Synthesis and In Vitro Studies of Novel Pyrimidinyl Peptidomimetics as Potential Antimalarial Therapeutic Agents

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A class of new pyrimidinyl peptidomimetic agents (compounds **1**−**6**) were synthesized, and their in vitro antimalarial activities against *Plasmodium falciparum* were evaluated. The core structure of the new agents consists of a substituted 5-aminopyrimidone ring and a Michael acceptor side chain methyl 2-hydroxymethyl-but-2-enoate. The synthesis of **1**−**6** featured a Baylis−Hillman reaction of various aldehydes with methyl acrylate catalyzed by 1,4-diazabicyclo[2.2.2]octane (DABCO) and a S_N2' Mitsunobu reaction under the conditions of diethyl azadicarboxylate (DEAD), triphenylphosphine (Ph_3P), and various acids. The new compounds exhibited potent in vitro growth inhibitory activity (IC_{50} = 10−30 ng/mL) against both chloroquine sensitive (D-6) and chloroquine resistant (W-2) *Plasmodium falciparum* clones. Compound **6** (IC_{50} = 6−8 ng/mL) is the most active compound of the class, the antimalarial efficacy of which is comparable to that of chloroquine. In general, this class of compound exhibited weak to moderate in vitro cytotoxicity against neuronal and macrophage cells with IC_{50} in the range of 1−16 μg/mL and showed less toxicity in a colon cell line. Preliminary results indicated that compounds **3** and **6** are active against *P. berghei*, prolonged the life span of parasite-bearing mice from 6 days for untreated control to 16−24 days for drug-treated animals.

Introduction

Malaria is one of the most common infectious diseases in over 100 countries in Africa, Southeast Asia, and South America. Every year, there are an estimated 300 million new cases of malaria throughout the world, and the mortality associated with malaria is estimated at 1.1 million deaths per year.[1] The increasing prevalence of multiple-drug-resistant (MDR) strains of *Plasmodium falciparum* in most malaria endemic areas has significantly reduced the efficacy of current antimalarial drugs for prophylaxis and treatment of this disease.[2] For instance, resistance to the inexpensive antimalarial mainstays, such as chloroquine, is worldwide. Similarly, resistance to mefloquine, which was proposed as the drug of choice for chloroquine-resistant malaria, has been reported from Africa and Southeast Asia.[3,4] Although drug resistance is a common problem in the treatment of most microbial infections, malaria, and many neoplasm, the impact is more acute for malaria chemotherapy because of the limited number of clinically useful antimalarial drugs. Thus, identification of new targets for antimalarial therapy and synthesis of new agents specifically against these targets are important tasks for scientists involved in malaria research.

Parasitic proteases, cysteine protease (falcipain), and aspartic protease (plasmepsin) are two of the many potential targets for malaria chemotherapy.[5] Both enzymes are involved in degradation of human hemoglobin as a source of amino acids for protein synthesis of the schizontes, resulting in destruction of the erythrocytes. Therefore, design and synthesis of selective inhibitors of aspartic protease and/or cysteine protease as potential malaria therapeutic agents are of significant interest to medicinal chemists, and the approach has attracted broad attention.[6−8]

Low molecular weight synthetic protease inhibitors are well documented, including peptidyl(acyloxy)methyl,[9] halomethyl,[10] and diazomethyl ketones[11] and various epoxysuccinyl peptide derivatives[12] as irreversible affinity labels, peptidyl aldehyde[13] and peptidyl nitrile[14] as reversible potent cysteine protease inhibitors, peptidyl Michael acceptors as substrate analogues,[15] vinyl sulfones as mechanism-based inhibitors,[16] and others.[17] However, the stability of these synthetic inhibitors in living organisms is poor, presumably because of the fact that peptide moieties can be readily cleaved by various enzymes in vivo. In addition, some of the compounds are so reactive that nonspecific side reactions often occur, resulting in host toxicity.

As part of an ongoing malaria chemotherapy project in our laboratory, we undertook the synthesis and antimalarial activity evaluation of a novel class of pyrimidinyl peptidomimetics (compounds **1**−**6**, Chart 1). The core structure of these new agents comprises a peptidomimetic segment[18,19] for recognition and binding to the target enzyme, and an active group, which can be considered a Michael acceptor for enzyme alkylation. The latter differs from ordinary Michael acceptor or vinyl sulfones because the new agent features a unique "addition−elimination" mechanism when it reacts with the cysteine residue of the target enzyme (Scheme 1), resulting in irreversible inhibition of the target enzyme. The use of a peptidomimetic segment in place of the natural peptides renders the compounds more stable to in vivo enzymatic degradation. The activity of the

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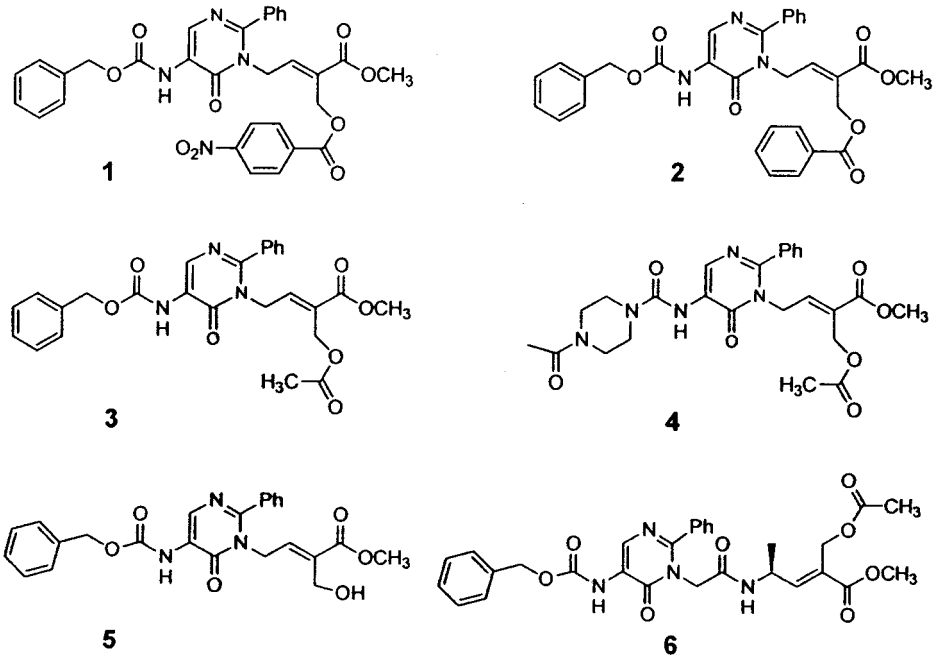
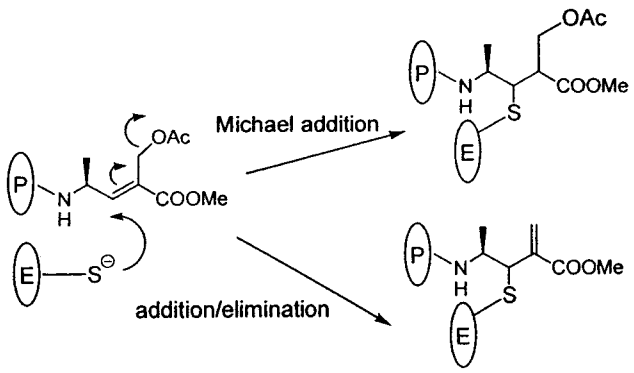
Chart 1



Scheme 1



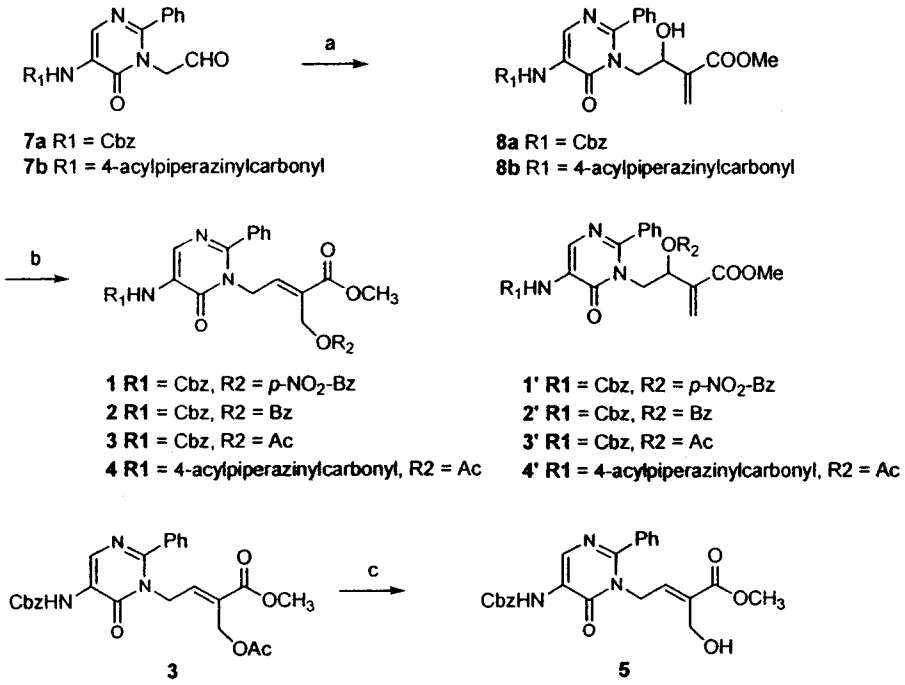
compounds as a Michael acceptor can be finely tuned by systematic modification of the Michael acceptor leaving group to balance the chemical reactivity versus cell growth inhibitory potency and host toxicity.

Chemistry

Scheme 2 illustrated the synthesis of compounds **1**−**5**. Aldehydes **7a** and **7b** were synthesized by the methods of Bernstein.[18a] Treatment of these aldehydes with methyl acrylate in the presence of 1,4-diazabicyclo[2.2.2]-octane (DABCO)[20] gave allylic alcohols **8a** or **8b** in good yield. Both **8a** and **8b** are mixtures of *R* and *S* enantiomers. Compound **8a** was subjected to the S_N2' Mitsunobu reaction[21a] in the presence of diethyl azadicarboxylate (DEAD), triphenylphosphine (Ph_3P), and *p*-nitrobenzoic acid or benzoic acid to yield compound **1** or **2**, respectively. Likewise, treatment of **8a** or **8b** with DEAD, Ph_3P, and acetic acid produced compound **3** or **4**. The products **1**−**4** were solely trans alkenes, a result of S_N2' displacement reactions. In all reactions, no S_N2 displacement products such as **1′**−**4′** were detected in the crude products by NMR. This observation fully agreed with the literature report that the Mitsunobu reaction of acyclic allylic alcohols bearing an ester group at the β position gave high regio- and stereoselective products, (*E*)-2-(hydroxymethyl) 3-substituted 2-alkenoate derivatives, in >70% yield with S_N2':S_N2 ratio of 22:1 to >50:1.[21a] Since S_N2 and S_N2' reactions take place in a competitive manner and are sensitive to steric hindrance, the bulky 2-phenylpyrimidinyl group in compounds **8a** and **8b** may have prevented the S_N2 and favored the S_N2' displacement reaction. Identification of the Mitsunobu reaction products was achieved by NMR studies. Distinctive differences in NMR spectra of S_N2 and S_N2' products involve the number of olefinic proton(s) (2H vs 1H), the split patterns (two doublets with a small $^2J_{HH}$ in the range of 0−3 Hz vs a triplet), and the chemical shift of the olefinic proton(s). The olefinic proton cis to the ester function showed a very downfield chemical shift (~7.0 ppm) on ^1H NMR, apparently caused by conjugative and anisotropic deshielding effects of the ester group. The exclusive formation of the (*E*) isomer suggested that this reaction proceeds through a stepwise mechanism involving carbonium ion formation, as suggested by Farina.[21b] Reaction by a concerted S_N2' mode would have furnished (*E*) and (*Z*) isomers in equal amounts, since the starting alcohols **8a** and **8b** are each a racemic mixture.

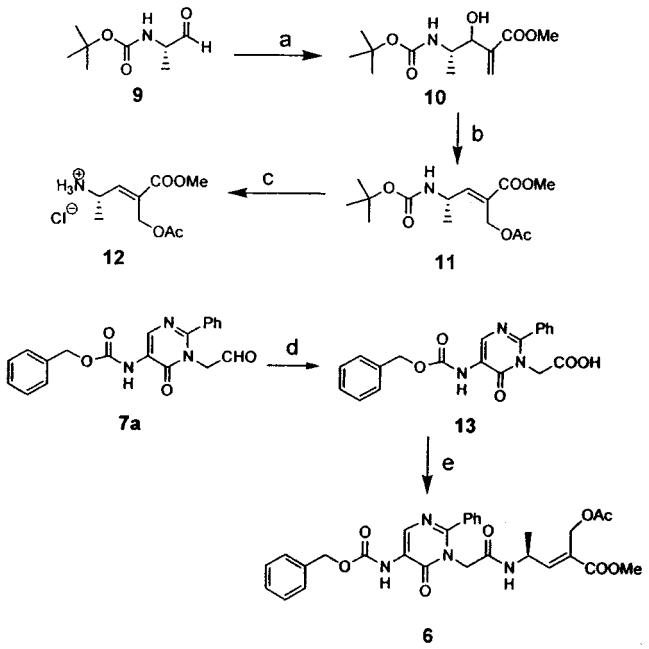
The synthesis of compound **6** is shown in Scheme 3. Chiral amino acid aldehyde **9**, reported by Fehrentz and Castro,[22] was treated with methyl acrylate in the presence of DABCO to yield methyl 4-*tert*-butoxycarbonylamino-3-hydroxyl-2-methylenepentanoate **10** in 78% yield. The S_N2' Mitsunobu reaction of compound **10** furnished methyl 4-*tert*-butoxycarbonylamino-2-acetoxy-*trans*-2-pentenoate **11**. Again, the trans alkene was the only isomer isolated and displacement occurred exclusively through the S_N2' mode. Removal of the BOC protecting group with dry HCl in diethyl ether gave the corresponding amine hydrogen chloride salt **12**. The intermediate pyrimidinyl acid **13**[18a] was prepared in good yield by oxidation of aldehyde **7a** with $NaClO_2$. Compound **6** can be readily anticipated from the coupling of acid **13** and the amine HCl salt of **12**. However,

Scheme 2[a]



[a] (a) DABCO, methyl acrylate, 72−91%; (b) DEAD, Ph_3P, AcOH (or benzoic acid or *p*-nitrobenzoic acid), THF, 59−75%; (c) MeOH, K_2CO_3, 71%.

Scheme 3[a]



[a] (a) DABCO, methyl acrylate, 88%; (b) DEAD, Ph_3P, AcOH, THF, 77%; (c) dry HCl, Et_2O, 99%; (d) $NaClO_2$, H_2O, *tert*-butyl alcohol, 62%; (e) **12**, DCC, HOBT, DMAP, $CHCl_3$, 79%.

several reaction conditions were attempted before conditions with satisfactory yield were found. We first investigated the mixed anhydride method for the coupling reaction, using isobutyl chloroformate and triethylamine. It turned out that the quickly formed mixed anhydride from compound **13** and isobutyl chloroformate was too stable to react with the free amine generated in situ from the amine salt **12**. The mixed anhydride can be purified over silica gel column chromatography. Dicyclohexylcarbodiimide (DCC) or 1-ethyl-3-(3-dimethylamino)propylcarbodiimide (EDC) alone also gave poor yield. Nevertheless, a combination of DCC and HOBT makes an excellent coupling reagent for this final reaction.

Table 1. In Vitro Activity against Malarial Parasite (IC_{50})

compound	IC_{50} (ng/mL) D6 clone	IC_{50} (ng/mL) W2 clone
1	24	24
2	27	28
3	17	17
4	17	18
5	16	17
6	9	10
chloroquine (control)	6	114

Results and Discussion

These compounds (**1**−**6**) exhibited potent in vitro growth inhibitory activity (IC_{50} = 10−30 ng/mL) against *Plasmodium falciparum* clones, D-6 and W-2 (Table 1). The former (D-6) is a chloroquine sensitive clone, and the latter (W-2) is a chloroquine-resistant cell line. These new compounds not only showed potent activity against chloroquine sensitive malarial strain (D-6) but are equally active against chloroquine-resistant malarial strain (W-2). Compound **6** is the most active compound of the class. The antimalarial efficacy of compound **6** is comparable to that of chloroquine with IC_{50} = 6−8 ng/mL against D-6. Compound **6** differs from compound **1**−**5** by having an extra unit of peptide, which may have contributed to the enhancement of its efficacy. However, no significant inhibitory activity against plasmepsin was observed, suggesting that plasmepsin may not be the primary target enzyme of the new peptidomimetic agents synthesized in this study.

This study has uncovered the core structure of a new class of peptidomimetic antimalarial agents, which

Table 2. In Vitro Cytotoxicity Study (IC_{50})

	IC_{50} (ng/mL)		
compound	neuronal	macrophage	colon
1	1750	3600	7600
2	2600	1600	13500
3	920	1080	>50000
4	13800	16000	>50000
5	950	780	>50000
6	1100	5400	1600

consists of a substituted 5-aminopyrimidone ring and a Michael acceptor side chain methyl 2-hydroxymethylbut-2-enoate. Acetylation of the hydroxymethyl function of the Michael acceptor of compound **5** to give compound **3** did not affect their antimalarial efficacy in the test system. Since acetyloxy is a better leaving group than the hydroxy function, one would expect that acetylation of the hydroxy group will enhance the efficacy substantially if addition−elimination is a mode of action. However, the results suggest that the addition−elimination mechanism may play a small or no role in antimalarial activity of this class of compounds. This contention is further substantiated by the observation that benzoyl esters (**1** and **2**), which are an even better leaving group than the acetyloxy group, are less active than acetyl esters **3** and **4**. Furthermore, the comparable activity of **3** and **4** indicated that the substituent on the amino group at the 5-position of the pyrimidone ring has minimum effect on the activity. However, elongation of the Michael acceptor side chain by one additional peptide unit, such as compound **6**, enhanced the antimalarial activity 1- to 2-fold.

The in vivo antimalarial activity of compounds **3** and **6** were assessed against *P. berghei* under the reported Thompson test protocol.[23a] The preliminary results indicated that both compounds exhibited antimalarial activity at 40−160 mg/kg dosages administered subcutaneously and prolonged the life span of parasite-bearing mice from 6 days for untreated control to 16−24 days for those treated with test compounds.

To evaluate the potential human toxicity of the new peptidomimetic agents, the cell growth inhibitory activity in three human cell lines (neuronal, macrophage, and colon) were assessed. The results are shown in Table 2. In general, this class of compound exhibited weak to moderate cytotoxicity against neuronal and macrophage cells with IC_{50} of ~1 μg/mL for **5** to 16 μg/mL for **4.** In most cases the toxicity to colon cell line were weak (IC_{50} = 1.6 μg/mL for **6**) to insignificant (>50 μg/mL for compound **3**−**5**). Compound **4**, the most polar among the compounds tested, was the least toxic compound.

Experimental Section

Chemistry. Melting points were determined on a Mettler FP62 melting point apparatus and are uncorrected. Unless otherwise noted, all nonaqueous reactions were performed under an oxygen-free atmosphere of nitrogen with rigid exclusion of moisture from reagents and glassware. Analytical thin-layer chromatography (TLC) was performed using EM Reagents 0.25 mm silica gel 60-F plates. Visualization of the developed chromatogram was performed with UV absorbance, aqueous potassium permanganate, or ethanolic anisaldehyde. Liquid chromatography was performed using a force flow (flash chromatography) of the indicated solvent system on EM Reagents silica gel 60 (70−230 mesh). Preparative TLC was performed using Whatman silica gel C8 TLC plates (PLK5F). Infrared spectra were recorded on a Bio-Rad FTS 3000 spectrophotometer and are reported in reciprocal centimeters (cm^{-1}). ^{1}H NMR and ^{13}C NMR spectra were recorded in deuteriochloroform, unless otherwise noted, on a Bruker Avance 600 spectrometer at a frequency of 600.1 MHz. Chemical shifts are reported in parts per million on the δ scale from an internal standard of tetramethylsilane. Combustion analyses were performed by Atlantic Microlab, Inc. (Norcross, Georgia). When necessary, solvents and reagents were dried as follows. Ether, tetrahydrofuran, benzene, and toluene were stored and distilled from sodium benzophenone ketyl; dichloromethane, triethylamine, pyridine, and hexane were distilled over calcium hydride. Unless otherwise stated, the reagents were purchased from Fisher Scientific, Aldrich Chemical Co., Lancaster, and Fluka and used as received.

2-(2-*tert*-Butoxycarbonylamino-1-hydroxy-propyl)acrylic Acid Methyl Ester (10). DABCO (150 mg) was added to a solution of aldehyde **9** (1.19 g) in methyl acrylate (5 mL), and the resulting mixture was stirred at ambient temperature for 6 days. Excess methyl acrylate was evaporated. The resulting oil was dissolved in 30 mL of EtOAc and washed successively with 0.5 N HCl (20 mL), saturated aqueous $NaHCO_3$ (20 mL), and brine (20 mL). The ethyl acetate solution was then dried over anhydrous Na_2SO_4 and evaporated in a rotary evaporator to give 1.57 g of compound **10** as a viscous oil. Yield: 88%. ^{1}H NMR: δ 6.32 (s, 1H), 5.91 (s, 1H), 4.78 (br, 1H), 4.42 (m,1H), 3.92(m, 1H), 3.79(s, 3H), 3.42(br, 1H), 1.41 (s, 9H), 1.24 (d, J = 6.9 Hz, 3H). ^{13}C NMR: δ 166.7, 156.3, 140.5, 126.2, 79.5, 74.2, 51.9, 49.8, 28.4, 15.4. Anal. ($C_{12}H_{21}NO_5$) C, H, N.

2-Acetoxymethyl-4-*tert*-butoxycarbonylaminopent-2-enoic Acid Methyl Ester (11). Triphenylphosphine (3.8 g) was added to a solution of 1.25 g of **10** in 15 mL of THF. The resulting solution was cooled to −78 °C, and acetic acid (0.83 mL) was added, followed by slow addition of DEAD (2.3 mL) via a syringe. The mixture was slowly warmed to −30 °C and stirred for 1 h. It was then further warmed to 0 °C and stirred for another hour. The mixture was then diluted with 40 mL of EtOAc and washed successively with saturated aqueous $NaHCO_3$ (20 mL) and brine (20 mL). The EtOAc solution was dried over anhydrous Na_2SO_4 and evaporated in a rotary evaporator to give an oil, which was loaded onto a silica gel column and eluted with 25% EtOAc/hexanes to give 1.12 g of compound **11** as a viscous oil. Yield: 77%. ^{1}H NMR: δ 6.75 (d, J = 9.2 Hz, 1H), 5.00 (d, J = 12.1 Hz, 1H, AB of A), 4.90 (d, J = 12.1 Hz, 1H, AB of B), 4.60 (br, 1H), 4.58 (m, 1H), 3.78-(s, 3H), 2.05 (s, 3H), 1.42 (s, 9H), 1.26 (d, J = 6.4 Hz, 3H). ^{13}C NMR: δ 170.6, 166.8, 154.8, 149.0, 126.9, 79.8, 58.3, 52.2, 44.7, 28.4, 20.9, 20.7. Anal. ($C_{14}H_{23}NO_6$) C, H, N.

2-(2-{5-[(4-Acetylpiperazine-1-carbonyl)amino]-6-oxo-2-phenyl-6*H*-pyrimidin-1-yl}-1-hydroxyethyl)acrylic Acid Methyl Ester (8b). The same procedure as for the synthesis of **10** was used to synthesize compound **8b**, starting from aldehyde **7b**, except the purification was achieved by elution from the column with 15% MeOH/EtOAc to give **8b** as a white solid, mp 211.7 °C. Yield: 72%. ^{1}H NMR: δ 8.30 (s, 1H), 7.52−7.47 (m, 5H), 6.25 (s, 1H), 5.80 (s, 1H), 4.62 (m, 1H), 4.47 (dd, J = 13.9, 6.8 Hz, 1H), 4.38 (dd, J = 13.9, 4.5 Hz, 1H), 3.78 (m, 4H), 3.59 (s, 3H), 3.46 (m, 4H), 2.16 (s, 3H). ^{13}C NMR: δ 167.9, 166.1, 157.8, 154.7, 152.0, 138.8, 136.5, 134.9, 131.8, 130.1, 128.5, 127.4, 125.7, 74.6, 56.9, 49.5, 45.9, 43.3, 21.8. Anal. ($C_{23}H_{27}N_5O_6$) C, H, N.

2-[2-(5-Benzyloxycarbonylamino-6-oxo-2-phenyl-6*H*-pyrimidin-1-yl)-1-hydroxyethyl]acrylic Acid Methyl Ester (8a). The same procedure as for the synthesis of **10** was used to synthesize compound **8a**, starting from aldehyde **7a**, except the purification was achieved by elution from the column with 25% EtOAc/hexanes to give **8a** as a white solid, mp 185.6 °C. Yield: 91%. ^{1}H NMR: δ 8.78 (br, 1H), 7.56 (s, 1H), 7.50−7.39 (m, 10H), 6.24 (s, 1H), 5.76 (s, 1H), 5.24 (s, 2H), 4.63 (m, 1H), 4.46 (dd, J = 13.8, 7.5 Hz, 1H), 4.39 (dd, J = 13.8, 4.4 Hz, 1H), 3.69 (d, J = 7.6 Hz, 1H), 3.57 (s, 3H). ^{13}C NMR: δ 167.5, 158.1, 152.7, 152.4, 139.6, 135.7, 134.9, 133.6, 131.0, 129.1, 128.8, 128.5, 128.2, 127.3, 125.4, 74.5, 68.1, 56.4, 49.7. Anal. ($C_{24}H_{23}N_3O_6$) C, H, N.

2-Acetoxymethyl-4-{5-[(4-acetylpiperazine-1-carbonyl)amino]-6-oxo-2-phenyl-6*H*-pyrimidin-1-yl}but-2-enoic Acid Methyl Ester (4). Compound **4** was obtained in 75% yield from **8b** according to the same procedure as for the synthesis of compound **11**. The purification was achieved using a silica gel column and elution with 15% MeOH/EtOAc, mp 231.3 °C. ^{1}H NMR: δ 8.24 (s, 1H), 7.55−7.41 (m, 5H), 6.24 (t, J = 5.3 Hz, 1H), 5.12 (d, J = 5.3 Hz, 2H), 4.72 (s, 2H), 3.75 (m, 4H), 3.65 (s, 3H), 3.42 (m, 4H), 2.14 (s, 3H), 2.10 (s, 3H). ^{13}C NMR: δ 169.2, 162.7, 153.2, 141.2, 133.7, 130.9, 129.5, 129.2, 128.9, 128.2, 127.9, 127.8, 124.6, 120.6, 63.4, 51.9, 47.1, 45.9, 42.3, 21.4, 21.0. IR (film), cm^{-1}: 2999, 2927, 1718, 1668, 1624, 1523, 1492, 1424, 1369, 1242, 1161, 1022, 996, 924, 778, 729, 704. Anal. ($C_{25}H_{29}N_5O_7$) C, H, N.

2-Acetoxymethyl-4-(5-benzyloxycarbonylamino-6-oxo-2-phenyl-6*H*-pyrimidin-1-yl)but-2-enoic Acid Methyl Ester (3). Compound **3** was obtained in 65% yield from **8a** according to the same procedure as for the synthesis of compound **11**. The purification was achieved by using a silica gel column and elution with 25% EtOAc/hexanes, mp 184.1 °C. ^{1}H NMR: δ 8.76 (br, 1H), 7.51−7.40 (m, 11H), 6.82 (t, J = 5.9 Hz, 1H), 5.24 (s, 2H), 4.88 (d, J = 5.9 Hz, 2H), 4.56 (s, 2H), 3.77 (s, 3H). ^{13}C NMR: δ 170.8, 166.4, 157.9, 152.8, 152.3, 138.5, 136.2, 129.2, 128.8, 128.4, 128.1, 125.5, 68.1, 62.3, 52.4, 44.8, 20.6. Anal. ($C_{26}H_{25}N_3O_7$) C, H, N.

Benzoic Acid 4-(5-Benzyloxycarbonylamino-6-oxo-2-phenyl-6*H*-pyrimidin-1-yl)-2-methoxycarbonylbut-2-enyl Ester (2). Compound **2** was prepared using the same method as for the synthesis of compound **3** to give white solid, mp 219.4 °C. Yield: 62%. ^{1}H NMR: δ 8.77 (br, 1H), 7.88 (d, J = 7.4 Hz, 2H), 7.55 (s, 1H), 7.54 (d, J = 7.1 Hz, 1H), 7.44−7.35 (m, 12H), 6.89 (t, J = 6.2 Hz, 1H), 5.24 (s, 2H), 4.96 (d, J = 6.2 Hz, 2H), 4.79 (s, 2H), 3.79 (s, 3H). ^{13}C NMR: δ 165.9, 165.7, 157.7, 153.0, 152.5, 141.2, 135.6, 134.8, 133.9, 133.2, 130.4, 129.64, 129.62, 129.55, 129.0, 128.7, 128.5, 128.4, 128.24, 128.21, 125.3, 67.6, 58.3, 52.4, 45.4. Anal. ($C_{31}H_{27}N_3O_7$) C, H, N.

4-Nitrobenzoic Acid 4-(5-Benzyloxycarbonylamino-6-oxo-2-phenyl-6*H*-pyrimidin-1-yl)-2-methoxycarbonylbut-2-enyl Ester (1). Compound **1** was prepared using the same method as for the synthesis of compound **3** to give a light-yellow solid, mp 234.9 °C. Yield: 59%. ^{1}H NMR: δ 8.77 (br, 1H), 8.25 (d, J = 8.9 Hz, 2H), 8.08 (d, J = 8.9 Hz, 2H), 7.54 (s, 1H), 7.47−7.30 (m, 10H), 6.92 (t, J = 6.3 Hz, 1H), 5.24 (s, 2H), 4.96 (d, J = 6.3 Hz, 2H), 4.86 (s, 2H), 3.80 (s, 3H). ^{13}C NMR: δ 165.5, 164.1, 157.7, 152.9, 152.3, 150.7, 141.7, 134.9, 134.8, 133.9, 130.8, 130.5, 129.1, 129.0, 128.9, 128.8, 128.7, 128.6, 128.3, 125.4, 123.6, 67.7, 59.0, 52.5, 45.2. IR (film), cm^{-1}: 3378, 3274, 3059, 2953, 1720, 1653, 1605, 1517, 1491, 1348, 1267, 1213, 1190, 1096, 1064, 967, 770, 736, 719, 699. Anal. $C_{31}H_{26}N_4O_9$) C, H, N.

4-(5-Benzyloxycarbonylamino-6-oxo-2-phenyl-6*H*-pyrimidin-1-yl)-2-hydroxymethylbut-2-enoic Acid Methyl Ester (5). Granular potassium carbonate (20 mg) was added into a stirred solution of compound **3** (100 mg) in 2 mL of methanol at ambient temperature. After being stirred for 30 min, the mixture was partitioned between EtOAc (20 mL) and H_2O (15 mL). The separated organic layer was washed with brine (10 mL), dried over anhydrous Na_2SO_4, and evaporated in a rotary evaporator to give 65 mg of compound **5** as a white solid, mp 188.5 °C. Yield: 71%. ^{1}H NMR: δ 8.79 (br, 1H), 8.54−7.38 (m, 11H), 6.63 (t, J = 6.9 Hz, 1H), 5.23 (s, 2H), 4.84 (d, J = 6.9 Hz, 2H), 4.21 (s, 2H), 3.77 (s, 3H), 3.00 (br, 1H). ^{13}C NMR: δ 166.7, 157.8, 152.9, 152.4, 138.6, 136.1, 135.6, 135.0, 134.6, 133.9, 130.5, 129.1, 128.7, 128.5, 128.2, 125.4, 67.6, 56.9, 52.3, 44.9. Anal. ($C_{24}H_{23}N_3O_6$) C, H, N.

2-Acetoxymethyl-4-[2-(5-benzyloxycarbonylamino-6-oxo-2-phenyl-6*H*-pyrimidin-1-yl)acetylamino]pent-2-enoic Acid Methyl Ester (6). Compound **11** (207 mg) was dissolved in 3.0 mL of Et_2O. To the solution was added 0.5 mL of 2.0 N HCl in ether, and the mixture was stirred for 15 min at ambient temperature. The solvent was removed in vacuo to give the amine salt **12**, which was dissolved in 2 mL of chloroform. The amine solution was added dropwise with stirring at room temperature to a mixture that consisted of acid **13** (260 mg), DCC (142 mg), HOBT (93 mg), and DMAP (910 mg) in 5 mL of chloroform. The resulting mixture was stirred overnight, then diluted with 40 mL of EtOAc, and washed successively with saturated aqueous $NaHCO_3$ solution (20 mL) and brine (20 mL). The ethyl acetate solution was then dried over anhydrous Na_2SO_4 and evaporated in vacuo to dryness to give a crude product as an oil. The crude product was purified by the use of a silica gel column and elution with 50% EtOAc/hexanes to give 306 mg of compound **6** as a white solid, mp 214.6 °C. Yield: 79%. ^{1}H NMR: δ 8.79 (br, 1H), 7.53−7.39 (m, 11H), 6.69 (d, J = 9.3 Hz, 1H), 6.10 (d, J = 7.0 Hz, 1H), 5.23 (s, 2H), 4.99 (d, J = 12.3 Hz, 1H), 4.93 (d, J = 12.3 Hz, 1H), 4.91 (m, 1H), 4.54 (d, J = 15.3 Hz, 1H), 4.45 (d, J = 15.3 Hz, 1H), 3.79 (s, 3H), 2.03 (s, 3H), 1.30 (d, J = 6.8 Hz, 3H). ^{13}C NMR: δ 170.8, 166.6, 165.5, 158.1, 153.0, 146.7, 136.0, 135.5, 134.3, 130.8, 129.23, 129.18, 129.09, 129.00, 128.91, 128.7, 128.4, 125.1, 73.6, 58.3, 52.3, 50.0, 44.3, 20.9, 20.4. IR (film), cm^{-1}: 3310, 3061, 2953, 1719, 1655, 1605, 1515, 1491, 1443, 1366, 1216, 1191, 1090, 1029, 980, 772, 735, 700. Anal. ($C_{29}H_{30}N_4O_8$) C, H, N.

In Vitro Antimalarial Studies. The in vitro assays were conducted by using a modification of the semiautomated microdilution technique of Desjardins et al.[23b] and Chulay et al.[24] Two *P. falciparum* malaria parasite clones, from CDC Indochina III (W-2) and CDC Sierra Leone I (D-6), were utilized in susceptibility testing. They were derived by direct visualization and micromanipulation from patient isolates.[25] The W-2 clone is susceptible to mefloquine but resistant to chloroquine, sulfadoxine, pyrimethamine, and quinine, whereas the D-6 clone is naturally resistant to mefloquine but susceptible to chloroquine, sulfadoxine, pyrimethamine, and quinine. Test compounds were initially dissolved in DMSO and diluted 400-fold in RPMI 1640 culture medium supplemented with 25 mM Hepes, 32 mM $NaHCO_3$, and 10% Albumax I (Gibco BRL, Grand Island, NY). These solutions were subsequently serially diluted 2-fold with a Biomek 1000 (Beckman, Fullerton, CA) to give over 11 different concentrations. The parasites were exposed to serial dilutions of each compound for 48 h and incubated at 37 °C with 5% O_2, 5% CO_2, and 90% N_2 prior to the addition of [^{3}H]hypoxanthine. After a further incubation of 18 h, parasite DNA was harvested from each microtiter well using Packard Filtermate 196 harvester (Meriden, CT) onto glass filters. Uptake of [^{3}H]hypoxanthine was measured with a Packard Topcount scintillation instrument. Concentration−response data were analyzed by a nonlinear regression logistic dose response model, and the IC_{50} values (50% inhibitory concentrations) for each compound were calculated (Table 1).

Toxicity Assay. Selected compounds were tested for toxicity in vitro against three mammalian cell lines. A subclone (G8) of the murine monocyte-like macrophage line J774 was obtained from Dr. Jose Alunda, Departmento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense, Madrid, Spain. Murine cells were maintained in 75 cm^2 tissue culture flasks in Dulbecco's modified Eagle medium (GIBCO) supplemented with 10% fetal calf serum, 2 mM l-glutamine, and 50 mg/mL gentamicin under humidified 5% CO_2/95% air at 37 °C. A subclone (NG-108-15) of a hybrid rat neruoblastoma/mouse glioma cell line was the gift of Dr. Marshall Nirenberg, National Heart Lung and Blood Institute, National Institutes of Health, Bethesda, MD. Neuronal cells were maintained in 75 cm^2 tissue culture flasks in Dulbecco's modified essential medium, including a hypoxanthine−aminopterin−thymidine (HAT) supplement, 10% fetal calf serum, and 50 mg/mL gentamicin under humidified 5% CO_2/95% air at 37 °C. The human colon cell line SW620 was provided by Robert Robey, National Cancer Institute, National Institutes of Health, Bethesda, MD. Colon cells were maintained and tested in RPMI-1640 supplemented with 10% fetal bovine serum. Toxicity tests were performed in 96-well tissue culture plates using the protein-binding dye sulforhodamine B (SRB) as described.[26] Test compounds were serially diluted and added to empty wells of the 96-well plate. Appropriate cells in their respective culture medium were immediately seeded into the

wells. Appropriate solvent blanks (no compound) were run in each test. After 72 h under culture conditions, cells were fixed to the plate by layering 50% TCA (4 °C) over the growth media in each well to produce a final TCA concentration of 10%. After incubating for 1 h at 4 °C, cultures were washed five times with tap water and air-dried. Wells were stained for 30 min with 0.4% (w/v) SRB in 1% acetic acid and washed four times with 1% acetic acid. Cultures were air-dried, and bound dye was solubilized with 10 mM Tris base (pH 10.5) for 15 min on a gyratory shaker at room temperature. A Spectra MAX Plus microtiter plate reader (Molecular Devices) was used to measure the optical density (OD) at wavelengths of 490−530 nm. The 50% cell growth inhibitory concentration (IC_{50}) of each compound is shown in Table 2.

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