2000	>2000
gossypol	2.64 ± 1.00	9.54 ± 2.87	2.03 ± 0.80	2.80 ± 1.40	5.80 ± 2.04
1	87.3 ± 1.90	>232	48.3 ± 0.30	64.0 ± 0.13	>232
2	>241	>241	>241	96.7 ± 1.95	NA
3	NA[g]	NA	NA	17.1 ± 2.19	13.6 ± 2.80
4	191 ± 1.37	>225	>225	>225	NA
5	3.13 ± 0.18	>187	6.76 ± 0.39	2.72 ± 0.13	4.88 ± 0.07
6	8.25 ± 0.50	>179	86.9 ± 0.46	5.91 ± 0.12	10.9 ± 2.67
7	1.75 ± 0.30	11.4 ± 2.89	2.66 ± 0.24	0.39 ± 0.01	6.31 ± 2.64

[a] pfLDH: *Plasmodium falciparum* lactate dehydrogenase. [b] mLDH: mammalian lactate dehydrogenase. [c] pfMDH: *Plasmodium falciparum* malate dehydrogenase. [d] MtMDH: mammalian mitochondria malate dehydrogenase. [e] cMDH: mammalian cytosolic malate dehydrogenase. [f] Mean ± SE. [g] NA=not active.

moiety with Arg171, Arg109, and Asn140 and hydrogen bonding of the chromene moiety with Gly29, Ile31, Met30, and Gly99.

It is noted that substitution *ortho* to the *N*-oxamic group influences the inhibition. A methoxy group (inhibitor **7**) at this position increases inhibitory activity against pfLDH by 5-fold compared to its absence (inhibitor **5**), whereas a cyano group slightly decreases activity (inhibitor **6**). These results agree with the docking scores in which **6** has the lowest score among these three compounds. In addition, these substituents (methoxy and cyano) are located near where the amide group of the nicotinamide ring of the cofactor is positioned as shown in Figure 3. The most important residue near the amide group of the nicotinamide ring is a leucine in pfLDH. Leu163 is replaced by serine in hLDH, which is considered to play a critical role in the mechanism of substrate inhibition in pfLDH and hLDH. The hydroxyl group of Ser163 in hLDH forms a hydrogen bond with the amide of nicotinamide through a water molecule, while the amide oxygen of Leu163 participates in hydrogen bonding with the amide of the nicotinamide due to steric hindrance. This suggests that introduction of a hydrogen bonding donor on the phenyl group of the acids **5**−**7** would improve the activity and selectivity when designing future pfLDH inhibitors.

Compounds **1**−**7** were also tested against the malarial parasite in vitro. However, these compounds showed no activity against the D2 and W2 clones in the whole cell assays. The inconsistency between the protein binding and the lack of in vitro activity may be attributed to solubility issues and an inability of these compounds to permeate the cell membrane.

In search of dual inhibitors, we synthesized and tested novel oxamate compounds against pfLDH, mLDH, pfMDH, MtMDH, and cMDH. As recently reported,[20] it is known that pfMDH is structurally similar to pfLDH and compliments pfLDH when pfLDH is inhibited, suggesting dual inhibitors that block both pfLDH and pfMDH would be potent antimalarials. It is also important to consider selectivity due to the existence of human LDHs with structural similarity. However, pfLDH has unique structural and kinetic properties when compared to the human LDH isoforms, which makes pfLDH a potential target for antimalarial drugs. Our study shows that pfLDH inhibitors may

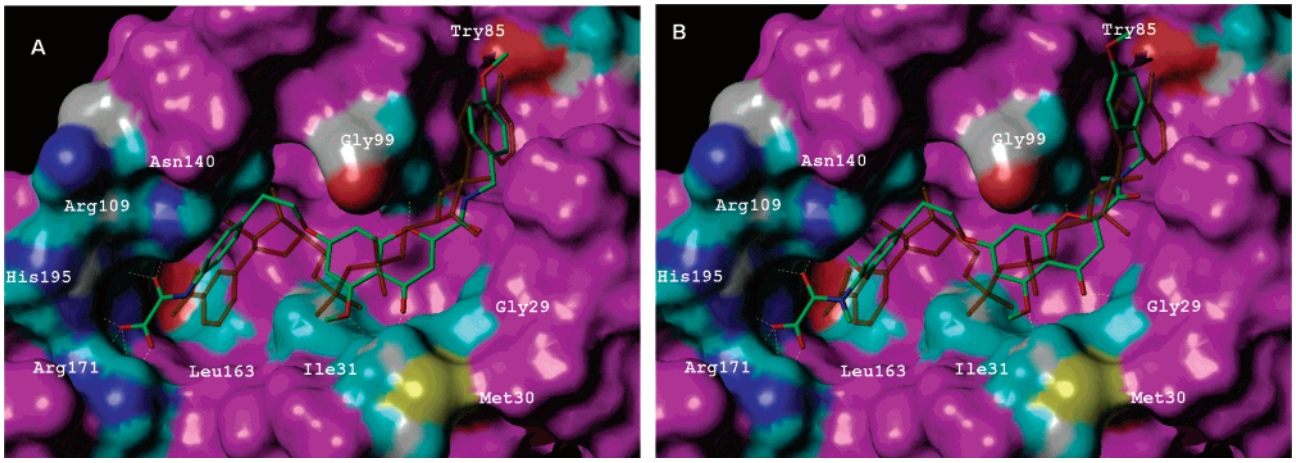


Figure 3. Molecular docking of **5** (A, green) and **7** (B, green) into the active site of pfLDH. The cofactor NADH (orange) is overlaid in the active site.

act in the same way as MDH inhibitors like gossypol. Specifically, the acids **5** and **7** are pfLDH inhibitors that ubiquitously show inhibitory activity against LDHs and MDHs. These two compounds can be used as lead structures for developing dual inhibitors for pfLDH and pfMDH because both enzymes may be important for the energy production of the malaria parasite. Thus, we have shown that the design and development of dual inhibitors for pfLDH and pfMDH may be achieved.

Experimental Section

Molecular Modeling: A Silicon Graphics Octane 2 workstation, equipped with two parallel R12000 processors, was used for all computational studies. InsightII (Accelrys), Sybyl (Tripos, Inc.), and GOLD (CCDC) were used for modeling. InsightII was used to refine the crystal structure of pfLDH complexed with oxamate and NADH, which was obtained from the PDB data bank (code 1LDG). After separation of the crystal structure from oxamate and the cofactor NADH, hydrogen atoms, water, the substrate, and the cofactor at pH 7 were added to the enzyme. Potentials were assigned to the ligands (oxamate and NADH), the enzyme, and water using the force field CVFF. Minimization using Discover_3 was performed to orient the hydrogen atoms on the water molecules in the presence of the enzyme and the ligands. Following addition of a 15 Å layer of water molecules around the protein, the outer 5 Å shell of the water layer was held fixed during minimization to prevent the water molecules from moving away from the water layer. Minimization and dynamics were performed to orient the crystallographic water's hydrogen atoms and the water molecules of the 5 Å inner shell of the water layer using Discover_3, while the enzyme and ligands remained fixed. The steepest descent and then the conjugate gradient algorithm were used to remove close atom contacts until the maximum derivative was less than 0.25 kcal/Å. An MD simulation was performed in two stages. The first stage was an equilibration phase of 1000 fs at 300 K, followed by a collection phase of 5000 fs. Subsequently, the side chains of the enzyme were minimized, while the backbone of the enzyme and all the heavy atoms of ligands were tethered. First, the system was minimized using 1000 steps of the steepest descent followed by 5000 steps of the conjugate gradients with a tethering force constant of 1000 kcal/Å. In subsequent minimizations, the conjugated gradients (5000 steps) were applied to the system at each tethering force constant, 100, 50, 15, and 2 kcal/Å, in a stepwise manner. Finally, minimization was performed on the fully relaxed system using with the conjugate gradient algorithm (5000 iterations). The refined pfLDH structure was aligned with the crystal structure of pfLDH, with rmsd = 0.5999 for the C-α atoms.

The CombiLibMaker tool in Sybyl 7.0 was used to generate a library of oxamic acids. Three-dimensional structures were generated using the Concord Standalone module with the default settings. A molecular docking study was performed using the standard default settings with 10 GA runs on each molecule. When the top three dockings were within 1.5 Å of each other, the docking was terminated. A spherical region with a 14 Å radius surrounding the carbonyl oxygen of Gly99 was defined as the binding site. The final selection was based on docking score, binding mode, and synthetic viability.

General Methods: All chemicals were purchased from Aldrich and Lanchester. Precoated silica gel G or GP Uniplates from Analtech were used for thin-layer chromatography (TLC). Column chromatography was performed on silica gel 60 (Scientific Adsorbents Incorporated (SAI)). ^{1}H and ^{13}C NMR spectra were obtained on a Bruker APX400 at 400 and 100 MHz, respectively. The high-resolution mass spectra (HRMS) were recorded on a Waters Micromass Q-Tof Micro mass spectrometer with a lock spray source. The liquid chromatography mass spectra (LCMS) were recorded on a WATERS ACQUITY Ultra Performance LC with ZQ detector in ESI or APCI mode. HPLC spectra were recorded on a Waters Alliance with Waters 996 Photodiode Array Detector with Symmetry C8 5 μm 3.9 × 150 mm column, and HPLC purification was performed using a Waters (Delta Prep 4000 with a dual detector), with Symmetry C18 5 μm 19 × 100 mm column eluting 30% water in AcCN or 20% water in MeOH. Chemical names were generated using Advanced Chemistry Development, Inc., software (ACD/Labs Release: 9.00 product version 9.04; Build 6293, 22 Jul 2005).

Bioassay: The recombinant pfLDH and pfMDH were prepared as described in detail elsewhere.[15] The purified mLDH (bovine heart) and MtMDH as well as cMDH (porcine heart) were purchased from Calzyme Laboratories, San Luis Obispo, California. The assay was performed in a 96-well plate. Initial rapid screening at concentrations of 50 μg/mL and 100 μg/mL was performed to check the activity of the synthesized compounds to all the battery of enzymes (pfLDH, mLDH, pfMDH, MtMDH, and cMDH). Briefly, the reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0) and pyruvate (200 μM) with the appropriate concentration of the test compounds along with the standards. The reaction was initiated by the addition of NADH (250 μM), and the decrease in absorbance at 340 nm was monitored for 5 min at 25 °C. The compounds showing inhibition of more than 50% at 50 μg/mL concentration were retested and their IC_{50} values were determined.

5,7-Dihydroxy-4-oxo-4*H*-chromene-2-carboxylic Acid (9): To an ice-cold pyridine solution (25 mL) of trihydroxyacetophenone **8** (2 g, 10.7 mmol), ethyl chlorooxoacetate (8.3 mL, 74 mmol) was added dropwise. The reaction was stirred at room temperature overnight. The slurry was poured into water and extracted with $CHCl_3$ (3 × 10 mL). The combined organic layers were washed with 10% HCl and evaporated. The residue was taken up in 50 mL of EtOH, and 100 mL of 5% Na_2CO_3 was added. The mixture

was stirred at 60∼70 °C for 2 h. After removal of ethanol, the aqueous phase was acidified to pH <2. The crude precipitate was collected and washed with ethyl acetate, which gave a yellow solid (0.99 g, 42%). ^{1}H NMR (DMSO) δ 12.39 (s, 1H), 11.1 (br, 1H), 6.78 (s, 1H), 6.39 (s, 1H), 6.22 (s, 1H).

5,7-Dihydroxy-*N*-(4-methoxybenzyl)-4-oxo-4*H*-chromene-2-carboxamide (10): 4-Methoxy benzylamine (0.16 g, 1.2 mmol) was added to a solution of the acid **9** (0.22 mg, 1 mmol), DCC (0.288 g, 1.4 mmol), and HOBt (0.19 g, 1.4 mmol) in 5 mL of 1:1 (v/v) CH_2Cl_2/DMF. The reaction was stirred at room temperature for 1 h. The reaction was washed with water and extracted with CH_2Cl_2 (3 × 10 mL). The organic layer was dried over $MgSO_4$. The crude product was crystallized from ethyl acetate, hexane, and EtOH to give a yellow solid (0.3 g, 88%). ^{1}H NMR (DMSO) δ 12.4 (s, 1H), 11.1 (s, 1H), 9.6 (s, 1H), 7.25 (d, 2H, J = 8.0 Hz), 6.91 (d, 2H, J = 8.0 Hz), 6.78 (s, 1H), 6.50 (s, 1H), 6.27 (s, 1H), 4.38 (d, 2H, J = 6.0 Hz), 3.80 (s, 3H); ^{13}C NMR (DMSO) δ 182.2, 165.4, 161.9, 158.9, 157.4, 156.3, 130.9, 129.3, 114.2, 109.7, 105.2, 99.9, 94.9, 55.5, 42.6; LC-MS (M^+ + 1) 342.00.

Ethyl 5,7-Dihydroxy-4-oxo-4*H*-chromene-2-carboxylate (11): Sulfuric acid (63 μL, 1.2 mmol) was added to a solution of the acid **9** (221 mg, 1 mmol) in EtOH. The reaction was refluxed overnight. The EtOH was removed under vacuum, and the residue was diluted with ethyl acetate and then washed with 10% $NaHCO_3$ and H_2O. The organic layer was dried over $MgSO_4$. Column chromatography was performed with 60% ethyl acetate in hexane to furnish a yellow solid (180 mg, 72%). ^{1}H NMR (DMSO) δ 12.34 (s, 1H), 6.83 (s, 1H), 6.41 (d, 1H, J = 2.0 Hz), 6.25 (d, 1H, J = 2.0 Hz), 4.38 (q, 2H), 1.34 (t, 3H); ^{13}C NMR (DMSO) δ 181.9, 165.8, 161.9, 159.8, 157.6, 152.8, 113.0, 105.5, 100.0, 94.7, 63.2, 14.2; LC-MS (M^+ + 1) 251.01.

4-{[Ethoxy(oxo)acetyl]amino}-3-methoxybenzoic Acid (13): To an ice-cooled solution of benzoic acid **12** (1.67 g, 10 mmol) and pyridine (1.6 mL, 20 mmol) in CH_2Cl_2 was added dropwise ethyl chlorooxoacetate (1.5 g, 11 mmol) in CH_2Cl_2. The mixture was allowed to warm to room temperature and stirred overnight. The precipitate was collected by filtration and washed with water. HCl (1 N) was added to the aqueous phase and extracted with chloroform (3 × 10 mL). The combined organic layers were dried over $MgSO_4$. Recrystallization from 2-propanol gave a while solid (2.35 g, 88%). ^{1}H NMR (DMSO) δ 9.75 (s, NH), 8.16 (d, 1H, J = 8.0 Hz), 7.60 (d, 1H, J = 8.0 Hz), 7.55 (s, 1H), 4.29 (q, 2H), 3.92 (s, 3H), 1.29 (t, 3H); LC-MS (APCI) 266.1 (M − 1)

Ethyl {[4-(Hydroxymethyl)-2-methoxyphenyl]amino}(oxo)-acetate (14): To a solution of **13** (1.79 g, 6.7 mmol) and trimethyl borate (40 mmol) in THF was added dropwise BH_3·THF (1 M in THF, 13.4 mmol). The mixture was stirred at room temperature for 6 h. After methanol was carefully added, the reaction was partitioned between saturated $NaHCO_3$ and CH_2Cl_2 (3 × 10 mL). The organic layer was dried over $MgSO_4$. Column chromatography was performed using 75% EtOAc in hexane to give a white solid (1.16 g, 69%). ^{1}H NMR ($CDCl_3$) δ 9.45 (s, H), 8.34 (d, 1H, J = 8.0 Hz), 6.96 (s, 1H), 6.94 (d, 1H, J = 8.0 Hz), 4.65 (s, 2H), 4.42 (q, 2H), 3.91 (s, 3H), 1.42 (t, 3H); ^{13}C NMR ($CDCl_3$) δ 160.8, 153.7, 148.6, 138.6, 125.3, 119.7, 119.3, 108.8, 64.9, 63.5, 55.8, 13.9; LC-MS (M^+ + 1) 254.09.

General Procedure I (Mitsunobu Reaction): DIAD (1.2 equiv) was added to a mixture of the hydroxyethyl compound (1 equiv), phenol (1.2 equiv), and triphenylphosphine (1.2 equiv) in dry THF or CH_2Cl_2 at 0 °C. The reaction was stirred at room temperature overnight. After completion of the reaction, the mixture was diluted with CH_2Cl_2 and washed with water. The organic layer was dried over $MgSO_4$ and concentrated under vacuum. The crude product was purified by flash column chromatography (ethyl acetate/hexane = 2:1) to afford the coupled product.

General Procedure II (Hydrolysis): NaOH (1 N) or LiOH (1 N, 1.2 equiv) was added to the solution of alkyl phenyl ether (1 equiv) dissolved in MeOH, EtOH, or THF/H_2O (2/1). The progress of the reaction was monitored by TLC. After completion of the reaction, the organic solvent was removed under vacuum. The aqueous phase was washed with ethyl acetate and was then acidified to pH <2 with 1 N HCl. Purification was performed by HPLC or recrystallization.

Ethyl 7-[(4-{[Ethoxy(oxo)acetyl]amino}-3-methoxybenzyl)-oxy]-5-hydroxy-4-oxo-4*H*-chromene-2-carboxylate (15): DIAD (39 μL, 0.2 mmol) was added to a mixture of compound **14** (43 mg, 1.73 mmol), triphenylphosphine (52 mg, 0.2 mmol), and compound **11** (50 mg, 0.2 mmol) in THF at 0 °C. After the reaction was completed, a yellow solid (47 mg, 57%) was obtained as described in general procedure I. ^{1}H NMR (DMSO) δ 12.3 (s,1H), 9.71 (s, 1H), 8.00 (d, 1H, J = 8.0 Hz), 7.24 (s, 1H), 7.09 (d, 1H, J = 8.0 Hz), 6.91 (s, 1H), 6.83 (d, 1H, J = 2.0 Hz), 6.55 (d, 1H, J = 2.0 Hz), 5.24 (s, 2H), 4.40 (q, 2H), 4.31 (q, 2H), 3.90 (s, 3H), 1.33(m, 6H); ^{13}C NMR (DMSO) δ 182.3, 165.4, 161.5, 160.9, 159.8, 157.5, 155.2, 153.1, 150.0, 134.0, 125.8, 121.1, 120.5, 113.2, 111.4, 106.6, 99.9, 94.3, 70.4, 63.2, 63.1, 56.5, 14.3, 14.2; LC-MS (M^+ + 1) 486.34, (M^+ + Na) 508.37.

7-({4-[(Carboxycarbonyl)amino]-3-methoxybenzyl}oxy)-5-hydroxy-4-oxo-4*H*-chromene-2-carboxylic Acid (1): LiOH (1 N, 0.11mmol) was added to a solution of **15** (25 mg, 49μmol) in THF/H_2O (2:1). The reaction was stirred at room temperature for 2 h. The product as a yellow solid was obtained as described in general procedure II (18 mg, 78%). ^{1}H NMR (DMSO) δ 12.3 (s, 1H), 9.64 (s, 1H), 8.09 (d, 1H, J = 8.0 Hz), 7.23 (s, 1H), 7.09 (d, 1H, J = 8.0 Hz), 6.85 (s, 1H), 6.78 (d, 1H, J = 2.0 Hz), 6.52 (d, 1H, J = 2.0 Hz), 5.21 (s, 2H), 3.82 (s, 3H); ^{13}C NMR (DMSO) δ 182.6, 165.4, 161.5, 161.2, 157.7, 153.1, 149.6, 147.1, 133.7, 126.1, 120.6, 120.5, 112.8, 111.3, 110.1, 106.6, 99.8, 94.3, 70.5, 56.5; HRMS calcd for $C_{20}H_{15}NO_{10}$, 430.0696; found, 430.0781 (M^+ + 1). HPLC (C8 column).

2-Amino-5-(2-hydroxyethyl)benzonitrile (17): NBS (0.17 g, 1 mmol) was added to an ice-cooled solution of 4-aminophenethanol **16** (0.18 g, 1 mmol) in dry DMF at 0∼5 °C. The reaction was stirred at room temperature for 1.5 h. The mixture was washed with water and extracted with ethyl acetate (3 × 5 mL). The combined organic layers were dried over $MgSO_4$. The crude yellow product was purified by column chromatography (ethyl acetate/hexane = 4:1) to afford 2-(4-amino-3-bromophenyl)ethanol as a pale yellow solid (200 mg, 95%). ^{1}H NMR ($CDCl_3$) δ 7.28 (s, 1H), 6.97 (d, 1H, J = 7.6 Hz), 6.72 (d, 1H, J = 7.6 Hz), 4.00 (br, 2H), 3.77 (t, 2H), 2.72 (t, 2H), 1.52 (br, 1H); ^{13}C NMR ($CDCl_3$) δ 142.5, 132.8, 129.7, 129.0, 115.9, 109.4, 63.5, 37.8; LC-MS (M^+ + 1) 216.19.

To a solution of 2-(4-amino-3-bromophenyl)ethanol (2.15 g, 10 mmol) in NMP in a microwave vial was added $Ni(CN)_2$·$4H_2O$ (0.6 equiv) and the vial was sealed. The microwave was set at 200 °C, 200 psi, and 120 W. The reaction was held at 200 °C for 10 min. The reaction was partitioned between water and ethyl acetate and extracted with ethyl acetate (3 × 10 mL). The combined organic layers were washed with water and dried over $MgSO_4$. The crude product was purified by column chromatography (ethyl acetate/hexane = 2/1) to afford a solid (1.01 g, 62%). ^{1}H NMR ($CDCl_3$) δ 7.28 (d, 1H, J = 8.4 Hz), 7.24 (s, 1H), 6.73 (d, 1H, J = 8.4 Hz), 4.35 (br, 1H), 3.82 (t, 2H), 2.76 (t, 2H); ^{13}C NMR ($CDCl_3$) δ 148.4, 135.0, 132.1, 128.4, 117.8, 115.6, 95.7, 63.1, 37.6, 28.2; LC-MS (APCI) 163.1; IR (cm^{-1}) 3450, 2208.

4-[2-(*tert*-Butyldimethylsilyloxy)ethyl]aniline (18a): Imidazole (0.74 g, 11 mmol) was added to a mixture of 4-aminophenethanol **16** (1.37 g, 10 mmol) and TBS-Cl (1.65 g, 11 mmol) in DMF (10 mL). The reaction was stirred at room temperature for 4 h. The reaction was partitioned between water and ethyl acetate and extracted with ethyl acetate (3 × 10 mL). The combined organic layers were washed with brine and dried over Na_2SO_4. The crude product was purified by flash column chromatography (EtOAc/hexane = 1/1) to furnish a yellow oil (2.4 g, 96%). ^{1}H NMR ($CDCl_3$) δ 7.03 (d, 2H, J = 8.0 Hz), 6.66 (d, 2H, J = 8.4 Hz), 3.77 (t, 2H), 3.59 (br, NH_2), 2.75 (t, 2H), 0.94 (s, 9H), 0.00 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 144.3, 129.9, 129.3, 115.2, 64.9, 38.8, 25.9, 18.3; LC-MS (M^+ + 1) 252.13.

2-Amino-5-[2-(*tert*-butyldimethylsilyloxy)ethyl]benzonitrile (18b): Imidazole (0.72 g, 10.6 mmol) was added to a mixture of compound **17** (1.57 g, 9.68 mmol) and TBS-Cl (1.59 g, 10.6 mmol)

in CH_2Cl_2 (10 mL). The reaction was treated as described for the preparation of **18a** to give a yellow solid (2.43 g, 88%). ^{1}H NMR ($CDCl_3$) δ 7.19 (s, 1H), 7.16 (d, 1H, J = 8.4 Hz), 6.67 (d, 1H, J = 8.0 Hz), 4.21 (br, NH_2), 3.71 (t, 2H), 2.65 (t, 2H), 0.84 (s, 9H), 0.00 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 148.1, 135.2, 132.3, 129.1, 117.8, 115.2, 95.8, 64.0, 38.0, 25.8, 18.2 0.00; LC-MS (APCI) 277.3.

Ethyl ({4-[2-(*tert*-Butyldimethylsilyloxy)ethyl]phenyl}amino)-(oxo)acetate (19a): To a mixture of the aniline **18a** (2.23 g, 8.9 mmol) and DIPEA (1.7 mL, 9.79 mmol) in dry CH_2Cl_2 was added ethyl chlorooxoacetate (1.09 mL, 9.79 mmol) at 0 °C. The reaction was stirred at room temperature for 2 h. The reaction was partitioned between water and CH_2Cl_2 (2 × 10 mL). The combined organic layers were dried over $MgSO_4$. The crude product was purified by flash column chromatography (ethyl acetate/hexane = 1/1) to afford a white solid (2.19 g, 70%). ^{1}H NMR ($CDCl_3$) δ 8.94 (s, NH), 7.55 (d, 2H, J = 8.4 Hz), 7.18 (d, 2H, J = 8.0 Hz), 4.36 (q, 2H), 3.76 (t, 2H), 2.76 (t, 2H), 1.37 (t, 3H), 0.83 (s, 9H), 0.00 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 161.0, 153.8, 136.6, 134.5, 129.8, 119.7, 64.2, 63.5, 39.0, 25.8, 18.2, 13.9, 0.00; LC-MS (APCI) 352.3.

Ethyl ({4-[2-(*tert*-Butyldimethylsilyloxy)ethyl]-2-cyanophenyl}-amino)(oxo)acetate (19b): To a mixture of 2-aminonitrile **18b** (2.37 g, 8.29 mmol) and DIPEA (1.64 mL, 9.45 mmol) in dry CH_2Cl_2 was added ethyl chlorooxoacetate (1.05 mL, 9.45 mmol) at 0 °C. The reaction was performed as described for the preparation of **19a** to afford a colorless oil (1.78 g, 55%). ^{1}H NMR ($CDCl_3$) δ 9.34 (s, NH), 8.35 (d, 1H, J = 8.4 Hz), 7.51 (s, 1H), 7.49 (d, 1H, J = 8.4 Hz), 4.46 (q, 2H), 3.80 (t, 2H), 2.81 (t, 2H), 1.44 (t, 3H), 0.85 (s, 9H), 0.00 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 159.8, 154.1, 137.5, 136.8, 135.1, 132.9, 120.7, 115.6, 102.8, 64.0, 63.3, 38.3, 25.7, 18.1, 13.9; DEPT 135 δ CH (135.1, 132.9, 120.7), CH_2 (64.0, 63.3, 38.3), CH_3 (25.8, 13.9); LC-MS (APCI) 377.3.

Ethyl {[4-(2-Hydroxyethyl)phenyl]amino}(oxo)acetate (20a): HF (70%) in pyridine (0.93 mmol) was added to a solution of TBS-protected **19a** (164 mg, 0.46 mmol) in dry CH_2Cl_2 at 0 °C. The reaction was stirred at 0 °C for 1 h, warmed, and stirred at room temperature for an additional 10 min. The reaction was quenched by addition of saturated $NaHCO_3$ and extracted with ethyl acetate (3 × 5 mL). The combined organic layers were dried over $MgSO_4$. The crude product was purified by flash column chromatography (ethyl acetate/hexane = 2/1) to afford a white solid (94 mg, 86%). ^{1}H NMR ($CDCl_3$) δ 8.90 (s, NH), 7.56 (d, 2H, J = 8.0 Hz), 7.22 (d, 2H, J = 8.0 Hz), 4.39 (q, 2H), 3.82 (t, 2H), 2.83 (t, 2H), 1.76 (br, OH), 1.40 (t, 3H); ^{13}C NMR ($CDCl_3$) δ 161.00, 153.9, 136.0, 134.7, 129.7, 120.1, 63.7, 63.4, 38.6, 13.9; DEPT 135 δ CH (129.0, 120.0), CH_2 (63.7, 45.1, 27.5), CH_3(13.9); LC-MS 238.16.

Ethyl {[2-Cyano-4-(2-hydroxyethyl)phenyl]amino}(oxo)acetate (20b): HF (70%) in pyridine (9.25 mmol) was added to a solution of the TBS-protected compound **19b** (1.74 g, 4.62 mmol) in dry CH_2Cl_2 at 0 °C. The reaction was treated as described for the preparation of **20a** to give a yellow solid **20b** (0.84 g, 70%). ^{1}H NMR ($CDCl_3$) δ 9.35 (s, 1H), 8.33 (d, 2H, J = 8.8 Hz), 7.51 (m, 2H), 4.42 (q, 2H), 3.84 (t, 2H), 2.85 (t, 2H), 1.42 (t, 3H); ^{13}C NMR ($CDCl_3$) δ 159.8, 154.1, 137.0, 136.9, 132.8, 121.1, 115., 103.1, 64.1, 62.7, 37.9, 13.9; LC-MS (M^+ + 1) 264.22, 185.07.

(3-Methoxy-4-nitrophenyl)acetonitrile (22): Trifluoroacetic anhydride (4.45 mL, 60 mmol) was added dropwise to a mixture of tetrabutylammonium nitrate (3.95 g, 13 mmol) and 18-crown-6 (50 mg, 1.9 mmol) in dry CH_2Cl_2 (90 mL) at 0 °C. The yellow solution was stirred for 15 min and was added to a solution of compound **21** (2 g, 13.5 mmol) in dry CH_2Cl_2 (200 mL). The reaction was stirred for 1.5 h at room temperature and diluted with CH_2Cl_2. The mixture was washed with saturated $NaHCO_3$ and H_2O and dried over $MgSO_4$. Column chromatography was performed with 50% ethyl acetate in hexane to give two major products, 2,6-dinitro and 4-nitro-3-methoxyphenyl acetonitrile (0.84 g, 32%). ^{1}H NMR ($CDCl_3$) δ 7.86 (d, 1H, J = 7.5 Hz), 7.07 (s, 1H), 7.0 (d, 1H, J = 7.5 Hz), 4.0 (s, 3H), 3.84 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 153.4, 139.2, 136.7, 126.4, 119.7, 116.5, 113.1, 56.7, 23.7; LC-MS (M − 1) 191.04.

(3-Methoxy-4-nitrophenyl)acetic Acid (23): 4-Nitrobenzylcyanide **22** (0.82 g, 4.26 mmol) was dissolved in 6 N HCl (35 mL) and refluxed at 100 °C for 3.5 h. After cooling to room temperature, the reaction was diluted with water. The precipitate was collected by filtration and washed with H_2O to give a yellow solid (0.5 g, 56%). ^{1}H NMR (MeOD) δ 8.02 (br, 1H), 7.83 (d, 1H, J = 8.0 Hz), 7.36 (d, 1H, J = 2.0 Hz), 7.08 (dd, 1H, J = 8.0, 2.0 Hz), 3.98 (s, 3H), 3.78 (s, 2H); ^{13}C NMR (MeOD) δ 176.07, 153.1, 140.0, 139.0, 126.0, 121.3, 114.6, 56.5, 40.9; LC-MS (M − 1) 210.09.

2-(3-Methoxy-4-nitrophenyl)ethanol (24): To a solution of the acid **23** (0.1 g, 0.47 mmol) was added BH_3−THF (1 M in THF, 0.84 mmol) at 0 °C. The reaction was stirred at 0 °C for 1 h, then at room temperature for 1.5 h. After 1 mL of MeOH was carefully added, the mixture was concentrated in vacuo. The residue was partitioned between water and ethyl acetate (2 × 10 mL). The combined organic layers were washed with saturated $NaHCO_3$ and dried over $MgSO_4$. Column chromatography was performed with 66% ethyl acetate in hexane to afford a yellow oil (87 mg, 94%). ^{1}H NMR ($CDCl_3$) δ 7.80 (d, 1H, J = 8.0 Hz), 6.94 (s, 1H), 6.88 (d, 1H, J = 8.0 Hz), 3.93 (s, 3H), 3.88 (t, 2H), 2.89 (t, 2H), 1.94 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 153.1, 147.1, 137.6, 125.9, 120.8, 114.2, 62.6, 56.4, 39.2; LC-MS (M^+ + 1) 198.00.

2-[2-(3-Methoxy-4-nitrophenyl)ethoxy]tetrahydro-2*H*-pyran (25): At room temperature, 3,4-dihydro-2*H*-pyran (0.6 mL, 6.64 mmol) was added to a mixture of compound **24** (0.43 g, 2.21 mmol) and PPTS (10 mol %) in 10 mL of dry CH_2Cl_2. The reaction was stirred at room temperature for 50 min, and saturated $NaHCO_3$ was added. The mixture was extracted with CH_2Cl_2 (3 × 5 mL), and the combined organic layers were dried over Na_2SO_4. The crude product was purified by column chromatography (EtOAc/hexane = 3/1) to afford a yellow oil (0.62 g, 99%). ^{1}H NMR ($CDCl_3$) δ 7.80 (d, 1H, J = 8.0 Hz), 6.98 (s, 1H), 6.89 (d, 1H, J = 8.0 Hz), 4.57 (m, 1H), 3.99 (m, 1H), 3.94 (s, 3H), 3.71 (m, 1H), 3.83 (m, 1H), 3.45 (m, 1H), 1.75 (m, 2H), 1.52 (m, 4H); ^{13}C NMR ($CDCl_3$) δ 153.0, 147.4, 137.7, 125.6, 120.8, 114.2, 98.8, 67.1, 62.2, 56.3, 36.4, 30.6, 25.3, 19.4; LC-MS (APCI) 181.2.

{2-Methoxy-4-[2-(tetrahydro-2*H*-pyran-2-yloxy)ethyl]phenyl}-amine (26): Pd/C (10%, 10 mg) was added to a solution of compound **25** (0.55 g, 1.95 mmol) in MeOH. The reaction was stirred under H_2 at room temperature overnight. The mixture was filtered through a pad of Celite, methanol was removed in vacuo, and the residue was purified by column chromatography (50% ethyl acetate in hexane) to afford a red oil (0.37 g, 76%). ^{1}H NMR ($CDCl_3$) δ 6.72 (m, 3H), 4.61 (m, 1H), 3.94 (m, 1H), 3.82 (m, 1H), 3.85 (s, 3H), 2,84 (t, 3H), 1.72 (m, 1H), 1.60 (m, 1H), 1.54 (m, 4H), ^{13}C NMR ($CDCl_3$) δ 147.5, 133.5, 129.9, 121.2, 121.2, 115.3, 111.5, 98.7, 68.7, 62.2, 55.4, 36.0, 30.7, 25.4, 19.5; LC-MS (dimer) 503.08.

Ethyl ({2-Methoxy-4-[2-(tetrahydro-2*H*-pyran-2-yloxy)ethyl]-phenyl}amino)(oxo)acetate (27): To a mixture of compound **26** (0.36 g, 1.4 mmol) and DIPEA (0.27 mL, 1.57 mmol) in dry CH_2Cl_2 was added ethyl chlorooxoacetate (0.17 mL, 1.57 mmol) at 0 °C. The reaction was performed as described for the preparation of **19a** to afford a colorless oil (0.49 g, 99%). ^{1}H NMR ($CDCl_3$) δ 9.41 (s, NH), 8.27 (d, 1H, J = 8.4 Hz), 6.82 (d, 1H, J = 8.4 Hz), 6.79 (s, 1H), 4.56 (m, 1H), 4.39 (q, 2H), 3.93 (m, 1H), 3.90 (s, 3H), 3.72 (m, 1H), 3.58 (m, 1H), 3.43 (m, 1H), 2.86 (t, 2H), 1.76 (m, 1H), 1.66 (m, 1H), 1.53 (m, 4H), 1.40 (t, 3H); ^{13}C NMR ($CDCl_3$) δ 160.9, 153.5, 148.5, 136.7, 124.4, 121.3, 119.7, 111.0, 98.7, 68.0, 63.3, 62.1, 55.8, 36.3, 34.6, 30.6, 25.4, 19.4, 13.9; LC-MS (M + 1) 352.34, (M^+ + Na) 374.43.

Ethyl {[4-(2-Hydroxyethyl)-2-methoxyphenyl]amino}(oxo)-acetate (20c): To a solution of compound **27** (0.45 g, 1.28 mmol) in dry MeOH was added *p*-toluenesulfonic acid (22 mg, 10 mol %) at room temperature. The reaction was heated to 55 °C and continued stirring for 1 h. After cooling to 0 °C, the mixture was quenched with saturated $NaHCO_3$ and extracted with ethyl acetate (2 × 10 mL). The organic layers were combined and dried over $MgSO_4$. The crude product was purified by flash column chromatography (EtOAc/hexane = 3/1) to afford a solid that was crystallized from ethyl acetate and hexane to give a white solid (0.26 g, 76%). ^{1}H NMR ($CDCl_3$) δ 9.39 (s, NH), 8.27 (d, 1H, J = 8.0 Hz), 6.82 (d, 1H, J = 8.0 Hz), 6.77 (s, 1H), 4.40 (q, 2H), 3.88

(s, 3H), 3.82 (t, 2H), 2.28 (t, 2H), 2.1 (s, 1H, OH), 1.40 (t, 3H); ^{13}C NMR ($CDCl_3$) δ 160.8, 153.5, 148.5, 136.2, 124.3, 121.2, 119.8, 111.0, 63.41, 55.8, 53.8, 39.1, 13.9; LC-MS (APCI) 268.2.

Ethyl 7-[2-(4-{[Ethoxy(oxo)acetyl]amino}phenyl)ethoxy]-5-hydroxy-4-oxo-4*H*-chromene-2-carboxylate (28a): DIAD (49 μL, 0.25 mmol) was added to a mixture of **20a** (50 mg, 0.21 mmol), triphenylphosphine (65 mg, 0.25 mmol), and compound **11** (62 mg, 0.25 mmol) in CH_2Cl_2 at 0 °C. After the reaction was completed, a yellow solid (60 mg, 61%) was obtained as described in general procedure I. ^{1}H NMR ($CDCl_3$) δ 12.2 (s, 1H), 8.89 (s, 1H), 7.61 (d, 2H, J = 8.4 Hz), 7.29 (d, 2H, J = 8.0 Hz), 6.96 (s, 1H), 6.50 (d, 1H, J = 2.0 Hz), 6.36 (d, 1H, J = 2.0 Hz), 4.45 (m, 4H), 4.21 (t, 2H), 3.10 (t, 2H), 1.42 (m, 6H); ^{13}C NMR ($CDCl_3$) δ 182.1, 165.4, 162.0, 160.9, 159.9, 157.5, 153.8, 152.4, 135.0, 134.8, 129.7, 120.0, 113.6, 106.8, 99.3, 93.5, 69.1, 63.7, 63.0, 34.8, 14.0, 13.9; LC-MS (M − 1) 470.42.

Ethyl 7-[2-(3-Cyano-4-{[ethoxy(oxo)acetyl]amino}phenyl)-ethoxy]-5-hydroxy-4-oxo-4*H*-chromene-2-carboxylate (28b): DIAD (43 μL, 0.22 mmol) was added to a mixture of compound **20b** (50 mg, 0.19mmol), triphenylphosphine (57 mg, 0.22 mmol), and compound **11** (55 mg, 0.22 mmol) in CH_2Cl_2 at 0 °C. After the reaction was completed, a yellow solid (65 mg, 69%) was obtained as described in general procedure I. ^{1}H NMR ($CDCl_3$) δ 12.2 (br, 1H), 9.39 (s, 1H), 8.42 (d, 1H, J = 8.0 Hz), 7.58 (m, 2H), 6.97 (s, 1H), 6.50 (d, 1H, J = 2.0 Hz), 6.35 (d, 1H, J = 2.0 Hz), 4.45 (m, 4H), 4.25 (t, 2H), 3.14 (t, 2H), 1.45 (t, 6H); ^{13}C NMR ($CDCl_3$) δ 182.1, 165.0, 162.1, 159.8, 159.7, 157.7, 154.2, 152.5, 137.7, 135.6, 134.9, 132.6, 121.1, 115.4, 113.6, 106.9, 103.2, 99.2, 93.5, 68.3, 64.1, 63.0, 34.4, 34.0, 14.0, 13.9; LC-MS (M − 1) 493.00.

Ethyl 7-[2-(3-Methoxy-4-{[ethoxy(oxo)acetyl]amino}phenyl)-ethoxy]-5-hydroxy-4-oxo-4*H*-chromene-2-carboxylate (28c): DIAD (43 μL, 0.22 mmol) was added to a mixture of compound **20c** (50 mg, 0.18mmol), triphenylphosphine (57 mg, 0.22 mmol), and compound **11** (55 mg, 0.22 mmol) in CH_2Cl_2 at 0 °C. After the reaction was completed, a yellow solid (42 mg, 45%) was obtained as described in general procedure I. ^{1}H NMR ($CDCl_3$) δ 12.2 (s, 1H), 9.45 (s, 1H), 8.36 (d, 1H, J = 8.0 Hz), 6.98 (s, 1H), 6.91 (d, 1H, J = 8.4 Hz), 6.85 (s, 1H), 6.52 (d, 1H, J = 2.0 Hz), 6.38 (d, 1H, J = 2.0 Hz), 4.44 (m, 4H), 4.24 (t, 2H), 3.94 (s, 3H), 3.10 (t, 2H), 1.43 (m, 6H); ^{13}C NMR ($CDCl_3$) δ 182.1, 165.4, 162.0, 160.8, 159.9, 157.5, 153.6, 152.4, 148.5, 134.9, 124.8, 121.3, 119.9, 113.6, 106.8, 99.3, 93.4, 69.2, 63.5, 63.0, 55.8, 35.3, 14.0, 13.9; LC-MS (M − H) 500.31; HRMS calcd for $C_{25}H_{25}NO_{10}$, 500.1512; found, 500.1522 (M^+ + 1).

7-(2-{4-[(Carboxycarbonyl)amino]phenyl}ethoxy)-5-hydroxy-4-oxo-4*H*-chromene-2-carboxylic Acid (2): LiOH (1 N, 0.32 mmol) was added to a solution of compound **28a** (75 mg, 0.16 mmol) in THF/H_2O (2 mL/1 mL). The reaction was performed as described in general procedure II to give a yellow solid (50 mg, 68%). ^{1}H NMR (DMSO) δ 12.35 (br, OH), 10.59 (br, NH), 7.66 (br, 2H), 7.24 (br, 2H), 6.73 (s, 1H), 6.53 (s, 1H), 6.25 (br, 1H), 4.20 (br, 2H), 2.95 (br, 2H); ^{13}C NMR (DMSO) δ 182.6, 165.3, 162.7, 161.4, 161.3, 157.6, 155.3, 136.5, 134.5, 129.6, 120.7, 112.2, 106.2, 99.2, 93.6, 69.4, 34.4; HRMS calcd for $C_{20}H_{15}NO_9$, 414.0747; found, 414.0826 (M + 1); HPLC (C_8 column).

7-(2-{4-[(Carboxycarbonyl)amino]-3-cyanophenyl}ethoxy)-5-hydroxy-4-oxo-4*H*-2-carboxylic Acid (3): By following procedure II, compound **3** was prepared as a yellow solid (27 mg, 77%). ^{1}H NMR (DMSO) δ 12.6 (s, 1H), 10.7 (br, 1H), 7.87 (s, 1H) 7.64 (m, 2H), 6.76 (s, 1H), 6.61 (s, 1H), 6.32 (s, 1H), 4.36 (t, 2H), 3.10 (t, 2H); ^{13}C NMR (DMSO) δ 182.5, 165.3, 161.8, 161.5, 161.2, 157.6, 154.3, 137.6, 137.4, 135.1, 133.7, 126.1, 116.9, 112.7, 108.4, 106.3, 99.3, 93.8, 68.7, 33.8; LC-MS (M + Na) 459.20; HRMS calcd for $C_{21}H_{14}N_2O_9$, 439.0699; found, 439.0763 (M^+ + 1); HPLC (C_8 column).

7-(2-{4-[(Carboxycarbonyl)amino]-3-methoxyphenyl}ethoxy)-5-hydroxy-4-oxo-4*H*-2-carboxylic Acid (4): By following procedure II, compound **4** was prepared as a yellow solid (29 mg, 77%). ^{1}H NMR (DMSO) δ 12.40 (br, OH), 9.55 (br, NH), 7.95 (br, 1H), 7.01 (br, 1H), 6.90 (br, 1H), 6.80 (s, 1H), 6.68 (s, 1H), 6.39 (s, 1H), 4.31 (br, 2H), 3.86 (s, 3H), 3.01 (br, 2H); ^{13}C NMR (DMSO) δ 182.6, 165.5, 162.3, 161.5, 161.2, 157.7, 156.1, 154.6, 149.4, 135.8, 124.5, 121.3, 120.4, 112.6, 112.4, 106.4, 99.4, 93.8, 69.4, 56.4, 34.9; DEPT 135 δ CH (121.3, 120.4, 112.7, 112.4, 99.4, 93.8), CH_2 (69.4, 34.9), CH_3 (56.4); HRMS calcd for $C_{21}H_{17}NO_{10}$, 444.0852; found, 444.0977 (M^+ + 1); calcd for $C_{21}H_{17}NO_{10}Na$, 466.0750; found, 466.0766 (M + Na); HPLC (C_8 column).

Ethyl ({4-[2-({5-Hydroxy-2-[(4-methoxybenzylamino)carbonyl]-4-oxo-4*H*-chromen-7-yl}oxy)ethyl]phenyl}amino)(oxo)-acetate (29a): Compound **29a** was prepared by following general procedure I, giving a yellow solid (48 mg, 41%). ^{1}H NMR (DMSO) δ 12.4 (s, 1H), 10.72 (s, NH), 9.51 (t, NH), 7.67 (d, 2H, J = 8.0 Hz), 7.29 (d, 2H, J = 8.4 Hz), 7.25 (d, 2H, J = 8.4 Hz), 6.88 (d, 2H, J = 8.4 Hz), 6.75 (s, 1H), 6.61 (s, 1H), 6.37 (d, 1H, J = 1.6 Hz), 4.40 (d, 2H, J = 2.4 Hz), 4.28 (m, 4H), 3.70 (s, 3H), 3.01 (t, 2H), 1.29 (t, 3H); ^{13}C NMR (DMSO) δ 182.4, 165.2, 161.6, 161.1, 158.9, 158.7, 157.2, 156.5, 155.8, 136.3, 134.7, 130.7, 129.7, 129.4, 120.9, 114.2, 109.8, 106.1, 99.2, 93.8, 69.4, 62.8, 55.5, 42.6, 34.4, 14.3; HRMS calcd for $C_{30}H_{28}N_2O_9$, 561.1795; found, 561.1860 (M + 1); calcd for $C_{30}H_{28}N_2O_9Na$, 583.1693; found, 583.1713 (M + Na).

Ethyl ({2-Cyano-4-[2-({5-hydroxy-2-[(4-methoxybenzylamino)carbonyl]-4-oxo-4*H*-chromen-7-yl}oxy)ethyl]phenyl}amino)-(oxo)acetate (29b): Compound **29b** was prepared by following general procedure I, giving a yellow solid (85 mg, 38%). ^{1}H NMR (DMSO) δ 12.4 (br, 1H), 10.9 (s, 1H), 9.52 (s, 1H), 7.84 (s, 1H), 7.68 (d, 1H, J = 8.0 Hz), 7.52 (d, 1H, J = 8.0 Hz), 7.25 (d, 2H, J = 8.0 Hz), 6.88 (d, 2H, J = 8.8 Hz), 6.75 (s, 1H), 6.63 (s, 1H), 6.41 (s, 1H), 4.40 (d, 2H, J = 5.2 Hz), 4.32 (m, 4H), 3.69 (s, 3H), 3.10 (t, 2H), 1.30 (t, 3H); ^{13}C NMR (DMSO) δ 182.4, 165.1, 161.6, 160.4, 158.9, 158.8, 157.2, 156.6, 156.4, 137.8, 137.4, 135.1, 133.8, 130.7, 129.4, 126.6, 116.9, 114.2, 109.8, 108.9, 106.2, 99.2, 94.0, 68.8, 63.1, 42.6, 33.8, 14.2; DEPT 135 δ CH (135.1, 133.8, 129.4, 126.6, 114.2, 109.8, 99.2, 94.0), CH_2 (68.8, 63.2, 42.6, 33.8), CH_3 (55.5, 14.2); HRMS calcd for $C_{31}H_{27}N_3O_9$, 586.1747; found, 586.1794 (M^+ + 1); calcd for $C_{31}H_{27}N_3O_9Na$, 608.1645; found, 608.1622 (M + Na).

Ethyl ({2-Methoxy-4-[2-({5-hydroxy-2-[(4-methoxybenzylamino)carbonyl]-4-oxo-4*H*-chromen-7-yl}oxy)ethyl]phenyl}-amino)(oxo)acetate (29c): Compound **29c** was prepared by following general procedure I, giving a yellow solid (80 mg, 68%). ^{1}H NMR (DMSO) δ 12.4 (br, 1H), 9.59 (s, NH), 9.56 (t, NH), 7.89 (d, 1H, J = 8.0 Hz), 7.26 (d, 2H, J = 8.0 Hz), 7.07 (s, 1H), 6.88 (m, 3H,), 6.68 (s, 1H), 6.57 (s, 1H), 6.38 (s, 1H), 4.39 (d, 2H), 4.28 (m, 4H), 3.85 (s, 3H), 3.70 (s, 3H), 3.02 (t, 2H), 1.28 (t, 3H); ^{13}C NMR (DMSO) δ 182.4, 165.2, 161.6, 160.9, 158.9, 158.7, 157.2, 156.5, 154.9, 149.9, 136.2, 130.7, 129.4, 124.3, 121.3, 121.2, 114.2, 112.5, 109.8, 106.1, 99.2, 93.8, 69.4, 63.1, 56.4, 55.5, 42.6, 34.9, 14.2; HRMS calcd for $C_{31}H_{30}N_2O_{10}$, 591.1900; found, 591.1982 (M + 1); HRMS calcd for $C_{31}H_{30}N_2O_{10}Na$, 613.1798; found, 613.1786 (M + Na).

({4-[2-({5-Hydroxy-2-[(4-methoxybenzylamino)carbonyl]-4-oxo-4*H*-chromen-7-yl}oxy)ethyl]phenyl}amino)(oxo)acetic Acid (5): By following general procedure II, compound **5** was prepared, giving a yellow solid (12 mg, 46%). ^{1}H NMR (DMSO) δ 12.4 (s, OH), 10.6 (br, 1H), 9.54 (t, NH), 7.68 (d, 1H), 7.31 (s, 1H), 7.28 (d, 2H, J = 8.8 Hz), 6.91 (d, 2H, J = 8.8 Hz), 6.79 (s, 1H), 6.67 (s, 1H), 6.44 (s, 1H), 4.43 (d, 2H, J = 5.6 Hz), 4.29 (t, 2H), 3.73 (s, 3H), 3.03 (t, 2H); ^{13}C NMR (DMSO) δ 182.4, 165.3, 161.6, 158.9, 158.8, 157.5,157.2, 156.6, 134.5, 130.8, 129.6, 129.4, 120.8, 114.2, 109.8, 106.2, 99.3, 93.9, 69.5, 55.5, 42.6, 34.4; HRMS calcd for $C_{28}H_{24}N_2O_9$, 533.1482; found, 533.1548 (M^+ + 1); HPLC (C_8 column).

({2-Cyano-4-[2-({5-hydroxy-2-[(4-methoxybenzylamino)carbonyl]-4-oxo-4*H*-chromen-7-yl}oxy)ethyl]phenyl}amino)(oxo)-acetic Acid (6): By following general procedure II, compound **6** was prepared, giving a yellow solid (29 mg, 62%). ^{1}H NMR (DMSO) δ 12.4 (s, OH), 10.9 (br, 1H), 9.52 (t, 1H), 7.84 (s, 1H), 7.69 (d, 1H, J = 8.0 Hz), 7.65 (d, 1H, J = 8.0 Hz), 7.28 (d, 2H, J = 8.8 Hz), 6.88 (d, 2H, J = 8.8 Hz), 6.79 (s, 1H), 6.68 (d, 1H, J = 2.0 Hz), 6.46 (s, 1H, J = 2.0 Hz), 4.43 (d, 2H, J = 6.0 Hz), 4.35 (t, 2H), 3.12 (t, 2H); ^{13}C NMR (DMSO) δ 182.5, 165.2, 161.6,

158.9, 158.8, 157.2, 156.6, 137.8, 135.1, 133.7, 129.4, 116.9, 114.2, 109.8, 106.8, 99.2, 94.0, 68.8, 55.5, 42.6, 33.8; DEPT 135 δ CH (135.1, 133.7, 129.4, 125.8, 114.2, 109.8, 99.2, 94.0), CH_2 (68.8, 42.6, 33.8), CH_3 (55.5); HRMS calcd for $C_{29}H_{23}N_3O_9$, 558.1434; found, 558.1503 ($M^+ + 1$); HPLC (C_8 column).

({2-Methoxy-4-[2-({5-hydroxy-2-[(4-methoxybenzylamino)-carbonyl]-4-oxo-4*H*-chromen-7-yl}oxy)ethyl]phenyl}amino)-(oxo)acetic Acid (7): By following general procedure II, compound **7** was prepared, giving a white solid (23 mg, 61%). ^{1}H NMR (DMSO) δ 12.35 (br, OH), 9.56 (d, 2H, J = 18.4 Hz), 7.97 (br, 1H), 7.23 (br, 2H), 7.06 (s, 1H), 6.87 (br, 3H), 6.74 (s, 1H), 6.60 (s, 1H), 6.37 (s, 1H), 4.39 (br, 2H), 4.25 (br, 2H), 3.84 (s, 3H), 3.68 (s, 3H), 3.01 (br, 2H); ^{13}C NMR (DMSO) δ 182.4, 165.2, 162.3, 161.5, 158.8, 158.7, 157.1, 156.4, 149.4, 135.7, 130.7, 129.4, 124.6, 121.3, 120.4, 114.2, 112.3, 109.8, 106.1, 99.2, 93.8, 69.4, 56.3, 55.4, 42.6, 34.9; DEPT 135 δ CH (129.4, 121.3, 120.4, 114.2, 112.3, 109.8, 99.2, 93.8), CH_2 (69.4, 42.6, 34.9), CH_3 (56.3, 55.4); HRMS calcd for $C_{29}H_{26}N_2O_{10}$, 563.1587; found, 563.1641 (M + 1); calcd for $C_{29}H_{26}N_2O_{10}Na$, 585.1485; found, 585.1417 (M + Na); HPLC (C_8 column).

Acknowledgment. We wish to thank the Center for Disease Control and Prevention, U.S.A., for providing financial assistance (CDC cooperative agreements 1UO1 CI000211-03 and 1UO1 CI000362-01). This Investigation was conducted in a facility constructed with support from Research Facilities Improvement Program Grant Number C06 Rr-14503-01 from the National Center for Research Resources, National Institutes of Health.

Supporting Information Available: Analytical and spectral characterization data (^{1}H, ^{13}C NMR, HRMS, LC-MS, and HPLC). This material is available free of charge via the Internet at http://pubs.acs.org.

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JM070336K