Synthesis and Evaluation of New Antimalarial Phenylurenyl Chalcone Derivatives

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Phenylurenyl chalcone derivatives have been synthesized and tested as inhibitors of in vitro development of a chloroquine-resistant strain of *Plasmodium falciparum***,** activity of the cysteine protease falcipain-2, in vitro globin hydrolysis, β-hematin formation, and murine *Plasmodium berghei* malaria. The most active antimalarial compound was 1-[3′-*N*-(*N*′-phenylurenyl)phenyl]-3(3,4,5-trimethoxyphenyl)-2-propen-1-one **49**, with an IC_{50} of 1.76 μM for inhibition of *P. falciparum* development. Results suggest that chalcones exert their antimalarial activity via multiple mechanisms.

Introduction

Malaria, the most severe human parasitic disease, causes 200−500 million cases and 0.7−2.7 million deaths each year.[1−4] Although the greatest burden of malaria and the focus of its control are in Africa, it is also an important problem in other tropical areas such as Asia, South America, and Oceania.

Plasmodium falciparum and *Plasmodium vivax* are the two major human malaria parasites. *P. falciparum* is responsible for most deaths, and it has developed resistance to nearly all available drugs.[5] The search for novel antimalarial drugs against specific parasitic targets is thus an urgent priority.[6, 7] The antimalarial activity of chalcones has generated interest.[8−12] Their antimalarial activity was first reported when licochalcone A, a natural product isolated from Chinese liquorice root, exhibited potent antimalarial activity.[8] Subsequently, a synthetic analogue was reported to have antimalarial activity.[9] Some chalcones inhibit falcipain cysteine proteases, but it is unclear if the antimalarial activity of this class is primarily due to protease inhibition.[10] Several oxygenated chalcones and bischalcones were reported to have antimalarial activity.[11] In a recent study the synthesis and antimalarial activity of some quinolinyl chalcone analogues have been reported.[12]

Our work now focuses on the synthesis and characterization of a series of novel phenylurenyl chalcone compounds, including evaluation of effects on development of cultured *P. falciparum* parasites, cysteine protease activity, globin hydrolysis, and heme polymerization and in a murine malaria model.

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Results and Discussion

Synthesis. A procedure based on a Claisen−Schmidt condensation was developed for syntheses of all phenylurenyl chalcones and their derivatives. Part of the work presented here was undertaken to elaborate structure−activity relationships (SARs) with respect to variations of R, R_5, and R_6 (Scheme 1). Syntheses of methyl ketone derivatives **6**−**9** were obtained by reaction of *p*- or *m*-amino acetophenones **4** and **5** with the corresponding isocyanate derivatives, where R = H, Cl, OMe. The subsequent treatment of these derivatives with solid sodium hydroxide as a catalyst in methanol at room temperature and the corresponding substituted aromatic aldehydes by the use of Claisen−Schmidt condensation[13] yielded **10**−**56**. The above reaction conditions were found to be optimal, whereas organic bases such as triethylamine or piperidine generally gave lower yields under the same reaction conditions. Sodium methoxide did not work as well as the sodium hydroxide pellet. If the starting material was insoluble in methanol, then tetrahydrofuran (THF) or 1, 4-dioxane was used as a cosolvent. The vinylic protons of cis alkenes have coupling constants smaller than those of their trans isomers. Some studies[12,14] on chalcone derivatives have shown exclusively *E* configuration; however, NMR techniques allowed us to conclude that the compounds obtained almost always yielded the trans alkenes (*E* form). To assess the specificity and selectivity of these derivatives, they were tested in different malaria assays.

Biological Evaluation. Inhibition of Parasite Development and Enzyme Inhibition. Compounds were tested for their ability to inhibit parasite development by incubating different concentrations with parasites for 48 h, beginning at the ring stage, counting new ring forms by fluorescence-activated cell sorting (FACS) analysis, and comparing parasitemias with those of untreated controls (Table 1). Twelve compounds inhibited parasite development at or below 10 μM (Table 1).

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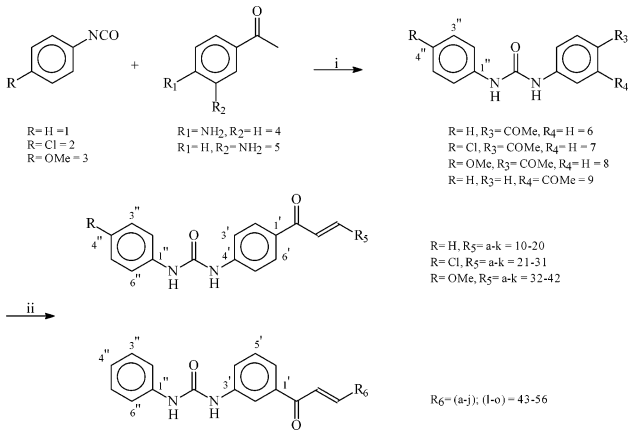
Scheme 1[a]



[a] R_5: (a) 2,4-di-$OMeC_6H_3$; (b) 4-FC_6H_4; (c) 3,4-$OCH_2OC_6H_3$; (d) 4-ClC_6H_4; (e) 2,4-di-ClC_6H_3; (f) 2,4-di-FC_6H_3; (g) 3,4,5-tri-$OMeC_6H_2$; (h) C_6H_5; (i) 4-MeC_6H_4; (j) 4-$OMeC_6H_4$; (k) C_5H_4N. R_6: (a–j) same as for R_5; (l) 2,3-di-$OMeC_6H_3$; (m) 3,4-di-$OMeC_6H_3$; (n) 4-BrC_6H_4; (o) N$(Me)_2C_6H_4$, (i) Me_2CO, room temp, (ii) MeOH, NaOH, R_5CHO or R_6CHO.

Table 1. Effect of Compounds on *P. falciparum* Development and Inhibition of Falcipain-2 Activity

compd[a]	FACS[b] IC_{50}, μM	falcipain-2[c] IC_{50}, μM
23	10	2.5
24	10	2.6
25	5	2.5
26	8	2.6
27	3	1.8
28	9	3.8
31	9	3.4
42	10	12.5
47	2.14	*d*
49	1.76	*d*
51	2.74	*d*
55	2.10	*d*
LEU	6.26	0.0058
E-64	2.50	0.058
CQ	0.059	*d*

[a] Only compounds expressing activity against *P. falciparum* parasites (IC_{50} < 10 μM) were screened for activity against recombinant falcipain-2. [b] IC_{50} for tested compounds as determined by flow cytometry. [c] IC_{50} of selected compounds active against recombinant falcipain-2. [d] There was no significant effect on inhibition of falcipain-2 probably because of limited solubility.

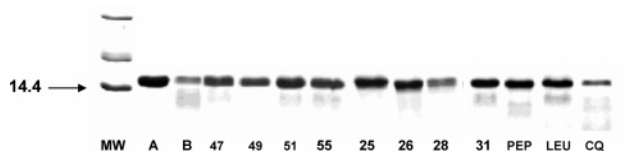


Figure 1. Effects on globin hydrolysis of chalcone derivatives. The samples were solubilized in SDS–sample buffer containing β-mercaptoethanol and boiled before electrophoresis in 15% SDS–PAGE gels. The gels were stained with Coomassie blue. The positions of molecular weight (MW) standards are shown in kDa. Undegraded globin appears as a dimer at 14 kDa: (A) control hemoglobin without enzyme; (B) control enzyme with hemoglobin. Also shown are **47**, **49**, **51**, **55**, **25**, **26**, **28**, and **31** (5 μM), PEP = pepstatin (10 μM), LEU = leupeptin (10 μM), and CQ = chloroquine (100 μM).

For the remaining compounds, the IC_{50} for the inhibition of parasite development was >10 μM.

Inhibition of Globin Hydrolysis. Selected compounds that inhibited parasite development were evaluated for the inhibition of globin hydrolysis (Figure 1). Compound **31** was most active, with 98% inhibition of hemoglobin degradation at of 5 μM. Six compounds (**47**, **49**, **51**, **55**, **25**, and **37**) inhibited hemoglobin degradation less completely (51–75%) (Figure 2, Table 2).

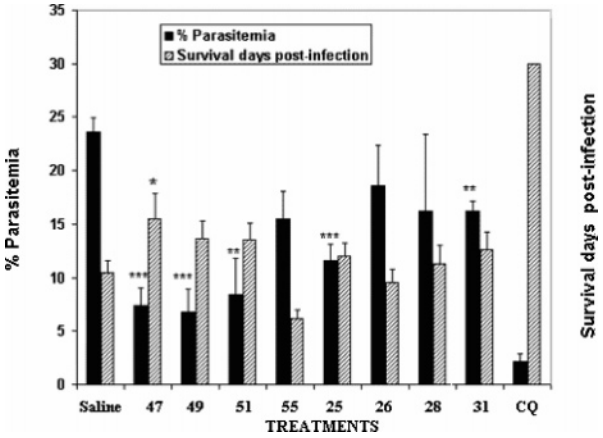


Figure 2. The % parasitemia at 4th day postinfection and survival days on mice treated with **47**, **49**, **51**, **55**, **25**, **26**, **28**, **31** (20 mg/kg) and CQ (25 mg/kg): (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, comparing with saline-treated mice ($n = 6$).

Table 2. Inhibition of Hemozoin Formation, Hemoglobin Hydrolysis, and Development in a *P. berghei* Murine Model (Peter's Test) by the Most Active Compounds[a]

compd	inhibition of hemozoin formation[b] (%)	inhibition of hemoglobin hydrolysis[c,e] (%)	Peter's test[d,e] (%P/SD)
saline			(23.62 ± 1.36)/(10.50 ± 1.12)
25	<5	60.31 ± 1.41	(11.60 ± 1.57)/(12.00 ± 1.26)
26	<5	33.04 ± 3.23	(18.60 ± 3.81)/(9.50 ± 1.25)
31	89.2	98.64 ± 0.73	(16.25 ± 0.84)/(12.66 ± 1.56)
47	<5	60.72 ± 1.73	(7.40 ± 1.64)/(15.50 ± 2.37)
49	<5	51.45 ± 2.26	(6.80 ± 2.17)/(13.66 ± 1.64)
51	<5	74.93 ± 2.53	(8.40 ± 3.40)/(13.50 ± 1.61)
55	*f*	66.58 ± 1.80	(15.50 ± 2.57)/(6.16 ± 0.77)
CQ	86.6	5.15 ± 1.85	
LEU	*f*	87.02 ± 0.69	
PEP	*f*	91.91 ± 0.67	

[a] CQ = chloroquine; LEU = leupeptin; PEP = pepstatin compared to LEU; P = parasitemia; SD= survival days. [b] % of inhibition of hemozoin formation. [c] Hemoglobin hydrolysis. [d] Peter's test results of the most active drugs. [e] The results are expressed by the mean ± standard error of the mean. $p < 0.0001$ [f] Unknown.

Inhibition of Hemozoin Formation. We tested the ability of the compounds to inhibit hemozoin formation. Compound **31** inhibited this process (IC_{50} = 0.73 mM) with activity somewhat greater than that of chloroquine (IC_{50} = 1.33 mM) (Table 2).

Activity in a Mouse Malaria Model. Selected compounds that were active in vitro (**47**, **49**, **51**, **25**, **26**, **28**, and **31**) were tested for activity in mice infected with *P. berghei* ANKA, a chloroquine-susceptible strain of murine malaria parasites. The mice were treated with compounds (20 mg/kg) or chloroquine (25 mg/kg) intraperitoneally once daily for 4 consecutive days (days 0–3 postinfection), and their survival times and parasitemia on day 4 were compared with those of control mice receiving only saline. A number of compounds significantly inhibited day 4 parasitemia and increased survival times (Figure 2).

In *P. falciparum*, a number of chalcones have been shown to exert antimalarial activity.[15] We have synthesized and evaluated the antimalarial activity of 47

new chalcone derivatives. We demonstrated in vitro and in vivo antimalarial activity of a number of compounds. To explore the mechanisms of action of these compounds, we considered the effects of compounds that inhibited parasite development on activity of the cysteine protease falcipain-2, catabolism of hemoglobin, and hemozoin formation and in a murine malaria model.

Nine compounds inhibited the development of cultured *P. falciparum* parasites with $IC_{50} < 10$ μM. The most active compounds were **47**, **49**, **51**, and **55**. Compounds **47** and **49** also showed good inhibition of globin hydrolysis. These compounds also were active against *P. berghei* infections in mice. However, the antimalarial effect in vivo was modest, and all treated mice eventually died because of lethal infections.

The data suggest that activity in most cases was governed to a large extent by groups attached to the substituted aromatic ring assigned as R_5 (difluoride, dichloride, and trimethoxy) and R_6 (dichloride and trimethoxy). These groups played an important role apparently because of electron-donating properties, yielding the most active antimalarial compounds. In general we speculate that electron-withdrawing groups on the substituted aromatic ring for these urenyl chalcones should favor the Michael addition to an available nucleophilic side chain on the enzyme. Compounds **23**−**28** and **31** were the most active as inhibitors of falcipain-2. It appears that their effects were due to inhibition of this important hemoglobinolytic enzyme (Table 1). We observed some correlation for compounds **25**−**28** and **31** between inhibition of falcipain-2 and of development of cultured parasites, suggesting that plasmodial cysteine proteases are involved in their antimalarial activity. None of the above active compounds were found to inhibit hemozoin formation in vitro in the same range compound **31**, and five among them, **25**, **31**, **47**, **51**, and **55**, displayed strong inhibition of globin hydrolysis (60−98%), suggesting a different mechanism of action. Considerable experimental evidence suggests that inhibition of hemozoin formation is central to the mechanism of action of the quinoline-containing antimalarial agents.[16] Some results indicate that the extent of drug accumulation at the site of the heme is also a regulator of antimalarial activity.[17] Within the range of solubility, hemozoin formation inhibitory activity could be measured for most of the studied chalcones. Only **31** showed an excellent inhibition of hemozoin formation (89.2%) and hemoglobin hydrolysis (98.64%). However, **31** failed to demonstrate a clear relationship between antiparasitic activity and inhibition of hemozoin formation. Therefore, the inhibition of heme formation does not guarantee antimalarial activity. Finally, it seems that para and meta positions in the urenyl ring play an important role in their antimalarial activity, as could be observed for aromatic substituted trimethoxyl **27** (IC_{50} for *P. falciparum* is 3 μM) vs **49** (1.76 μM) and for dichloride **25** (5 μM)) vs **47** (2.14 μM). These results offer new possibilities for further improvements in the antimalarial performance of urenyl chalcones.

Conclusion

A series of phenylurenyl chalcone derivatives have been synthesized and evaluated for in vitro antimalarial activity. Selected compounds were also tested in vivo in *P. berghei* infected mice. We found that compounds **47**, **49**, **51**, and **55** were excellent inhibitors against cultured *P. falciparum* parasites, with a good correlation with activity in a murine malaria model except for **55**. The most active derivative was 1-[3'-*N*-(*N*′-phenylurenyl)phenyl]-3(3,4,5-trimethoxyphenyl)-2-propen-1-one **49** with an IC_{50} of 1.76 μM against cultured *P. falciparum*. Four other chalcone derivatives, **25**−**28**, had IC_{50} below 9 μM, and the most active of these compounds against cultured *P. falciparum* parasites and the cysteine protease falcipain-2 was 1-[4′-*N*-(*N*′-*p*-chlorophenylurenyl)-phenyl]-3(3,4,5-trimethoxyphenyl)-2-propen-1-one **27** (IC_{50} of 3 and 1.8 μM, respectively). However, its biological study was incomplete because of poor solubility in polar solvents. A very interesting result was obtained for compound 1-[4′-*N*-(*N*′-*p*-chlorophenylurenyl)phenyl]-3-(3-pyridinyl)-2-propen-1-one **31**, which showed a good antimalarial effect in vitro and in vivo. Nevertheless, this compound was able to interfere with heme detoxification (inhibition 89.2%) and globin hydrolysis (inhibition 98.64%) (Table 2). This property could be attributed to derivative **31** having an aromatic ring that has been substituted by an aromatic pyridinyl group and refers to a nitrogen-containing heterocyclic (Scheme 1) that resembled the nitrogen of chloroquine and may be somewhat concentrated in the food vacuoles of malaria parasites for a given result.[18] The comprehensive relationship between antiparasitic activity, inhibition of hemozoin formation, and hemoglobin hydrolysis for this type of compound has been observed. Therefore, a potent inhibition of heme formation and hemoglobin hydrolysis does not guarantee antimalarial activity. These results provide an understanding of the structural features that influence functional activity for this class of compounds and also offer new possibilities for improvements in the antimalarial performance of urenyl chalcones. We concluded from our data that derivative **49** has the best antimalarial properties, but its mechanism of action does not appear to be related to hemoglobin hydrolysis or heme detoxification, and if we compare it against derivative **31**, we found that at the same concentration **49** was the best to arrest the development of *P. falciparum* in culture ($IC_{50} = 1.76$ μM) and to reduce the parasitemia of *P. berghei* infected mice (from control 23−6.8%). Further studies are necessary to improve its antimalarial activity.

Experimental Section

General Procedure for Preparation of 6−9. A mixture of the corresponding aminoacetophenone **4** or **5** (1 mmol) and phenylisocyanates **1**−**3** recently distilled (1 mmol) was dissolved in dry acetone (5 mL). The mixture was stirred under nitrogen atmosphere for 3−7 h at room temperature. The resulting solid was filtered, and crystallization with the appropriate solvent afforded the desired urenylacetophenone in pure form.

4′-*N*-(*N*′-Phenylurenyl)acetophenone (6). Following the general procedure, a white solid was obtained that was recrystallized in EtOH: yield 52%; mp 175−177 °C; IR 3360 (NH), 1685 (CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.51 (s, 3H, COMe), 6.98 (q, 2H, $H_{4''}$, $J = 8.91$ Hz), 7.28 (q, 4H, $H_{3''5''}$, $J = 8.91$ Hz), 7.43−7.48 (m, 4H, $H_{2''-6''}$), 7.58 (d, 2H, $H_{3'-5'}$, $J = 8.88$ Hz), 7.91 (d, 2H, $H_{2'-6'}$, $J = 8.88$ Hz), 8.64 (s, OH), 8.79 (br s, NH), 9.09 (br s, NH); ^{13}C NMR (DMSO-d_6) δ 26.87 (COMe), 117.69 ($C_{3'-5'}$), 118.76 ($C_{2''-6''}$), 119.02 ($C_{2''6''}$), 122.38 ($C_{4''}$), 122.80 ($C_{4''}$), 129.33 ($C_{3''-5''}$), 129.39, ($C_{3''-5''}$), 130.20

($C_{2'-6'}$), 130.98 ($C_{1'}$), 139.81 ($C_{4'}$), 140.23 ($C_{1''}$), 144.91 ($C_{1''}$), 152.72 ($CO(NH)_2$), 153.09 (N=COH−NH_2), 196.86 (CO).

4′-*N*-(*N*′-*p*-chlorophenylurenyl)acetophenone (7). The solid was recrystallized in EtOH: yield 65%; mp 224−225 °C; IR 3376 (NH), 1715 (COMe) cm^{-1}, 1648 (CO); ^1H NMR (DMSO-d_6) δ 2.51 (s, 3H, COMe), 7.33 (d, $H_{3''-5''}$, J = 8.67 Hz), 7.49 (d, $H_{2''-6''}$, J = 8.67 Hz), 7.57 (d, $H_{3'-5'}$, J = 8.64 Hz), 7.90 (d, $H_{2'-6'}$,J = 8.64 Hz), 8.94 (br s, NH), 9.13 (br s, NH); ^{13}C NMR (DMSO-d_6) δ 26.89 (COMe), 117.82 ($C_{3'-5'}$), 120.56 ($C_{2''-6''}$), 126.35 ($C_{4''}$), 129.22 ($C_{3''-5''}$), 130.19 ($C_{2'-6'}$),131.13 ($C_{1'}$),138.84 ($C_{1''}$), 144.70 ($C_{4'}$), 152.65 ($CO(NH)_2$), 196.89 ($CO_{\alpha,\beta}$).

4′-*N*-(*N*′-*p*-Methoxyphenylurenyl)acetophenone (8). The solid was recrystallized in a mixture of EtOH/H_2O: yield 82%; mp 210−212 °C; IR 3376 (NH), 1706 (CO), 1642 (CO), 1581 (C=C Ar) cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.51 (s, 3H, COMe), 3.72 (s, 3H, 4″-OMe), 6.88 (d, $H_{3''-5''}$, J = 8.91 Hz), 7.37 (d, $H_{2''-6''}$, J = 8.91 Hz), 7.57 (d, $H_{3'-5'}$, J = 8.91 Hz), 7.89 (d, $H_{2'-6'}$, J = 8.91 Hz), 8.61 (br s, NH), 9.02 (br s, NH); ^{13}C NMR (DMSO-d_6) δ 26.86 (COMe), 56.60 (4″-OMe), 114.55 ($C_{3'-5'}$), 117.55 ($C_{2''-6''}$), 120.86 ($C_{3''-5''}$), 130.20 ($C_{2'-6'}$), 130.76 ($C_{1'}$), 132.78 ($C_{1''}$), 145.12 ($C_{4'}$), 152.88 ($CO(NH)_2$), 155.28 ($C_{4''}$), 196.83 (CO).

3′-*N*-(*N*′-Phenylurenyl)acetophenone (9). The solid was recrystallized in EtOH and afforded the title compound: yield 65%: mp 172−173 °C: IR 3.296 (NH), 1.680 (CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.50 (s, 3H, $COCH_3$), 6.99 (t, 1H, $H_{4'}$, J = 6.43 Hz), 7.28 (t, 2H, $H_{3'-5'}$, J = 6.94 Hz), 7.46 (d, 2H, $H_{2'-6'}$, J = 6.94 Hz), 7.58 (d, 1H, H_4, J = 6.94 Hz), 7.66 (d, 1H, H_6, J = 6.94 Hz), 8.06 (s, 1H, H_2), 8.69 (br s, 1H, NH), 8.88 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ 117.89, 1218.92, 122.48, 122.58, 123.34, 129.35, 129.74, 137.94, 140.05, 140.69, 153.10, 198.35.

General Procedure for Preparation of 10−56. Title compounds were prepared by reacting equimolecular quantities of urenylacetophenone **6**−**9** and the corresponding substituted aldehydes in the presence of an excess sodium hydroxide (2.5 mmol) in dry methanol (5 mL). The mixture was stirred at room temperature, and the resulting solids were collected on a filter and washed three times with cold methanol. In most cases, off-white to bright-yellow solids were formed within 3−5 h. The product was recrystallized from appropriate solvents whenever necessary as indicated in Table 3. The yield of pure product ranged from 54% to 99%.

1-[4′-*N*-(*N*′-*p*-Chlorophenylurenyl)phenyl]-3-(3-pyridinyl)-2-propen-1-one (31). ^1H NMR (DMSO-d_6) δ 7.32 (d, $H_{3''-5''}$, J = 8.91 Hz), 7.46−7.52 (m, $H_{2''-6''}$), 7.67 (d, $H_{3'-5'}$, J = 8.40 Hz), 7.73 (d, H_α, J = 15.82 Hz), 8.14 (d, $H_{2'-6'}$, J = 8.40 Hz), 8.34 (d, H_4, J = 7.91 Hz), 8.60 (d, H_6, J = 8.70 Hz), 9.01 (s, H_2), 9.63 (br s, NH); ^{13}C NMR (DMSO-d_6) δ 118.05 ($C_{3'-5'}$), 120.65 ($C_{2''-6''}$), 124.45 (C_α), 124.57 (C_5), 126.30 ($C_{4''}$), 129.14 ($C_{3''-5''}$), 130.71 ($C_{2'-6'}$), 131.20 (C_4), 135.63 (C_1), 138.97 ($C_{1'}$), 140.14 ($C_{1''}$), 145.35 (C_β), 150.63 (C_2), 151.30 (C_6), 152.88 (CO-$(NH)_2$), 187.77 ($CO_{\alpha,\beta}$); CIMS (*m*/*z*) 379 [M + H]; IR 3344 (NH), 1654 (CO) cm^{-1}. Anal. ($C_{21}H_{16}ClN_3O_2$) C, H, N.

1-[3′-*N*-(*N*′-Phenylurenyl)phenyl]-3(3,4,5-trimethoxyphenyl)-2-propen-1-one (49). ^1H NMR: (DMSO-d_6) δ 3.71 (s, 3H, 4-OCH_3), 3.86 (s, 6H, 3,5-OCH_3), 6.97 (t, 1H, $H_{4''}$, J = 7.43 Hz), 7.22 (s, 2H, H_2, H_6), 7.28 (t, 2H, $H_{3''-5''}$, J = 7.43 Hz), 7.48 (d, 2H, $H_{2''-6''}$, J = 7.43 Hz), 7.66−7.85 (m, 3H, $H_{6'}$, $H_{4'}$, Hβ), 8.12 (s, 1H, $H_{2'}$), 9.01 (br s, 1H, NH), 9.19 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ 56.58, 60.81, 106.75, 119.31, 121.97, 123.02, 123.14, 129.39, 129.91, 130.67, 138.65, 139.58, 140.16, 140.43, 145.37, 153.49, 153.54, 190.59; CIMS (*m*/*z*) 433 [M + H]; IR 3.296 (NH), 1.638 (CO) cm^{-1}. Anal. ($C_{25}H_{24}N_2O_5$) C, H, N.

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Supporting Information Available: Synthesis and analytical data for **10**−**56** and biological techniques. This material is available free of charge via the Internet at http://pubs.acs.org.

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